UDC 575.633.11
https://doi.org/10.2298/GENSR1702583P
Original scientific paper

ASSESSMENT OF MOLECULAR AND PHENOTYPIC DIVERSITY AMONG WINTER WHEAT CULTIVARS

Sonja PETROVIĆ[1], Sonja MARIĆ[1], Tihomir ČUPIĆ[2], A. REBEKIĆ[1], Ivana RUKAVINA[3]

[1]Faculty of Agriculture, University J.J. Strossmayer, Osijek, Croatia
[2] Institute of Agriculture, Osijek, Croatia
[3] Croatian Centre for Agriculture, Food and Rural Affairs, Institute for Seeds and Seedlings, Osijek, Croatia

Petrović S., S. Marić, T. Čupić, A. Rebekić, I. Rukavina (2017): *Assessment of molecular and phenotypic diversity among winter wheat cultivars* - Genetika, vol 49 no. 2, 583-598.

Comparing results of different genetic diversity estimates can be useful in parental selection for plant breeders. Forty winter wheat cultivars, from three Croatian breeding centres and four foreign countries, were used to utilize and compare agronomic, morphologic and molecular based genetic diversity estimates. Ten morphologic descriptors according to UPOV guidelines and eight agronomic traits were used to establish phenotypic data. Molecular data consisted of 26 SSR and four combinations of AFLP markers, covering all three wheat genomes. Agro-morphologic data showed variability especially regarding plant height (CV=18.44%), yield (CV=22.02%), and ear emergence (range=8). Discriminant analysis confirmed grouping among cultivars was mostly influenced by number of days to heading and yield. The four AFLP primer combinations and 26 SSR markers yielded 108 polymorphic bands. The UPGMA based on phenotypic data, arranged cultivars in four clusters, with one distinctive outlier, cultivar U1. The UPGMA based on molecular data also arranged cultivars in four clusters, with one distinctive outlier, cultivar Antonius. The similarities based on all four genetic diversity estimates reflected, on average, the degree of relatedness of cultivars used. No correlations between phenotypic and molecular data were found implying that both types of data should be used for genetic diversity estimates in order to cover wider variability between tested cultivars.

Keywords: wheat; genetic diversity; phenotypic traits; molecular markers

Corresponding author: Sonja Petrović, Faculty of Agriculture in Osijek, University J.J. Strossmayer, Vladimira Preloga 1, Croatia, Telephone: 385-31-554-923, Fax: 385-31-554-853
E-mail: spetrovic@pfos.hr

INTRODUCTION

Plant breeding is based on detection and use of genetic variation. Analysis of genetic diversity among elite germplasm can offer predictive estimates of genetic variability between segregating progenies for pure line cultivar development (MANJARREZ-SANDOVAL *et al*., 1997). Improvement of crop genetic resources depends on continuous utilisation of wild relatives, local cultivars and the use of modern breeding techniques (MONDINI *et al*., 2009). Nevertheless, genetic variability of a crop plant population is also associated with responses to selection pressures. There are several methods to estimate genetic diversity: morphological, agronomical, pedigree, biochemical and molecular. The first markers used were morphologic ones, as they are visible and most likely qualitative (e.g. flower colours, presence of awns, grain shape, grain colour, etc.). Morphologic markers and agronomic traits are irreplaceable and used in purpose of evaluation, hybridisation and control of segregating populations as well as in monitoring of breeding progress during vegetation years (DREZNER, 1995; DENČIĆ *et al*., 1997; FUFA *et al*., 2005; ALI *et al*., 2008). Today they are still widely used in breeding programs and especially as descriptive tools in gene banks, genetic mapping in order to protect existing diversity and contribute efforts of conservation (DE LACY *et al*., 2000), preservation and utilisation (DOS SANTOS *et al*., 2009), and are essential in disease tolerance and in detecting phenotypic response on abiotic stress (SHAHRYARI *et al.,* 2011). Morphologic evaluation of phenotype traits is described in International Union for the Protection of new Cultivars of Plants (UPOV), utilised for testing the distinctness, uniformity and stability (DUS) of newly created cultivars, and widely used for cultivar identification and genetic diversity studies (LAW *et al*., 1998; JONES *et al*., 2003; RUKAVINA *et al*., 2013).

Although the morphologic descriptors are very useful, they are limited in number and may be affected by environment. In addition, they can lead to redundancies in phenotype based germplasm collections. Molecular markers represent effective complementing tools to morphologic descriptors as they are plentiful, independent of tissue or environment and allow cultivar identification in the early stages of development (POWELL *et al*., 1996; GUPTA *et al*., 1999; LANDJEVA *et al*., 2006; KONDIĆ-ŠPIKA *et al*., 2014). In many studies combinations of several diversity criteria are used for more accurate estimations (SOLEMANI *et al*., 2002; MARIĆ *et al*., 2004; KOTZAMANIDIS *et al*., 2011). 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), have very high level of polymorphism and thus suitable to distinguish closely related cultivars (MORGANTE and OLIVIERI, 1993; PLASCHKE *et al*., 1995). AFLP method (VOS *et al*., 1995) proved to be effective and reproducible (BARETT and KIDWELL, 1998; BUERSTMAYR *et al*., 2002) and it also gives a very large number of scorable bands which enhances the power to detect polymorphism (ROY *et al*., 2004). 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; MARIĆ *et al*., 2004; STODART *et al*., 2005; NAJAPHY *et al*., 2012). 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). These genetic diversity and variability estimates enhance conservation of plant genetic resources of all cultivated plants by storing in gene banks. Possibility of searching through wheat collections enables the utilisation of variability with the purpose of creating new wheat cultivars with improved disease tolerance and more adaptable to

climate changes. With the help of new technologies, it is necessary to develop conservation strategy of agronomically desired genes in gene banks using the molecular population genetics and genetic potential of wild relatives of modern cultivars (PRADA, 2009; GLASZMANN *et al*., 2010). Most of the cultivars used in this study were used directly in breeding programme as parental components in crossings, registered and bred cultivars in Croatia representing the ongoing working set of winter wheat germplasm. The aims of this study were a) to evaluate genetic diversity of hexaploid wheat cultivars and b) to evaluate and compare molecular and phenotypic diversity criteria.

