Original Scientific Paper
10.7251/AGRENG1701075D
UDC 633.1:631.5

GENETIC VARIABILITY OF THE CEREAL (POACEAE) GERMPLASM COLLECTION MONITORED BY PROTEIN AND MOLECULAR MARKERS

Miodrag DIMITRIJEVIĆ[1*], Sofija PETROVIĆ[1], Borislav BANJAC[1], Goran BARAĆ[1], Aleksandra YURIEVNA DRAGOVIĆ[2], Alexander MIHAILOVICH KUDRYAVTSEV[2], Desimir KNEŽEVIĆ[3]

[1]Faculty of Agriculture, University of Novi Sad, Serbia
[2]Vavilov Institute of General Genetics Russian Academy of Sciences, Moscow, Russia
[3]Faculty of Agriculture, University of Priština, Lešak, Serbia
*Corresponding author: mishad@polj. uns. ac. rs

ABSTRACT

All the new challenges that food production has been experienced, requires adequate response not only in wide agricultural practice, but also in modern breeding programs. Broadening genetic variability is indispensable to meeting the border set by climatic changes, land erosion, human population growth, and sustainable agriculture. Gathering genetic variability, forming and examining genetic collection are integral part of the task. A germplasm collection of 220 entries, consisting of cereal (*Poaceae*) genotypes has been formed. Genetic variability of wheat (*Triticum* sp.), barley (*Hordeum* sp.), and goat grass (*Aegilops* sp.), samples from the germplasm collection was analysed using gliadin blocks as protein markers, as well as, Random Amplified Polymorphic DNA (RAPD) markers. Gliadin allelic variation was notable within *Triticum* sp. samples, revealing not only genetic divergence, but also the origin and the structure of populations. Genotype variation and structure of populations of *Hordeum* sp. was followed by hordein allelic variation. Sampled population expressed heterogeneity from two to five genotypes per population sample. Landraces, old and modern varieties were separated in collection using hordein allelic variation, as well. A small, pilot, investigation was conducted on *Aegilops* sp. polymorphism using RAPD primers. Number and percentage of polymorphic loci, effective number of alleles, expected heterozygosity and Shannon's information index were used to estimate genetic variation.

Key words: *biodiversity, wheat, barley, Aegilops* sp., *genetic marker.*

INTRODUCTION

Genetic resources, in the part of wild relatives, have always been important for broadening genetic variability in contemporary breeding programs, as well as, for food production for special requrements. Green revolution, the industrialization of primary food production, would not be possible without gene introduction from a

wide range of local populations, wild relatives and other plant genetic resources that changed the phenotype of cultivars dramatically (Hedden, 2003). Finally, intergenus hybridization that spontaneously occurred in nature has led to cultivated plants we are using in food production of today. Bread wheat (*Triticum aestivum ssp. vulgare* L.) has, for example, only one third of its genome originated from direct ancestors of *Triticum* genus (Arrigo *et al*., 2011; Matsuoka, 2011). Two third of wheat genome came from wild relatives belonging to *Agilops* genus that are absolutely non-edible. The "secret" of gene recombination of genotypes qualitative and quantitative non-suitable as food that gave the revolutionary outcome in hexaploid wheat genome is still the subject of investigations (Gogniashvili, 2016). However, a task that modern age and the future set up to agriculture requires new "green revolution". In order to take a food production to higher level a new plant ideotype is required (Petrović et al.,2008). Hence, new genetic variability requires new gene recombination and new sources of genetic variability (Dimitrijević and Petrović, 2015). That is the reason genetic resources have come in focus in the last decades (Monneveux *et al*., 2000; Colmer *et al*., 2006; Dimitrijević *et al.*, 2011). An *ex situ* conservation gene bank has been established consisting of about 200 entries of landraces and wild relatives in cereals, collected at SW Balkans during the first decade of XXI Century (Petrović et al., 2006).

The aim of the article is to present results of genetic variability study using protein and molecular marker analysis.

