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Original scientific paper

EFFICIENCY OF THE DIFFERENT MARKER SYSTEMS FOR ESTIMATION OF DISTINCTNESS BETWEEN SISTER LINE WHEAT CULTIVARS

Srbislav DENČIĆ[1,*], Ron DePAUW[2], Vojislava MOMČILOVIĆ[1], and Ankica KONDIĆ-ŠPIKA[1]

[1]Institute of Field and Vegetable Crops, Novi Sad, Serbia,
[2]Semiarid Prairie Agricultural Research Centre, AAFC, Swift Current, SK, Canada

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A set of 42 winter wheat cultivars developed from nine populations were evaluated for distinctness. Three marker systems: morphological markers, gliadin allele profiles and microsatellites were used to analyze distinctness between sister cultivars. The morphological based distinctness tests for wheat are based on a crop-specific set of characters that comply with UPOV guidelines. The morphological markers were quite informative although they were not capable to estimate the distances/distinctness between two pair of sister cultivars Evropa 90 and Evropa and Novosadska rana 3 and Novosadska rana 2. The gliadins allele profiles were the least efficient to estimate distinctness between sister line cultivars. This system was not capable to distinguish even cultivars developed from different populations. Sister line wheat cultivars were fingerprinted with 19 wheat microsatellites markers. A total of 106 alleles were detected at 19 wheat microsatellite loci, resulting in an average allele number per marker of 5.6. The number of markers was sufficient to distinguish among most sister line cultivars. Only one pair of sister cultivars, Lozničanka and Kosovka, derived from the same cross could not be distinguished. Comparing all marker systems to evaluate distinctness of sister line wheat cultivars the most efficient was microsatellite markers while gliadin allele profiles was the least efficient. Correlations between matrices based on pedigree data and morphological marker, gliadin profiles and microsatellites were significant but not large.

Key words: Distinctness, Gliadin allele profiles, Morphological markers, Microsatellite markers, Sister line cultivar, Wheat

Corresponding author: Srbislav Denčić, Institute of Field an Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia; Phone: 381214898233; Fax: 381214898227 E-mail: srbislav.dencic@nsseme.com

INTRODUCTION

Differences between two living organisms can be assessed on the basis of phenotypic characteristics, biochemical characteristics, loci, alleles, and the end of the nucleotide sequence of individual alleles. The generally accepted categories of markers are morphological, biochemical and molecular to distinguish between two biological entities.

Most agricultural species, including wheat, generally exist as a number of genetically distinct cultivars but with varying degrees of relatedness. Today, cultivar identification is usually carried out using morphological and physiological markers. Morphological markers are the most frequent type of descriptors used for registration of cultivars and for awarding Plant Breeders` Rights (PBR) based on distinctness, uniformity and stability (DUS) criteria. The criteria for distinctness, uniformity and stability are established by guidelines determined by The International Union for the Protection of New Varieties of Plants (UPOV 2009). New candidate cultivars have to be distinct from all cultivars "in common knowledge" by the expression of at least one character and also be sufficiently uniform and stable with respect to the characters used to demonstrated distinctness. Since many of the morphological characters for DUS testing are multigenic, quantitative or continuous characters, their phenotypic expression can be altered by environmental conditions. The fact is that morphological characters are limited in number, they are not reliable and their relationship to other data may be influenced by environmental conditions and epistatic interactions, and their genetic control is largely unknown. Furthermore as the number of cultivars released increases over time it is becoming more challenging to compare each new submitted cultivar against all cultivars of common knowledge. Furthermore, morphological markers established by guidelines determined by UPOV for wheat (UPOV 2009) are internationally accepted procedure for registration of new cultivars.

In wheat, gliadins which are alcohol-soluble seed storage proteins, show high level of inter-varietal polymorphism (METAKOVSKI, 1991). The gliadin polymorphism is controlled by multiple alleles of six main unlinked loci: *Gli-A1, Gli-B1, Gli-D1, Gli-A2, Gli-B2,* and *Gli-D2,* located on the short arms of chromosomes of homoeological groups 1 and 6. Variability of the *Gli* loci (*Gli-1 and Gli-2*) is characterized by 120 alleles, whose combination lead to high levels of variation of seed storage proteins (METAKOVSKI, 1991). Combinations of different alleles at six primary loci ensure a great diversity and therefore make it possible to distinguish a number of wheat cultivars (genotypes) in terms of gliadin allele composition (JONES *et al.* 2003; KONAREV *et al.* 2005; NOVOSELSKAYA-DRAGOVICH *et al.* 2011). Gliadins currently are not used for DUS testing, although their value for wheat cultivar identification is established and internationally accepted.