MATERIALS AND METHODS

Plant material and field trial

Total of 40 Croatian and foreign winter bread wheat cultivars (PFOS collection), registered from 1931 to 2008, were used in this study (Table 1). Cultivars originating from Croatia (CR), Austria (AU), France (FR), Italy (IT) and Russia (RU) were sown during two years (2007/2008; 2008/2009) on the test fields at Institute of Agriculture, Osijek. Experimental design was complete block design with three replicates where each plot area was 6.5 m^2. The cultivars were sown based on breeders' instructions (sowing density ranged from 400 to 600 seeds per m^2); fertilization and plant protection was carried out to an optimum.

Phenotypic data analysis

Diversity based on morphologic markers was evaluated according to UPOV guidelines for testing distinctness, uniformity and stability of wheat (UPOV/TG 3/1, 1996). The following eight descriptors were used: growth habit, time of ear emergence, glaucosity of sheath of flag leaf, ear glaucosity, ear density, presence of awns or scurs, ear colour and grain colour. Diversity based on agronomic data was measured using eight traits: plant height (cm), spike length (cm) and number of plant per m^2 were measured during dough stage. Just before harvest we collected spikes for determining the number of spikelets per spike, number of grains per spike. Measurement of the hectolitre weight (hl/kg) and moisture was taken after harvest on field, after which we determined thousand grain yield (g) and yield (t/ha). For every trait 25 random spikes were taken of each cultivar in every replicate, in total 24 000 individual measurements have been made. Analysis of agronomic data was conducted for each parameter by analysis of variance (ANOVA) in a factorial design. Least significant differences (LSD) were calculated at the 5% and 1% probability level using SAS software 9.1.3 (2002-2003).

Cluster analysis based on agronomic data was calculated after standardisation by subtracting the mean value from the observed value and dividing by the standard deviation. Based on these standardised agronomic values and morphologic data Euclidian distances between cultivar *i* and *j* (D*ij*) was calculated following ROLDAN–RUIZ *et al*. (2001). Unweighted Pair Group Method Using Arithmetic Average (UPGMA) cluster analysis was performed in NTSYS ver.2.2 (ROHLF, 1998). Subsequently we used discriminant analysis to justify the grouping of the tested cultivars as Croatian (29), old (four registered from 1936 to 1971) and foreign (five from AUS and two from FRA) (Table 1), and to determine which of the agronomic traits were the most discriminant between groups.

Table 1. Name, origin and pedigree of tested cultivars

Cultivar	Origin	Reg. year	Pedigree	Cultivar	Origin	Reg. year	Pedigree
U1	CR	1936	Carlotta Strampeli/Marquis	Janica	CR	2003	Osk. 5.36-9-91/Srpanjka
Os.crvenka	CR	1976	Libellula/Bezostaja	Barbara	CR	1997	GO 3135/Žitarka
Os.20	CR	1978	Osk. 6.9-1-64/V-188-M	Katarina	CR	2006	Osk.5.B.4-1-94/Osk. 5.140-22-91
Slavonija	CR	1984	Osječka 20/Osk.4.216-2-76	Alka	CR	2003	Osk. 5.140-22-91/Sana
Zitarka	CR	1985	Osk. 6.30-20/Slavonka/3/Eph. M68/Osk.154-19/Kavkaz	Seka	CR	2006	Srpanjka/Demetra
Srpanjka	CR	1989	Osk. 4.50-1-77/Zg 2696	Lela	CR	2006	Srpanjka/Osk. 5.136-8-90
Demetra	CR	1991	Osk. 4.216-2-76/Zg 2877-74	Sana	CR	1983	Mura/CI14123//Zg241372
Su.žitarka	CR	1997	GO 3135/Žitarka	Adriana	CR	1988	ZG 1758-70/TpR-349
Lucija	CR	2001	Srpanjka/Kutjevčanka	Divana	CR	1995	Favorit/5/Cipriz/4/J.Kwang/2/ Atlas66/Comanc./3/Velvet
Renata	CR	2006	Žitarka /2/Osk.7.5-4-82/KB160-86/3/Srpanjka	Libellula	IT	1965	Tevere/Giuliari//San Past.
Aida	CR	2006	Srpanjka/Rialto	Bezostaja	RU	1963	Skorospelka2/Lutescens17
Pipi	CR	2006	Soissons/Osk. 6.83-5-91	BC Patria	CR	1994	Odesskaya-51/ZG-IPK-8210/2/GK-32-82
Ilirija	CR	2008	Osk.14.294-16-95/Soissons	BC Elvira	CR	2002	Bc 2377-79/MV-C2-33//Irena
Felix	CR	2008	Srpanjka/K160/86	Soissons	FR	1987	Iena/HN 35
Zlata	CR	2008	Srpanjka/Demetra	Valerius	AU	2006	Carolus//Monopol/Karat// Ekspert/Severin
Anđelka	CR	2008	Srpanjka/Demetra	Antonius	AU	2006	Pokal/Karat//Ekspert/Severin
Mihaela	CR	2008	Srpanjka/Osk. 5.136-11-90	Bastide	FR	2006	Fertil / Arche
Ružica	CR	2008	Osk. 5.36-9-91/Srpanjka//Brea	Edison	AU	2003	Agron/Regent//Capo
Zl.dolina	CR	1971	Zg 414-57/Leonardo	Eurofit	AU	2005	Pegassos/Kontrasst

DNA extraction and molecular data analysis

Twenty plants per cultivar were grown in greenhouse during 20 days. DNA was isolated with 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. Twenty-six microsatellite primer pairs (PETROVIC *et al*. (2012a) created by RÖDER *et al*. (1998) were used. PCR reactions were carried out in GeneAmp® Thermocycler 9700, while PCR amplification was carried out according to RÖDER *et al*. (1998) modified for LI-COR® Biosciences 4200 DNA Analyzer. For AFLP procedure genomic DNA was digested using restriction enzymes Sse8371 and Msel. 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). 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 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® 350 bp sizing standard, and for AFLP fragments IRDye® 700bp sizing standard. 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 a binary matrix.

Genetic diversity and polymorphism of microsatellites was analysed based on total and average allele number per marker (N_a). For each microsatellite loci and every AFLP marker combination polymorphic information content (PIC) was estimated using formula: $PIC = 1 - \sum_{i=1}^{I} p_i{}^2 - 2\sum_{i=j+1}^{I}\sum_{j=1}^{I-1} p_i{}^2 p_j{}^2$ as well as Genetic diversity (H_E) or expected heterozygosity (Nei) using formula $H_E = 1 - \sum_{i=1}^{I} p_i^2$. Above mentioned parameters were calculated with Powermarker (LIU, 2002). Combined binary matrices of allelic frequencies based on SSR data and AFLP data were used to calculate similarity coefficient (S_{ij}) according to Dice: $S_{ij} = \frac{2N_{ij}}{N_i + N_j}$. Similarity matrices are used to make dendrogram using UPGMA. Calculations were carried out in NTSYS ver.2.2 (ROHLF, 1998).