MATERIALS AND METHODS

The germplasm collection has been gathered for several years in Southern Adriatic, consiting of cereal landraces and wild relatives Aegilops sp. (goat grass). Samples of Triticum sp.,and Hordeum sp. collection has been examined for genetic variation using gliadin blocks. The screening and the results analysis was done at the Vavilov Institute of General Genetics, Russian Academy of Science, Moscow. Gliadin seed storage proteins were extracted from single seed wheat meal by 70% ethanol for 30min at 400oC. Gliadin separation was conducted using 8. 33% polyacrylamide gel electrophoresis (12. 5g acrilamid, 0. 62g N, N'-methylenebisacrylamide, 0. 15g ascorbin acid, 200µl 10% ferosulfate heptahydrate, diluted in 150 ml Al-lactate buffer pH=3. 1) after Novoselskaya et al. (1983), and Metakovsky and Novoselskaya (1991). Genetic variation in barley samples was followed by electrophoretic spectra of storage protein grains - hordein (HRD), using Pomortsev and Lyalina (2003) method. A discontinuous genetic variation of Aegilops sp.,was examined using 6 RAPD primers. DNA extraction has been done using the method of Somma (2004). In order to test amplification profiles for polymorphism, readability and reproducibility, six decamer (10 nucleotides length) primers from ROTH®GmbH kits were tested. PCR was carried out in a 25-µL reaction volume containing 2. 5 µL buffer; 0. 2 mM of each dNTP; 0. 5 µM of primer; 2 units of Taq polymerase (Fermentas) and 30 ng of DNA. Reactions were performed in Tpersonal PCR (Biometra) and Mastercycler ep gradient S (Eppendorf) thermocyclers with amplification profile: denaturation at 94°C for 4

min, followed by 40 cycles with 94°C for 2 min, 36°C for 1 min and 72°C for 2 min, with final elongation on 72°C for 10 min. PCR products were separated on 1. 2% or 1. 7% agarose gels containing 0. 005% ethidium bromide and visualized under UV light. Each fragment amplified using RAPD primers was treated as binary unit character and scored "0" for absence and "1" for presence. Estimation of genetic variation was carried out by using the POPGENE software package version 1. 32 for calculation of the following parameters: number of polymorphic loci and their percentage, effective number of alleles per loci, expected heterozygosity based on allelic frequencies and Shannon's index of phenotypic diversity based on marker frequencies. Calculations of all parameters were done separately for each species, and overall for all samples.

RESULTS AND DISCUSSION

Genetic variation within samples of *Triticum sp*. Several samples chosen from germplasm collection have been screened for genetic variation using gliadin loci. The sample arbitrary labeled as No. 1, represents the spring wheat (*T. aestivum*) landrace collected in Western Montenegro, the area of Piva River, municipality of Plužine, in the village of Zabrđe(43, 13°N+18, 76°E) at an altitude of 1381m. That wheat has been traditionally sown for decades using seed inherited from the ancestors. Harvest is in the middle of August. The host claimed that the yield was low, but the quality of flour and bread is quite good. According to gliadin allelic formula, this landrace appeared to be one of the oldest and unique genotype. The allelic variation of special interest comprises two alleles, *Gli-B1s*, and *Gli-A2z*, that were not originated anywhere in Europe and Caucasus, but had been found in the Mid-Asia and the Palestine. Another sample (No. 2) was the landrace "Grbljanka", collected under the very top of the Rumija Mountain (SE-Montenegro) nested the small settlement Lunje (42, 04°N+19, 18°E, alt. 718m) consisting of three houses of Lunjic family. That remote and hard accessible place grew the last remnants of landrace Grbljanka (*T. turgidum*) that had been grown all along the Montenegrin coast, from Ulcinj to Lastva Grbaljska, decades ago (Petrović, andDimitrijević, 2012). Gliadin allelic composition reveals that the population that was gathered consisted of 70% *T. turgidum* (4x), and 30% of *T. aestivum* (6x). Within *T. turgidum*, four biotypes were detected. A hexaploid component of the mixture showed alleles - *Gli-B1k*, *Gli-A2new2*, *Gli-B2t* that occur in landraces, old varieties and are rarely found in modern varieties. It is believed that such a mixture of tetraploid and hexaploid wheat sown by the ancient farmers. The wheat sample No. 3, represents the wheat population from the small settlement of about 30 inhabitants, named Ujniče (43. 09°N+19, 70°E, alt. 1157m) near the town of Bijelo Polje at the Northern Montenegro. The wheat (*T. aestivum*) sample, exhibited about 60% of the allele *Gli-B1g* within total allelic variation of this locus. The *Gli-B1g* is typical for *T. macha*, Caucasus endemic, and could be often found in *T. spelta*, but very rarely in *T. aestivum.* The allele *Gli-D1b* (80% of allelic variation on this locus) appears, as a rule, in landraces, as well as, very old varieties (tab. 1).