Currently, molecular marker techniques are widely applied in germplasm characterization to assist and complement phenotypic assessment. Their main advantage over morphological markers is that variation can be measured directly at the DNA level, which makes them insensitive to environmental influence. Over the last few decades, a number of different molecular marker systems have emerged (COLLARD *et al.* 2005; SPOONER *et al.* 2005). Marker systems which have been used for assessing genetic diversity in wheat include restriction fragment length polymorphism (RLFP) (KIM *et al*. 2000), random amplification of polymorphic DNA (RAPD) (MALAKI *et al*. 2008), sequence tagged site (STS) markers (CHEN *et al*. 2003), amplified fragment length polymorphism (AFLP) (MALAKI *et al*. 2008) and simple sequence repeats (SSR) also called microsatellite (KOBILJSKI *et al.* 2002; RÖDER *et al.* 2002; NEUMANN *et*

al. 2011; BRBAKLIĆ *et al.* 2015), through to markers consisting of smaller pieces of nucleotide sequences such as sequence characterized amplified region (SCAR); single nucleotide polymorphism (SNP); sequence tagged site (STS) (COLLARD *et al.* 2005). SSR markers can detect high levels of polymorphism even in closely related wheat cultivars (NOLI *et al.* 2012). These various simple and or single nucleotide markers can be used to construct a large marker database for wheat cultivars (RÖDER *et al.* 2002). Another novel approach to distinguish between individuals is diversity arrays technology (DArT) which provides a high-throughput whole-genome genotyping platform for the detection and scoring of hundreds of polymorphic loci without any need for prior sequence information (SCHOUTEN *et al.* 2012).

Morphological markers established by guidelines determined by UPOV for wheat (UPOV 2009), despite their drawbacks, such as, impact of environmental conditions, setting up field trials and evaluation of the characteristics throughout the growing season, remain as the main criteria for accessing distinctness between cultivars in the process of registration of varieties. On the other hand, molecular markers such as SSRs can be highly effective in assessing genetic distinctness between different wheat cultivars (DREISIGACKER *et al.* 2004; BANAYI *et al.* 2006; STĘPIEŃ *et al.* 2007;).

The main types of closely related cultivars in wheat are: (i) sister lines that derive from the same cross (WARBURTON *et al.* 2002); (ii) multilines which are mixtures of pure lines that have different genes for a particular trait backcrossed into a common parent (BRUNNER *et al.* 2012), and (iii) essentially derived cultivars, which derive from an original cultivar (NOLI *et al.* 2012). It is logical that assessing the degree of distinctness between closely related cultivars is more difficult compared to unrelated cultivars.

The objective of this study was to assess the effectiveness of morphological, biochemical (gliadins) and molecular (SSR) markers in estimating distinctness among sister line wheat cultivars developed from the same population.

MATERIALS AND METHODS

Plant materials

The plant material used in this study included 42 winter hexaploid wheat cultivars developed by the Institute of Field and Vegetable Crops in Novi Sad, Serbia. These cultivars were developed from nine populations, forming nine groups of sister line cultivars (Table 1). The cultivars have been registered in former Jugoslavija or in Serbia in the period from 1962 to 2009.

Each year (2007- 2011) all cultivars were planted by utilizing the method spike/row, on the experimental field of the Institute of Field and Vegetable Crops in Novi Sad. Three hundred spikes per cultivar were collected, threshed individually, and planted to establish 300 rows. During the growing season atypical rows were removed: just before heading, milking stage and few days before harvest. The detection of atypical rows is very rare.

Morphological characteristics

Morphological characteristics are assessed at the optimal stage for each characteristic according to UPOV guidelines for wheat, TG/3/11 (UPOV, 2009). Morphological data were scored as either present (1) or absent (0) to create binary matrices. The morphological characters were scored using a method developed by BENESI (2002) in which a trait with more than two

categories of description, for example lower glume: shoulder shape, ranges from 1 – sloping, 3 - slightly sloping, 5 – straight, 7 – elevated, 9 – strongly elevated with second point present. If a cultivar had slightly sloping glume shoulder, 1 was scored and the rest of the attributes were scored as 0.