Comparison of phenotypic and molecular data

Correspondence between phenotypic (Euclid) and SSR and AFLP (Dice) matrix was tested with the Mantel Z test (MANTEL, 1967). Before calculations of correlation coefficients genetic distance matrix based on phenotypic data was transformed to similarity matrix. The significance of Z based on following formula $Z = \sum_{i \triangleright j}^{n} X_{ij} Y_{ij}$ was determined by comparison of Z value with 1000 permuted variants. Calculations were carried out in NTSYS ver.2.2 (ROHLF, 1998).

RESULTS

Diversity of agronomic and morphological traits

ANOVA showed highly significant differences (P<0,001) for each agronomic trait measured between all tested cultivars in both vegetation years (Table 2). The highest coefficient of variation (CV) was established for yield (22.02%) followed by plant height (18.44%), while lowest CV value was obtained for the thousand grain yield (2.66%). Plant height ranged from 63.89 cm to 141.87 cm in average, spike length varied significantly from 6.11 cm to 9.69 cm, while highest and lowest recorded grain yield were 3.97 and 9.22 t/ha, respectively.

Table 2. Data evaluation of eight agronomic traits on 40 winter wheat cultivars

Agronomic trait	Range	Mean	F-value	CV %
Plant height (cm)	63.89-141.87	87.83	85.40**	18.44
Plant per m^2	117.17-203.33	160.06	9.85**	10.96
Spike length (cm)	6.11-9.69	7.50	12.67**	13.24
Number of spikelets per spike	15.76-20.87	17.76	17.09**	8.60
Number of grains per spike	33.02-54.79	43.68	19.93**	12.76
Hectolitre weight (hl/kg)	40.41-51.81	44.50	13.93**	7.48
Thousand grain yield (g)	78.52-85.75	82.98	18.35**	2.66
Yield (t/ha)	3.97-9.22	7.75	20.54**	22.02

Variability of morphological traits was illustrated in Figure 1. Largest variability between cultivars was covered with ear emergence expressing almost all grades from very early to very late. Similar variability was established in glaucosity of sheath, grouping most of the

cultivars between grades 4 (medium weak) and 7 (strong) followed by glaucosity of ear, mostly grouping the cultivars with weak (grade 3) to medium ear glaucosity (grade 5). Majority of cultivars expressed semi-errectum to intermediate growth habit, with only two cultivars with semi-prostrate and prostrate growth type. Lowest variability was expressed in ear and grain colour. White ear colour character was present in all cultivars except two, while only seven cultivars had white and 33 had red grain colour.

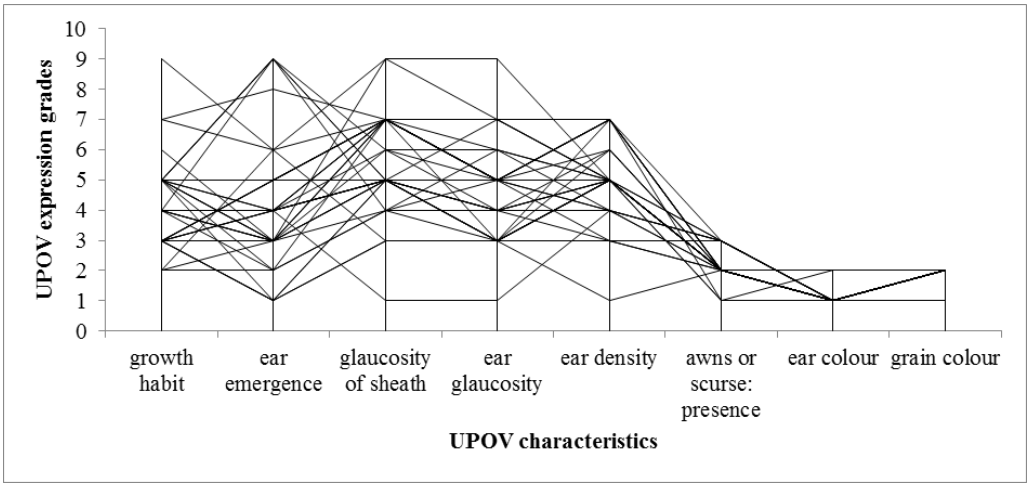

Figure 1. Variability of morphologic characters between cultivars

Based on eight morphologic and agronomic traits calculated Euclid diversity coefficient outlined cultivar U1, as morphologically and agronomical most diverse (E=0.149) from all tested cultivars belonging to the cluster 1 (Figure 2). Average genetic distance was E=0.149, while the largest calculated distance was between Croatian cultivar U1 and French cultivar Bastide (E=0.197). Most similar cultivars were Anđelka and Demetra (E=0.026), and the Croatian cultivar Aida had the average minimum genetics distance of E=0.0701 in comparison with all tested cultivars.

The cluster 2 consisted of one solitary cultivar, Bastide from France, and the cluster 3 consisting of two cultivars (Libellula and Osječka crvenka). Cluster 4 is further divided in two subclusters 4a (five cultivars) and 4b consisting of 36 cultivars. According to the similar agronomic and morphologic traits all the Austrian cultivars were clustered together (4a), while some very old cultivars, like Bezostaja and Libellula, and all Croatian cultivars share more or less similar agro-morphological characters. As the results fulfilled all four presumptions (normal distribution, homogeneity of variances/covariances, no correlation between means and variances) we applied discriminant analysis to predict whether a wheat genotype can be classified as Croatian, foreign or an old genotype on the basis of agronomic traits. Stepwise selection was used and predictor variables retained in analysis were days to heading and average yield.

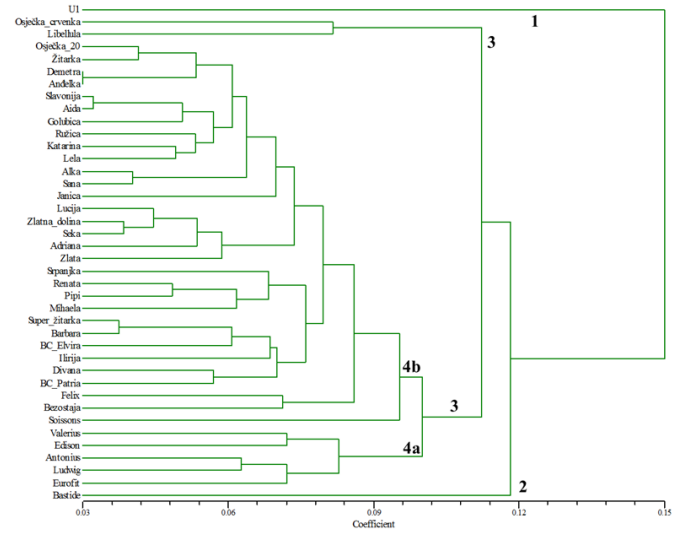

Figure 2. Dendrogram based on Euclid genetic distance matrix based on agro-morphological data for 40 bread wheat cultivars using the UPGMA clustering method

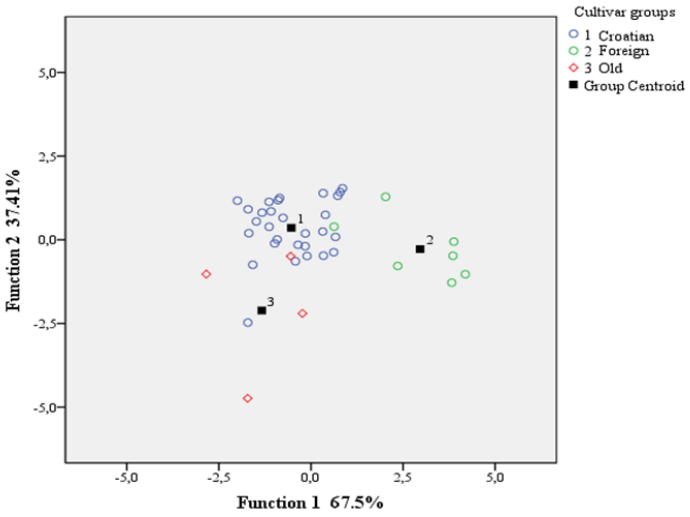

Figure 3. Discriminant analysis of 40 winter wheat cultivars based on agronomic traits

Log determinants were not quite similar but Box's M indicated that the assumption of equality of covariance matrices was not violated (F = 0.184). First two canonical discriminant functions were used in the analysis, accounting for 67.5% (Function 1) and 37.41% (Function 2) of between group variability. According to Wilks' Lambda coefficient Function 1 (0.203) is better predictor of group membership in compare to Function 2 (0.626). Structure matrix revealed that

in Function 1 only predictor days to heading (0.863) was significant predictor of group membership while in Function 2 both, days to heading (-0.505) and average yield (0.924) were significant predictors of group membership (Figure 3). For classification of winter wheat into different groups (Y1 – Croatia, Y2 - Foreign, Y3 – Old) we can calculate each discriminant function and assign the wheat genotype to the group with the highest value, as follows: Y1 = (27,881*av_yield) + (28,988*days_to_heading) - 2920,148; Y2 = (29,520*av_yield) + (30,318*days_to_heading) - 3198,658; Y3 = (24,464*av_yield) + (29,065*days_to_heading) - 2913,670. The cross validated classification showed that overall 87.5% cases were correctly classified.

Variability based on molecular markers

Set of 26 microsatellite primer pairs were used to profile 40 cultivars yielded total of 108 different alleles. Gene diversity (Nei) ranged from 0.255 to 0.814, while PIC value ranged from 0.222 to 0.787 (Supplement 2, Table 3). Largest calculated distance was between cultivars Zlatna Dolina and Lela (d_{ij}=0.98), while most similar cultivars were Super Žitarka and Barbara with distance value of d_{ij}=0.2, results were previously reported by Petrovic et al. (2012a). The set of 26 SSR markers were subsequent to differentiate all cultivars and are in line with origin and pedigree information, except sister lines Slavonija and Golubica. Primer combination SseTC/MseGA produced highest number of polymorphic bands (33), while the first primer combination (SseTC/MseAT) produced lowest (20). On average 27 polymorphic bands was generated with average PIC value of 0.34 (Table 4). Individual dendrograms for all 40 cultivars based on SSR and AFLP data are available in PETROVIC *et al*. (2012a). The dendrogram based on genetic similarity (combined SSR and AFLP data) confirmed high level of genetic diversity within the tested cultivars (Figure 4) calculating average similarity of d_{ij}=0.495. All cultivars could be discriminated except two cultivars Super Žitarka and Barbara with the highest genetic similarity coefficient of d_{ij}=0.811.

Table 4. AFLP primer combinations, number of polymorphic and specific bands, and PIC

AFLP primer combination	No. polymorphic bands (np_i)	No. specific bands	PIC	H_e
SseTC/MseAT	20	1	0.34	0.44
SseGC/MseAT	29	4	0.33	0.42
SseTC/MseGA	33	3	0.35	0.45
SseGC/MseGA	26	5	0.34	0.43

In most cases clear clustering of cultivars by breeding program or registration year was observed. The four major clusters can be distinguished. The cluster I consisted of only Austrian cultivar Antonius followed by cluster 2 with Croatian cultivar Lela. The third cluster can be further divided in two subclusters, 3a consisted of six cultivars and 3b of seven. The biggest fourth cluster (25 cultivars) is divided into two separate subclusters: 4a consisted of only two cultivars (Bezostaja and Divana) and 4b, further distributed into three branches. The first branch IVb1 is consisted of seven cultivars, second, 4b2, of eight genetically very similar cultivars, with average highest genetic similarity compared to all cultivars, Barbara d_{ij}=0.548 and Osječka20 d_{ij}=0.419. The final 4b3 branch is consisted of six cultivars (U1, Bastide, Eurofit, Pipi, Ilirija and Soissons).