Table 1. Population and parentage from which sister cultivars derived and their generation of separation and year of registration

Population	Pedigree	Name of cultivar	Generation	Year of
I	Heine 7/129 Genus	Bačka	F_5	1964
		Panonija	F_5	1964
		Novosadska 32	F_5	1967
		Dunav	F_6	1968
II	Fortunato*2/Red coat	Sava	F_5	1970
		Biserka	F_6	1972
		Drina	F_5	1973
III	Bezostaja 1/NS 262// Mironovska 808 /3/NS 435	Novosadska rana 1	F_6	1975
		Novosadska rana 2	F_7	1975
		Novosadska rana 3	F_7	1975
		Novosadska rana 4	F_7	1978
IV	Argelato/KS56-R-386// 2*Bezostaja 1 /3/NS 422	Bečejka	F_5	1976
		Zrenjaninka	F_5	1976
		Nizija	F_6	1979
V	NS 646/Bezostaja 1//Aurora	Jugoslavija	F_6	1980
		Kozara	F_6	1980
		Zelengora	F_5	1982
		Kolubara	F_5	1984
		Pomurka	F_5	1984
		Apatinka	F_6	1986
VI	NS 646/Bezostaja 1// Aurora /3/Partizanka	Partizanka niska	F_6	1984
		Banatka niska	F_5	1985
		Staparka	F_6	1986
		Jednota	F_6	1987
		Subotičanka	F_5	1987
		Rodna	F_6	1988
		Tanjugovka	F_7	1988
		Kosovka	F_7	1988
		Lozničanka	F_7	1988
		Rudničanka	F_6	1989

VII	Novosadska rana 2/Aurora //Nova Banatka	Somborka	F_6	1986
		Pančevka	F_5	1987
		Panonka	F_6	1988
VIII	Talent/Novosadska rana 2	Evropa	F_8	1988
		Francuska	F_6	1988
		Italija	F_7	1989
		Evropa 90	F_8	1990
		Novosadska rana 6	F_6	1991
		Atina	F_6	1993
IX	Rodna/Pobeda//Milica	Janja	F_6	2005
		Srma	F_6	2006
		Biljana	F_7	2009

The trait "seasonal type" was not included since all cultivars were winter type. A few days before harvest, 400-500 spikes of each variety were collected, analyzed in a laboratory, atypical types were removed, and 10 spikes were selected for evaluation of morphological characteristics. Three hundred spikes were threshed for planting the following year.

Gliadins

Electrophoresis of gliadins was conducted as described by METAKOVSKI (1991). Identification of alleles was done in accordance with the protocol reported by METAKOVSKI (1991). The gliadin allele profiles of the cultivars were done at the Vavilov Institute of General Genetic, Russian Academy of Sciences, Moscow, Russia, on ten seeds per cultivar, in 2009 and 2010 years. Seed samples were provided by the Institute of Field and Vegetable Crops from Novi Sad, Serbia.

Microsatellites

Total genomic DNA was extracted from leaves, as described in DOYLE and DOYLE (1990). For molecular assessment of wheat cultivars 19 wheat microsatellites and one secalin-specific marker were selected in accordance with suggestions and results of RÖDER *et al.* (2002) (Table 2).

Polymerase Chain Reactions (PCR) were carried out in 20 µl reaction mixtures, each µl containing 1xPCR buffer, 2 mM MgCl2, 0.2 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 0.5 µM of each primer (fluorescently labeled forward and unlabeled reverse primer), 0.1 units of Taq polymerase (Applied Biosystems) and 1.25 ng of template DNA. After 5 min at 94°C, 45 cycles were performed with 30 sec at 94°C, 30 sec at either 52, 55, 57, 60, 62°C (depending on individual microsatellite), 30 sec at 72°C, and a final extension step of 10 min at 72°C. Reactions were performed in PCR System 9700 gold (Applied Biosystems). Amplified fragments were detected by capillary electrophoresis on ABI Genetic Analyzer 3130 and analyzed using the GeneMapper Software version 4.0.