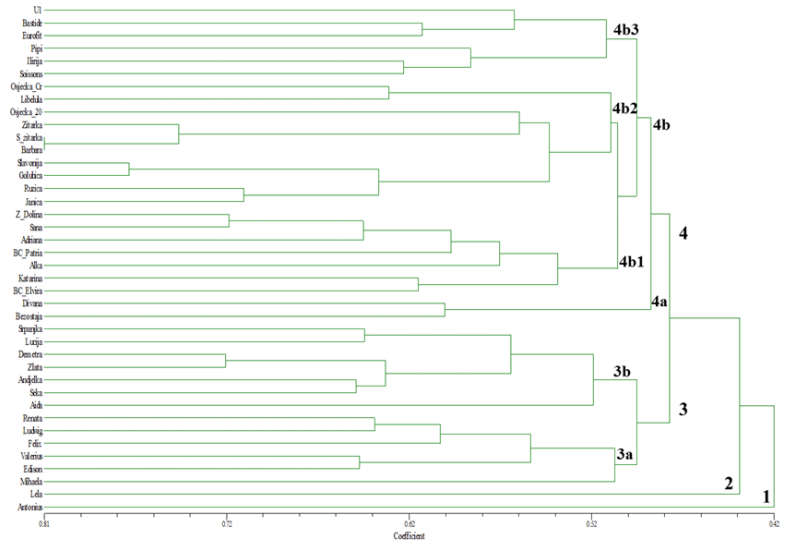

Figure 4. Dendrogram based on Dice genetic similarity matrix based on SSR and AFLP data for 40 winter wheat cultivars using the UPGMA clustering method

Correlation coefficients were calculated between phenotypic and both molecular markers methods separately. Mantel's test showed significant and high correlation between agronomic and morphologic data matrix ($r=0.854$; $P<0.001$), while correlation coefficient between SSR and AFLP data matrix was low ($r=0.191$) but also significant (data not shown). Based on these results we compared two types of genetic diversity criteria and compared their genetic similarity data matrices. No correlation was found between phenotypic and molecular data showing very low correlation coefficient of $r=0.115$.

DISCUSSION

Relatively high average genetic distance ($E=0.149$) between tested cultivars was recorded in this study confirming the large morphologic variability among them. Similar results were obtained by MARIĆ *et al*. (2004) and SALEM *et al*. (2008) founding the high average genetic distance between cultivars distinguishing all cultivars by UPGMA method. Opposite results were reported by MACCAFERRI *et al*. (2007), who determined high level of phenotypic similarity (0.73), distinguishing only very divergent lines. Great variability is mirrored in different goals of each breeding programme in the period of creation, regarding specific features and traits. The cultivars included in this study represent germplasm registered from 1930 till 2008; certain cultivars were (Zlatna dolina) and still are at grown on significant part of the production fields in Croatia (Žitarka, Srpanjka, Soissons). Agro-morphological diversity showed highly variable germplasm, outlining the old Croatian cultivar U1, registered in 1936, as the most diverse. Cultivar U1 was sown until the beginning of 1960 on 50 000 ha, with low fertilizing practice, 450 do 550 grains/m^2 and plant height over 140 cm (DREZNER, 1995). Traits like plant height and ear emergence were the main grouping tools in agro-morphologic dendrogram (Figure 2). Clustering of Croatian cultivars Osječka 20, Slavonija, Demetra, Žitarka, Aida, Alka, Golubica, Katarina, Ružica and Lela reveals high yielding cultivars with semi dwarf plant height, good bread making

quality and lodging resistance (BEDE, 1994; DREZNER, 1995). Other cluster consisting of five Croatian cultivars (Srpanjka, Renata, Pipi, Anđelka and Mihaela) represents very early and early heading cultivars with average plant height of 67 cm (PETROVIĆ, 2011), linking the cultivar Srpanjka, as one of the parents in pedigree of Mihaela, Renata and Anđelka (DVOJKOVIĆ, 2009; PETROVIĆ, 2011). These specific agro-morphologic features were also confirmed by grouping all Austrian cultivars in one cluster. Cultivars like Antonius and Ludwig were specially created in Probstdorf breeding programme for organic field production in Panonic region (LÖSCHENBERGER *et al*., 2008). The main prerequisite for molecular screening is estimation of phenotypic variability based on agronomic and morphologic traits (KOBILJSKI *et al*., 2002). Though statistical differences between variance and covariance were not determined, cross validation proved high similarity with the original distribution of cultivars. Discriminant analysis confirmed selection of agronomic traits that discriminated cultivars putting them in three consecutive groups Croatian (Y1), Foreign (Y2) and Old (Y3). Wilkins lambda coefficients point out that discrimination of tested wheat cultivar groups were mostly influenced by the number of days to heading and yield, very clearly grouping cultivars to Croatian and foreign, whilst group of old cultivars was slightly separated due to yield differences. Cross validation also confirmed the grouping, outlining three old cultivars: U1, Bezostaja, Libellula, while the fourth was clustered in Croatian (new) cultivar group because it was frequently used in pedigree as one of the parent. That small difference across first canonical axis between some Old and Foreign, and new Croatian cultivars also confirm the origin of tested cultivars. The Old cultivars were the source of stability and broad adaptability, while others were used as highyileding cultivars (like French cultivar Soissons). Remaining traits did not contribute to clear discrimination in sense of maximising diversity criteria between dispersion range among groups and between groups. Based on presented agro-morphologic results, and low average similarity between tested cultivars, further genetic diversity estimates included molecular markers. UPGMA analysis based on SSR and AFLP data produced results that compared to those obtained with agro-morphologic traits, more faithfully reflect genetic structure of tested cultivars, confirming the more suitable tool in genetic relationship studies (NOLI *et al*., 1997, 2008; MANIFESTO *et al*., 2001; ROY *et al*., 2004). Results stresses that utilisation of combination of both molecular marker methods were able to distinguish highly related cultivars (Žitarka, Super Žitarka and Barbara) in subcluster 4b2 (Figure 4). The last two originate from crossing 'GO 3135/Žitarka', similar results were also obtained by DVOJKOVIĆ (2009). Estimation of efficiency of different markers system was done by DENČIĆ *et al*. (2015), SSR used were sufficient to distinguish between most of the sister line cultivars. Distinction of cultivars with small genetic distance justifies selection of DNA samples and high polymorphism of used microsatellites and specific AFLP primer combinations in the analysis of genetic diversity of related cultivars which is in accordance with results by MORGANTE and OLIVIERI (1993), PLASCHKE *et al.* (1995) and BANAYI *et al*. (2006). Similar common genetic background was determined in subcluster 4b1, consisted of Slavonija, Golubica, Ružica and Janica, which relates with results reported by PETROVIĆ *et al.* (2012) and by MARIĆ *et al*. (2004) who established great similarity between Slavonija and Golubica in just a few RAPD bands. Some of the tested cultivars were not clustered according to pedigree information probably because of insufficient wheat genome coverage with four AFLP primer combinations and 26 SSRs. Similar discrepancies in 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.

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). Microsatellite markers are more efficient in determination of population structure, grouping the cultivars almost perfectly according to pedigree, while AFLP are more adjusted to detect genetic relation at individual level (NEIGEL, 1997; SCHUT *et al*., 1997). 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. The study by KONDIĆ-ŠPIKA *et al.* (2014) showed that by using a smaller number of highly-polymorphic microsatellites (four primer pairs), genetic variability was determined very effectively, grouping the cultivars according to similarity.

Comparison of different genetic similarity matrices showed correlations between two phenotypic diversity criteria and two molecular diversity criteria. Correlations between genetic diversity by SSR and AFLP data were also reported by MANIFESTO *et al.* (2001). Justification of these correlations shows that results obtained are partially reflected in pedigree of tested cultivars, and that these two methods supplement each other. Nevertheless, no significant correlations with low correlation coefficient r=0.115 was found among matrices based on agro-morphologic and SSR/AFLP data. Very weak correlations between morphologic and molecular data were also reported by SCHUT *et al.* (1997), MANIFESTO *et al*. (2001), MARIĆ *et al*. (2004), MACCAFERRI *et al*. (2007) and ČUPIĆ *et al*. (2009). Differences between these two genetic diversity criteria occur because of bias and environmentally influenced phenotypic traits and possible unreliable pedigree data.

Results confirm significant genetic diversity estimated for tested Croatian and foreign cultivars. Discriminant analysis validated clustering of tested genotypes with 87.5% certainty outlining days to heading and average yield as traits that influence the most to clustering of Croatia, foreign and old cultivars. Distinction of the cultivars with small genetic distance (Super Žitarka and Barbara) justifies the selection of DNA samples and high polymorphism of 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 between tested cultivars. Simultaneously assessing the genetic diversity by molecular and phenotypic traits we could discriminate groups of cultivars with similar phenotype and different genotype and vice versa. These results could be useful indicators and potentially valuable source for selecting parents which can be used in future crossings, and by that creating new and broader genetic base in wheat breeding programs.

ACKNOWLEDGEMENT

Research work is part of the research project 079-0730718-0268 financed by the Ministry of Science and Education, and Research Installation Project PHENOWHEAT (No.2000) financed by Croatian Science Foundation of the Republic of Croatia.

Received October 17th, 2016
Accepted March 25th, 2017

REFERENCES

AHMAD, M. (2002): Assessment of genomic diversity among wheat cultivars as determined by simple sequence repeats. Genome, *45*:646-651.

ALI, Y., B.M. ATTA, J. AKHTER, P. MONNEVEUX, Z. LATEEF (2008): Genetic variability, association and diversity studies in wheat (*Triticum aestivum* L.) germplasm. Pak. J. Bot., *40*(5):2087-2097.

BANAYI, J., P. SZUCS, I. KARSAI, K. MESZAROS, C. KUTI, L. LANG, Z. BEDO (2006): Cultivar identification by molecular markers. Cereal Res. Commun., *34*(2-3):865-870.

BARRETT, B.A., K.K. KIDWELL (1998): AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. Crop Sci., *38*:1261-1271.

BEDE, M. (1994): New trends in wheat improvement. Sjemenarstvo, *11*(1-2):5-13.

BUERSTMAYR, H., M. LEMMENS, L. HARTL, L. DOLDI, B. STEINER, M. STIERSCHEIDER, P. RUCKENBAUER (2002): Molecular mapping of QTLs for Fusarium head blight resistance in spring wheat. I. Resistance to fungal spread (Type II resistance). TAG, *104*:84–91.

CHRISTIANSEN, M.J., S.B. ANDERSEN, R. ORTIZ (2002): Diversity changes in intensively bred wheat germplasm during the 20th century. Mol. Breed., *9*:1-11.

CORBELLINI, M., M. PERENZIN, M. ACCERBI, P. VACCINO, B. BORGHI (2002): Genetic diversity in bread wheat, as revealed by coefficient of parentage and molecular markers, and its relationships to hybrid performance. Euphytica, *123*:273-285.

ČUPIĆ, T., M. TUCAK, S. POPOVIĆ, S. BOLARIĆ, S. GRLJUŠIĆ, V. KOZUMPLIK (2009): Genetic diversity of pea (*Pisum sativum* L.) genotypes assessed by pedigree, morphological and molecular data. J. Food Agric. Environ., *7*(3-4):343-348.

DENČIĆ, S., N. PRŽULJ, R. PROTIĆ, M. SIMOVIĆ, M. SIMOVIĆ, Ž. STOJANOVIĆ, D. PEROVIĆ (1997): Genetic resources in cereals. Contemp. Agric., *46* (1-2):87-98.

DENČIĆ, S., R. DE PAUW, V. MOMČILOVIĆ, A. KONDIĆ-ŠPIKA (2015.): Efficiency of the different marker systems for estimation of distinctness between sister line wheat cultivars. Genetika, *47* (1):219-232.

DICE, L.R. (1945): Measures and amount of ecologic association between species. Ecology, *26*:297-302.

DOYLE, J.J., J.L. DOYLE (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytoch. Bull., *19*:11-15.

DREISIGACKER, S., P. ZHANG, M.L. WARBURTON, B. SKOVMAND, D. HOISINGTON, A.E. MELCHINGER (2004): Genetic diversity among and within CIMMYT wheat landrace accessions investigated with SSR's and implications for plant genetic resources management. Crop Sci., *45*:653–661.

DREZNER, G. (1995): Wheat breeding at the Institute of Agriculture in Osijek. Sjemenarstvo, *12*(1):13-38.

DVOJKOVIĆ, K. (2009): Genetska raznolikost hrvatskih kultivara pšenice. Doktorska disertacija, Sveučilište u Zagrebu, Agronomski fakultet, Zagreb, Hrvatska.

FUFA, H., P.S. BEAZINGER, B.S. BEECHER, R.A., GRAYBOSCH, K.M. ESKRIDGE (2005): Genetic improvement trends in agronomic performances and end-use quality characteristics among hard red winter wheat cultivars in Nebraska. Euphytica, *144*:187-198.

GLASZMANN, J.C., B. KILIAN, H.D. UPADHYAYA, R.K. VARSHNEY (2010): Accessing genetic diversity for crop improvement. Curr. Opin. Plant Biol., *13*:167-173.

GUPTA, P.K., R.K. VARSHNEY, P.C. SHARMA, B. RAMESH (1999): Molecular markers and their application in wheat breeding. Plant Breed, *118*:369-390.

JONES, H., R.J. JARMAN, L. AUSTIN, J. WHITE, R.J. COOK (2003): The management of variety testing reference collection in distinctness, uniformity and stability testing of wheat. Euphytica, *132*:175-184.

KARSAI, I., G.Y. VIDA, S. PETROVIC, E. PETCU, B. KOBILJSKI, S. IVANOVSKA, Z. BEDŐ, O. VEISZ (2011): Assessment of the spatial genotypic and phenotypic diversity present in the various winter wheat breeding programs in Southeast Europe. Euphytica, *186*(1):139-151.