Table 2. Description of microsatellite markers employed, the number of alleles detected, and polymorphism information content (PIC) values

Marker	Chromosome	Variation in product size (bp)	Predominating allele (bp) and its frequency	Total no. of alleles	PIC
Xgwm3	3D	71-75	73/75 (37.2)	5	0.66
Xgwm18	1B	null, 186-196	186 (72.1)	3	0.44
Xgwm46	7B	null, 167-177	173 (42.3)	7	0.75
Xgwm95	2A	114-118	118 (55.8)	3	0.53
Xgwm155	3A	null, 134-150	138 (34.1)	8	0.79
Xgwm190	5D	202-214	212 (75)	4	0.42
Xgwm261	2D	null, 175-192	192 (79.1)	4	0.35
Xgwm325	6D	null, 133-143	137 (40.0)	7	0.74
Xgwm357	1A	117-121	119 (60.5)	3	0.51
Xgwm389	3B	null, 98-136	136 (31.3)	9	0.79
Xgwm408	5B	null, 145-191	159 (40.0)	8	0.80
Xgwm 437	7D	81-113	87 (27.3)	11	0.83
Xgwm 458	1D	null, 107-111	107 (51.2)	4	0.64
Xgwm513	4B	141-143	143 (65.1)	2	0.45
Xgwm577	7B	null, 126-207	161 (44.2)	6	0.69
Xgwm619	2B	133-158	139 (33.3)	5	0.77
Xgwm631	7A	null, 189-206	191 (31.8)	8	0.81
Xgwm680	6B	null, 105-119	119 (67.4)	4	0.47
Taglgap	1B	null, 195-239	217 (88.9)	5	0.67
Total	19			106	
Average				5.6	0.64

Data analysis

Pedigree data analysis and clustering of sister line cultivars was performed by Peditree software -program (VAN BERLO and HUTTEN, 2005). The main feature of the program is analysis of a complete pedigree tree structure. This is done recursively i.e. if genotype A has parents B and C, the program will look up the parents of B and C, and so on, as far as information is available. Our wheat sister line cultivars database pedigree has 9 to 13 levels deep.

Data of morphological characteristics, gliadin allele profiles and SSRs were transformed to binary matrices. Subsequently, Jaccard`s coefficients (JACCARD, 1912) were calculated and used to construct dendograms based on unweighted pair group method with arithmetical average (UPGMA) clustering. The reliability of the morphological, gliadin and SSR dendrogrames were tested by bootstrap analysis. Bootstrap analysis, which is a method for determining confidence limits of clusters produced by UPGM-based dendrograms, was performed using the FreeTree programme (PAVLICEK *et al.* 1999). In this study the threshold of 50% was used to assess the grouping of taxa statistically significant (CAPO-CHICHI *et al.* 2001). Correspondence between pairs of matrices was tested with Mantel test (MANTEL, 1967).

PCR data analysis was performed using Microsoft Excel-Software, and the polymorphism information content (PIC) was calculated according to ANDERSON *et al.* (1993).

RESULTS

Nine groups of sister line cultivars (Table1) were assessed for distinctness by morphological characteristics, gliadin allele profiles and microsatellites.

According to pedigree data all nine groups of sister lines cultivars were clearly separated (Fig. 1).

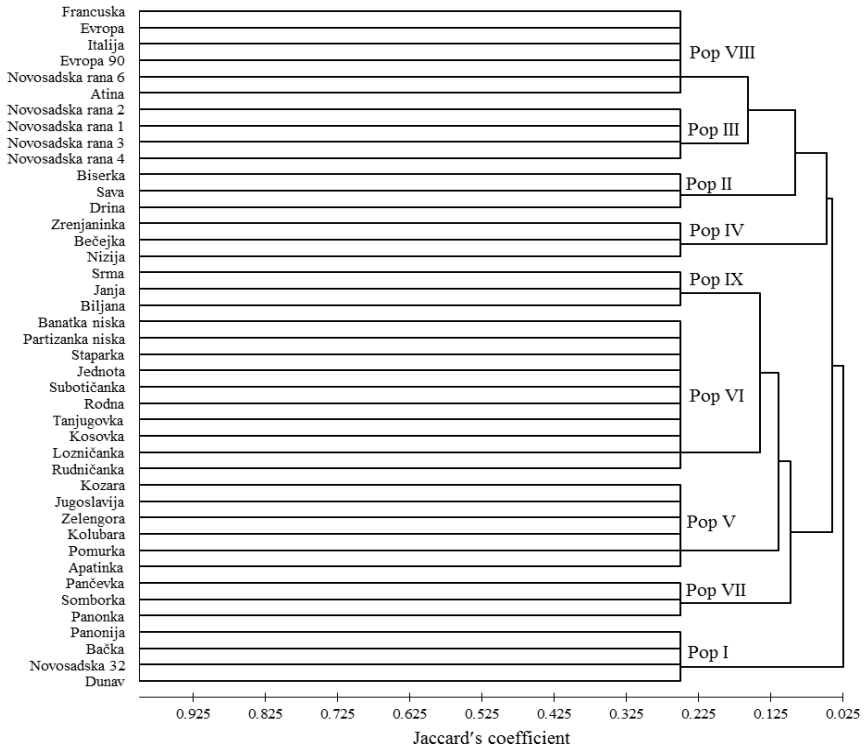

Figure 1. Dendogram of 42 sister cultivars based on pedigree data.

Morphological characteristics

Cluster analysis based on morphological characteristics separated the 42 sister lines cultivars into 7 clusters: A, B, C, D, E, F and G (Fig. 2). The clusters based on morphological characteristics generally disagree with the pedigree (genetic background) of the cultivars. The morphological characteristics were not capable to estimate the distances/distinctness between the pair of sister cultivars Evropa 90 and Evropa from population number VIII and Novosadska rana 3 and Novosadska rana 2 from population number III. In cluster C twenty cultivars from eight populations are included. Cultivars Sava and Panonija which are not related to each other (belong to different pedigree groups) exhibited the greatest distance to all other cultivars (Fig. 2).

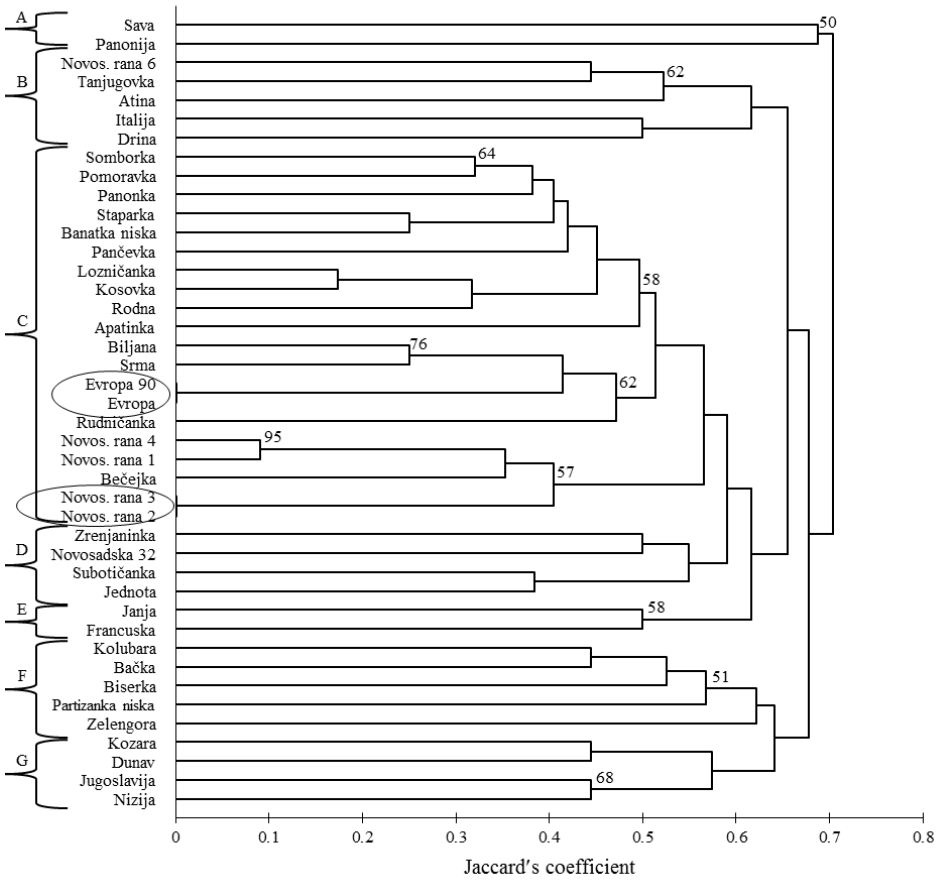

Figure 2. Dendogram of 42 sister cultivars based on morphological markers. Number at node indicate bootstrap values in percentage