KHLESTKINA, E.K., M.S. RÖDER, T.T. EFREMOVA, A. BÖRNER, V.K. SHUMNY (2004): The genetic diversity of old and modern Siberian cultivars of common spring wheat as determined by microsatellite markers. Plant Breed, *123*:122-127.

KOBILJSKI, B., S. QUARRIE, S. DENČIĆ, J. KIRBY, M. IVEGEŠ (2002): Genetic diversity of the Novi Sad wheat core collection revealed by microsatellites. Cell Mol. Biol. Lett., *7*:685-694.

KONDIĆ-ŠPIKA, A., M. NIČIĆ, LJ. BRBAKLIĆ, D. TRKULJA, D. MILADINOVIĆ, S. JOCIĆ, M. BRDAR, N. HRISTOV (2014): Microsatellites in the analysis of wheat genetic diversity. Genetika, *46* (3):1047-1063.

KOTZAMANIDIS, S., C. IPSILANDIS, A. MAVROMATIS, A. KORKOVELOS, A. LITHOURGIDIS, M. IRAKLI, M. PAPAGEORGIOU, D. ROUPAKIAS (2011): Prediction of pedigree relationships in durum wheat cultivars based on agronomic, morphological and molecular traits. AJCS, *5*(7):809-814.

LAW, J.R., P. DONINI, R.M.D. KOEBNER, J.C. REEVES, R.J. COOKE (1998): DNA profiling and plant variety registration. III: The statistical assessment of distinctness in wheat using amplified length polymorphism. Euphytica, *102*:335-342.

LANDJEVA, S., V. KOZUN, G. GANEVA (2006): Evaluation of genetic diversity among Bulgarian winter wheat (*Triticum aestivum* L.) cultivars during the period 1925-2003 using microsatellites. Genet Resour. Crop Evol, *53*:1605-1614.

LIU, J. (2002): POWERMARKER – A powerful software for marker data analysis. North Carolina State University, Bioinformatics Research Center, Relaigh, NC (http/powermarker.net)

LÖSCHENBERGER, F., A. FLECK, H. GRAUSGRUBER, H. HETZENDORFER, G. HOF, J. LAFFERTY, M. MARN, A. NEUMAYER, G. PFAFFINGER, J. BIRSCHITZKY (2008): Breeding for organic agriculture: the example of winter wheat in Austria. Euphytica, *163*(3):469-480.

MANIFESTO, M.M., A.R. SCHLATTER, H.E. HOPP, E.Y., SUAREZ, J. DUBCOVSKY (2001): Quantitative evaluation of genetic diversity in wheat germplasm using molecular markers. Crop Sci., *41*:682-690.

MACCAFERRI, M., S. STEFANELLI, F. ROTONDO, R. TUBEROSA, M.C. SANGUINETI (2007): Relationships among durum wheat accessions. I. Comparative analysis of SSR, AFLP, and phenotypic data. Genome, *50*:373-384.

MANJARREZ-SANDOVAL, P., T.E. CARTER, D.M. WEBB, J.W. BURTON (1997): RFLP genetic similarity estimates and coefficient of parentage as genetic variance predictors for soybean yield. Crop Sci., *37*:698-703.

MANTEL, N. (1967): The detection of disease clustering and a generalized regression approach. Cancer Res., *27*:209-220.

MARIĆ, S., S. BOLARIĆ, J. MARTINČIĆ, I. PEJIĆ, V. KOZUMPLIK (2004): Genetic diversity of hexaploid wheat cultivars estimated by RAPD markers, morphological traits and coefficients of parentage. Plant Breed. *123*:366-369.

MARTOS, V., C. ROYO, Y. RHARRABTI, L.F. GARCIA DEL MORAL (2005): Using AFLPs to determine phylogenetic relationships and genetic erosion in durum wheat cultivars released in Italy and Spain throught the 20th century. Field Crops Res., *9*:107-116.

MONDINI, L., A. NOORANI, M.A. PAGNOTTA (2009): Assessing Plant Genetic Diversity by Molecular Tools. Diversity, *1*:19-35.

MORGANTE, M., A.M. OLIVIERI (1993): PCR amplified microsatellites as markers in plant genetics. Plant J., *3*(1):01-08.

NAJAPHY, A., R.A. PARCHIN, E. FARSHADFAR (2012): Comparison of phenotypic and molecular characterizations of some important wheat cultivars and advanced breeding lines. AJCS, 6(2):326-332

NIEGEL, J.E. (1997): A comparison of alternative strategies for estimating gene flow from genetic markers. Ann. Rev. Syst., *28*:105-28.

NOLI, E., M.S. TERIACA, M.C. SANGUINETI, S. CONTI (2008): Utilization of SSR and AFLP markers for the assessment of distinctness in durum wheat. Mol. Breed., *22*:301-313.

PLASCHKE, J., M.W. GANAL, M.S. RÖDER (1995): Detection of genetic diversity in closely related bread wheat using microsatellite markers. TAG, *91*:1001-1007.

PETROVIĆ, S. (2011): Genetska različitost germplazme ozime krušne pšenice (*Triticum aestivum ssp. vulgare*). Doktorski rad, Sveučilište Josipa Jurja Strossmayera, Poljoprivredni fakultet u Osijeku. Osijek. Hrvatska.

PETROVIĆ, S., S. MARIĆ, T. ČUPIĆ, G. DREZNER, I. KARSAI (2012): Assessment of genetic diversity in Croatian winter wheat cultivars using SSR and AFLP markers. Poljoprivreda/Agriculture, *18* (2):18-24.

POWELL, W., G.C. MACHRAY, J. PROVAN (1996): Polymorphism revealed by simple sequence repeats. Trends Plant Sci., *1*(7):215-222.

PRADA, D. (2009): Molecular population genetics and agronomic alleles in seed banks: searching for a needle in a haystack? J. Exp. Bot., *60* (9):2541-2552.

RÖDER, M.S., V. KORZUN, K. WENDEHAKE, J. PLASCHKE, M.H. TIXIER, P. LEROY, M.W. GANAL (1998): A microsatellite map of wheat. Genetics, *149*:2007-2023.

ROLDAN-RUIZ, I., F.A. VAN EEUWIJKT, J. GILLILAN, C. DUBREUIL, C. DILLMANN, J.M. LALLEMAND, M. DELOOSEAND, C.P. BARIL (2001): A comparative study of molecular and morphological methods of describing relationships between perennial ryegrass (*Lolium perenne* L.) cultivars. TAG, *103*:1138–1150.