Gliadins

The gliadin allele profiles were the less efficient to estimate distinctness between sister line cultivars. According to gliadin alleles, two cultivars Novosadska rana 4 and Novosadska rana 3 from population number III were identical, as well as two cultivars (Zelengora and Jugoslavija) from population number V, three cultivars (Kosovka, Rodna and Lozničanka) from population number VI, two cultivars (Atina and Italija) from population number VIII, and three cultivars (Srma, Janja and Biljana) from population number IX. Furthermore cultivar Banatka niska from population number VI was identical with two cultivars from population number V. Cultivar Bečejka was clearly separated from all other cultivars (Fig. 3). Only 27 (64%) out of 42

cultivars were characterized by unique gliadin composition, and therefore permitted distinguishing among these cultivars.

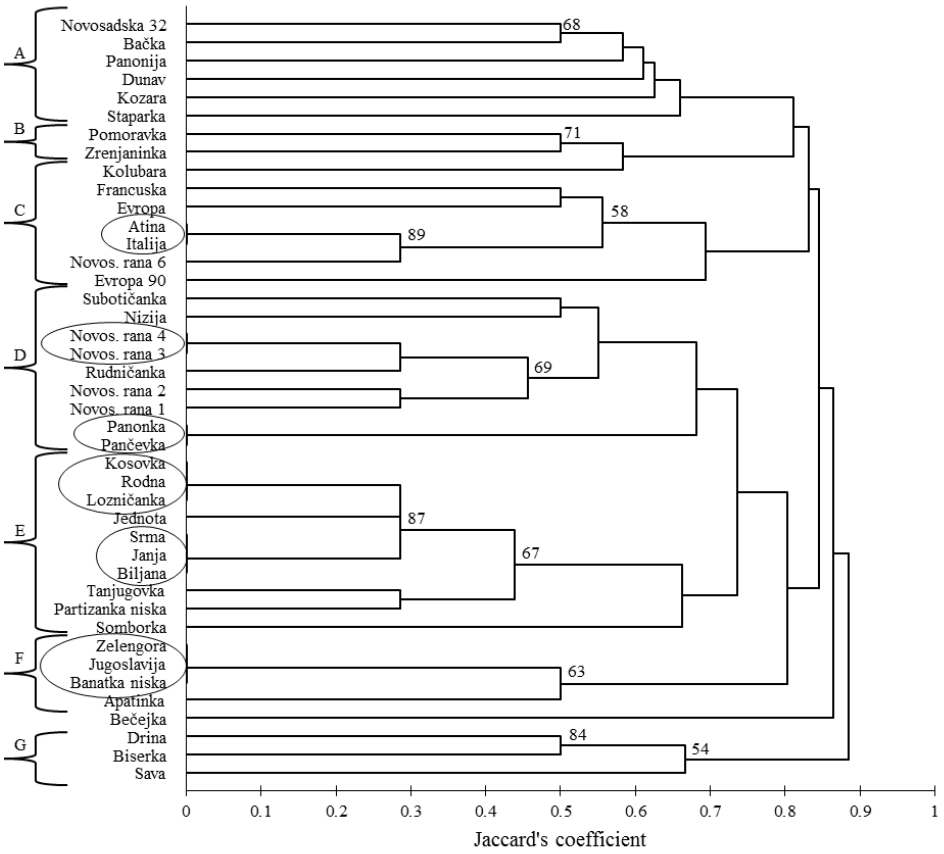

Figure 3. Dendogram of 42 sister cultivars based on gliadin alleles. Number at node indicate bootstrap values in percentage

Microsatelites

The sister line wheat cultivars were fingerprinted with 19 wheat microsatellite markers and the secalin-specific (*Secal*) marker. A total of 106 alleles were detected at 19 wheat microsatellite loci, resulting in an average allele number per marker of 5.6. The presence of the 1B-1R wheat-rye translocation was verified with the secalin-specific primers. The results have shown that PCR product of 96 bp (*Secal)* was detected in 40 out of 42 cultivars, indicating the presence of the 1B-1R translocation in most of the tested varieties .

The number of alleles for the individual markers ranged from two (*Xgwm513*) to 11 (*Xgwm437*). For 12 markers, null alleles were detected. The values of polymorphism information content (PIC) ranged from 0.35 for *Xgwm261* to 0.83 for *Xgwm437*. An average PIC value of 0.64 for all markers indicated a high level of detected polymorphism (Table 2).

The dendogram, which was constructed on the basis of binary molecular data, clearly discriminated all varieties from each other, except two sister varieties Lozničanka and Kosovka from the population number VI (Fig.4). The dendogram divided into 9clusters. All sister cultivars from populations number II grouped in cluster (C) as well as sister cultivars from population number IX (cluster D), indicating compatibility with pedigree based clusters (Fig. 4).

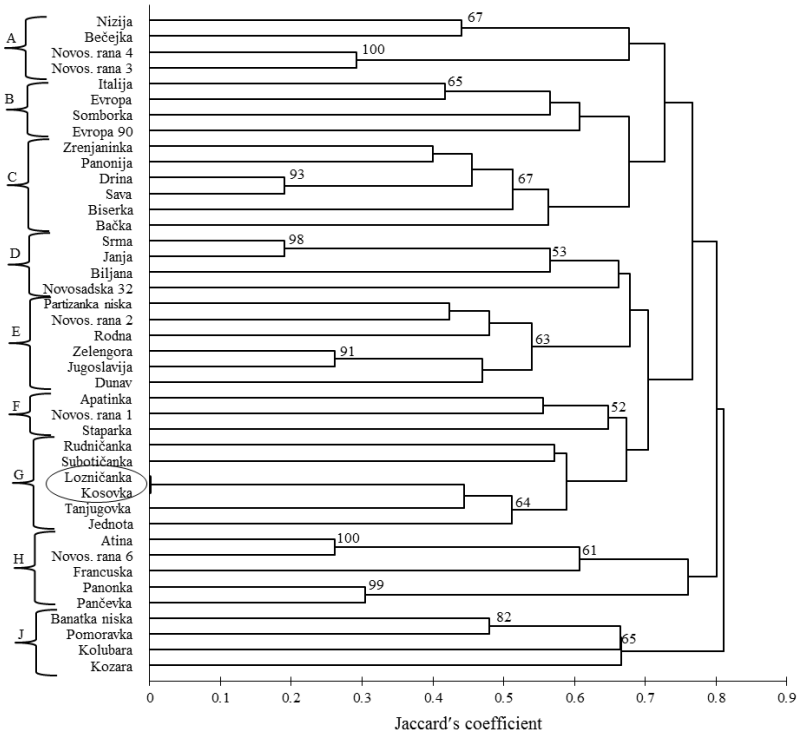

Figure 4. Dendogram of 42 sister cultivars based on microsatellite markers. Number at node indicate bootstrap values in percentage

Correlations

Mantel`s test showed weak but significant correlations between pedigree data on one side and matrices obtained by SSR markers (r =0.293, P≤ 0.05), gliadin profiles (r=0.416, P≤ 0.01), and morphological markers (r=0.244, P≤ 0.05). Significant but also weak correlations (0.269, P≤ 0.05) were detected between SSR and gliadin matrices.

DISCUSSION

In this paper, the distinctness between sister line wheat cultivars were assessed using: morphological markers, gliadin allelic profiles and microsatellites. Using morphological markers only, distinctness between the sister cultivars Evropa 90 and Evropa as well as between sister cultivars Novosadska rana 3 and Novosadska rana 2 was not possible (Fig. 2). How then were these varieties accepted to be registered? At thetime when they were registered, morphological markers specified by UPOV were not used as criteria. Wheat cultivar registration was based on differences in properties such as grain yield, bread making quality parameters, plant height, test weight, 1000 kernel weight, etc. The fact that these varieties are not identical was confirmed by differences between them for gliadin allelic profiles (Fig. 3) and microsatellites markers (Fig. 4). RUSSELL *et al.* (2000) also confirmed that SSR analysis provide better resolution and discrimination between individual cultivars compared to morphological data.

The different gliadins alleles at the six main loci theoretically give numerous combinations. However, a gliadin based marker system was the least efficient to evaluate distinctness between sister cultivars. Futhermore, varieties from different populations Zelengora, Jugoslavija and Banatka niska were in identical clusters (Fig. 3). The reason for the lower efficiency of gliadin allele profiles in determining distinctness between varieties is likely that in most populations there are a few common parents (Bezostaja 1, Aurora and Novosadska rana 2). For example, Russian cultivar Bezostaja 1 was one of the parent on first, second or third pedigree level in 7 out of 9 populations. Furthermore, gliadin seed storage proteins interact with glutenins to form gluten whose elasticity determines suitability to make a diversity of wheat based products. The functionality of gliadin results in preferential alleles being selected and perpetuated in cultivars.