ROHF, F.J. (2009): NTSYS-pc. Numerical Taxonomy System, ver. 2.21c. Exeter Software, Setauket, New York, USA.

ROY, J.K., M.S. LAKSHMIKUMARAN, H.S. BALYAN, P.K. GUPTA (2004): AFLP-based genetic diversity and its comparison with diversity based on SSR, SAMPL, and phenotypic traits in bread wheat. Bioch. Gen., *42*(1/2):43-59.

RUKAVINA, I., S. MARIĆ, T. ČUPIĆ, V. GUBERAC, S. PETROVIĆ (2013): Diversity of ear characteristics of Croatian wheat gernplasm. Poljoprivreda/Agriculture, *19*(1):3-10.

SALEM, K.F.M., A.M. EL-ZANATY, R.M. ESMAIL (2008): Assessing wheat (*Triticum aestivum* L.) genetic diversity using morphological characters and microsatellite markers. World J. Agric. Res., *4*(5):538-544.

DOS SANTOS, T.M.M., F. GANANÇA, J.J. SLASKI, M.Â.A. PINHEIRO DE CARVALHO (2009): Morphological characterization of wheat genetic resources from the Island of Madeira, Portugal. Genet. Res. Crop Evol., *56*(3):363-375.

SHAHRYARI, R., B. MAHFOOZI, V. MOLLASADEGHI, M. KHAYATNEZHAD (2011): Genetic diversity in bread wheat for phonological and morphological traits under terminal drought stress condition. Adv. Environ. Biol., *5*(1):169-172.

SMALE, M. (1997): The green revolution and wheat genetic diversity: some unfounded assumptions. World Develop. *25*(8):1257-1269.

SOLEIMANI, V.D., B.R. BAUM, D.A. JOHNSON (2002): AFLP and pedigree-based genetic diversity estimates in modern cultivars of durum wheat [*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.]. TAG, *104*:350–357.

STĘPIEN, Ł., V. MOHLER, J. BOCIANOWSKI, G. KOCZYK (2007): Assessing genetic diversity of Polish wheat (*Triticum aestivum*) cultivars using microsatellite markers. Gen. Res. Crop Evol., *54*:1499-1506.

STODART, B.J., M. MACKAY, H. RAMAN (2005): AFLP and SSR analysis of genetic diversity among landraces of bread wheat (*Triticum aestivum* L. em. Thell) from different geographic regions. Aust. J. Agric. Res., *56*:691-697.

SCHUT, J.W., X. QI, P. STAM (1997): Association between relationship measures based on AFLP markers, pedigree data and morphological traits in barley. TAG, *95*:1161-1168.

UPOV (1996): Guideline for the distinctness, uniformity and stability wheat (*Triticum aestivum* L.). TG/3/11.

VIEIRA, E.A., F.I.F. DE CARAVALHO, I. BERTAN, M.M. KOPP, P.D. ZIMMER, G. BENIN, J.A.G. DA SILVA, I. HARTWIG, G. MALONE, A.C. DE OLIVEIRA (2007): Association between genetic distances in wheat (*Triticum aestivum* L.) as estimated by AFLP and morphological markers. Genet. Mol. Biol., *30*(2):392-399.

VOS, P., R. HOGERS, M. BLEEKER, M. REIJANS, T. VAN DE LE, M. HORNES, F. JERINA, J. PELEMAN, M. KUIPER, M. ZABEAU (1995): AFLP: a new technique for DNA fingerprinting. Nuc. Acids Res., *23*(21):4407-4414.

YOU, G.X., X.Y. ZHANG, L.F. WANG (2004): An estimation of the minimum number of SSR loci needed to reveal genetic relationships in wheat cultivars: Information from 96 random accessions with maximized genetic diversity. Mol. Breed., *14*:397-406.

ZHANG, X.Y., C.W. LI, L.F. WANG, H.M. WANG, G.X. YOU, Y.S. DONG (2002): An estimation of the minimum number of SSR loci needed to reveal genetic relationships in wheat cultivars. I. Information from large-scale planted cultivars and cornerstone breeding parents in Chinese wheat improvement. TAG, *106*:112-117.

MOLEKULARNI I FENOTIPSKI DIVERZITET IZMEĐU KULTIVARA OZIME PŠENICE

Sonja PETROVIĆ[1], Sonja MARIĆ[1], Tihomir ČUPIĆ[2], A. REBEKIĆ[1], Ivana RUKAVINA[3]

[1]Poljoprivredni fakultet, Univerzitet J.J. Štrosmajer, Osijek, Hrvatska
[2] Poljoprivredni Institut, Osijek, Hrvatska
[3] Hrvatski Centar za Poljoprivredu, hranu i selo, Zavod za seme i rasadničarstvo, Osijek, Hrvatska

Izvod

Upoređivanje rezultata različitih kriterijuma genetske različitosti može da bude veoma korisna oplemenjivačima prilikom odabira roditelja za ukrštanja. Ukupno 40 kultivara ozime pšenice, poreklom iz tri hrvatska oplemenjivačka centra i iz inostranstva, korišćena su u svrhu upoređivanja genetske različitosti na temelju agronomske, morfološke i molekularne procene. Fenotipski podaci dobijeni su korišćenjem deset morfoloških i osam agronomskih svojstava. Molekularni podaci uključivali su 26 SSR i četiri kombinacije AFLP markera, koji su pokrili sva tri genoma pšenice. Veoma velika varijabilnost utvrđena je za agro-morfološka svojstva, posebno za visinu biljke, prinos, tip rasta i datum klasanja. Diskriminantna analiza potvrdila je da visina biljke, prinos i datum klasanja imaju najveći uticaj na grupisanje kultivara pšenice. Četiri AFLP kombinacije i 26 SSR markera proizveli su 108 polimorfnih produkata. UPGMA je prema fenotiskim podacima razvrstala kultivare u četiri, a prema molekularnim podacima u pet različitih klastera, pri čemu se kultivar U1 izdvojio kao poseban klaster u oba dendrograma. Sličnosti temeljene na sva četiri kriterijuma genetske različitosti u proseku se podudaraju s pedigreima korišćenih kultivara pšenice. Nije utvrđena korelacija između fenotipskih i molekularnih podataka ukazujući da treba koristiti obe vrste procene u svrhu utvrđivanja genetske različitosti i obuhvatanja veće varijabilnosti između kultivara.

Primljeno 17.X.2016.
Odobreno 25.III. 2017.