Gliadin profile based classification showed the highest compatibility with pedigree data. Sister cultivars from populations I, VIII, III, IX and II grouped in separated clusters A, C, D, E and G respectively (Fig. 3). The Mantel test confirms compatibility between gliadin and pedigree matrices by the highest correlation coefficient among the various parameters to measure distinctiveness.

Nineteen microsatellite markers from most of the wheat chromosomes were used. The number of markers was sufficient to distinguish between most of the sister line cultivars. Only one pair of sister cultivars Lozničanka and Kosovka derived from population VI could not be distinguished (Fig.4). Reason for this is probably that cultivars bred from the same population differ only in a limited number of genomic regions and such differences cannot be identified with a small number of markers. In this case the two sisters might have derived from a nearly inbred line, F_7. For sister line cultivars derived from a near inbred line or other type of genetically similar entities a large number of polymorphic markers from different chromosomal regions are required to detect differences. Preferably the SSR markers should be randomly distributed across all chromosomes and not be linked to genomic regions with high breeding value.

For the marker *gwm261* four alleles were observed with highest dominance of 192-bp allele (79.1%). The SSR marker, *gwm261*, is tightly linked to the dwarfing gene *Rht8* on chromosome 2DS, a chromosomal region also carrying the gene *Ppd D1* for photoperiod insensitivity (LIU *et al.* 2014). All 42 cultivars were characterized by semi dwarf stature and day length insensitivity. These two traits are the most important for wheat adaptation in central and

southern part of Europe. The dominant presence of *Rht8* in Novi Sad`s winter wheat varieties previously reported by WORLAND *et al.* (1988).

All correlations between pedigree data and all other marker systems as well as between gliadins and SSR markers were low but statistically significant. The highest correlation, but still relatively small (r = 0.42) was observed between matrices based on pedigree data and gliadins profiles.

It is very likely that the effect of selection during the development of the cultivars contributed to the lack of a higher correlations between pedigree data and the different marker systems.

Comparing all used marker systems to evaluate distinctness between sister line wheat cultivars the most efficient was microsatellite markers while gliadin allele profiles was the least efficient.

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EFIKASNOST RAZLIČITIH MARKER SISTEMA U OCENI DISTINKTNOSTI IZMEĐU SESTRINSKIH LINIJA PŠENICE

Srbislav DENČIĆ[1,*], Ron DePAUW[2], Vojislava MOMČILOVIĆ[1] i Ankica KONDIĆ-ŠPIKA[1]

[1]Institut za ratarstvo i povrtarstvo, Novi Sad, Srbija
[2]Semiarid Prairie Poljoprivredni istraživački centar, AAFC, Swift Current, SK, Kanada

Izvod

Cilj rada je bio da se testira distinktnost (različitost) između 42 sorte ozime pšenice nastale iz 9 različitih ukrštanja. Korišćena su tri sistema markera: morfološki markeri, glijadinski alelni profili, i mikrosateliti (SSRs). Kao morfološki markeri korišćeni su UPOV deskriptori namenjeni za pšenicu. Iako su ovi markeri veoma informativni ipak pomoću njih nije mogla da se ustanovi distinktnost/različitost između dva para sestrinskih sorti Evrope 90 i Evrope te Novosadske rane 3 i Novosadske rane 2. Glijadinski alelni profili su bili najmanje efikasni u oceni distinktnosti između sestrinskih sorti. Ovaj tip markera nije čak mogao da ustanovi razliku ni među pojedinih sorti nastalih iz različitih ukrštanja. Fingerprint sestrinskih sorti je urađen sa 19 mikrosatelitskih markera. U 19 mikrosatelitskih lokusa identifikovano je 106 alela što je u proseku 5,6 alela po markeru. Korišćeni broj makera je bio dovoljan da utvrdi dinstinkciju između svih sestrinskih sorti izuzev jednog para (Lozničanka i Kosovka). Upoređujući sve marker sisteme u oceni dinstinkcije (različitosti), između sestrinskih sorti pšenice, proizilazi da su najefikasniji bili mikrosateliti a najmanje efikasni glijadinski profili.

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