Table 1. Identified gliadin allelic variation of the samples from cereal germplasm collection

Sample No.	Genotype	Alleles of gliadin coding loci and their frequencies [%]					
		Gli-A1	***Gli-B1***	***Gli-D1***	***Gli-A2***	***Gli-B2***	***Gli-D2***
1	***T. aestivum***	q-50 i-36 m-14	s-48 b-42 f-5 e-5	a-100	z-45 t-32 k-13 c-5 f-5	?	a-85q-10 w-5
General formula		**q+i+m**	**s+b+f+e**	**a**	**z+t+k+c+f**	**?**	**a+q+w**
2	***T. turgidum*** (70%) 4x	4 biotypes					
	T. aestivum (30%) 6x	f-100	k-100	a-90f-10	f-90 new2-10	t-100	f-100
General formula		**f**	**k**	**a+f**	**f+new**	**t**	**f**
3	***T. aestivum***	f-75a-25	g-60f-15b-15 e-10	b-80 k-15 d-5	g-100	b-60 ?-40	b-50a-30 new1-20
General formula		**f+a**	**g+f+b+e**	**b+k+d**	**g**	**b+?**	**b+a+new1**

Genetic variation within samples of *Hordeum sp*. A seed sample of spring barley that goes under the local name "Bushket", was obtained in a small household in a village of Palež (43, 18°N and 19, 14°E) at 1431m of altitude, in Montenegro. That particular barley has been grown on that household for 70 years, according to the host. The sampled population consists of three genotypes where genotypes 1 and 2 are old varieties, and the genotype 3 is a modern barley variety (fig. 2A). A barley sample collected at the site of the village Kovčica (43, 09°N and 19, 12°E), at an altitude of 1437m, in Montenegrin mountain Durmitor area, appeared to be a mixed population of local cultivars (1 and 3), and HRD allelic composition (2) that could be found in modern varieties (fig. 2B). A barley sample obtained from Šćepan polje, Montenegro, located at 43. 35°N + 18. 85°E (alt. 764m), exhibited the highest genetic variability consisting of 5 genotypes with different hordein loci allelic variation where genotypes marked as 2 and 3 refer to modern varieties, while the rest (1, 4, and 5) originated from old local populations (fig. 2C). In the village of Pitomine, Montenegro, at the position (43, 09°N and 19, 06°E, alt. 1536m), a population of spring two-rowed barley was found. The host claimed that the seed had been inherited from his father. However, according to hordein allelic variation results, the sample belongs to modern barley variety (fig. 2D).

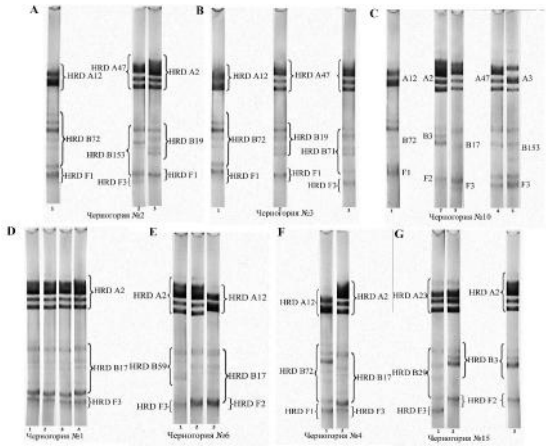

Figure 1. Seven samples of barley out of germplasm collection were analyzed using hordein allelic variation

In the village of Gradina (43, 10°N + 19, 29°E, alt. 1232m), the slopes of Kričaĸ Mountain, Montenegro, samp-les of barley, beside rye and buckwheat, were collected. The sampled population appe-ared to be a mixture of modern varieties (genotypes 2 and 3), and genotype labeled as 1, showing the pattern that corresponds to an old barley population (fig. 2E). The sample of two-rowed barley collected in Montenegro at the site of Mala Crna Gora (43, 12°N i 19, 00°E), at an altitude of 1932m - the population consists of two genotypes; the first one appeared to be old local variety (1), the second (2) refers to modern barley variety, (fig. 2F). A seed sample of barley obtained on the small farm in scattered Montenegrin village Podgora (43, 07°N + 18, 18°E, alt. 1456m), that the farmer claimed to be an old inherited seed, appeared to be a mixture of three varieties of modern barley according to hordein allelic variation (fig. 2G).

Table 2. RAPD primers used for screening goat grass genotypes

Primers	**Nucleotide sequence**	**Usability**
OPA-02	5'-TGCCGAGCTG-3'	+
OPA-05	5'-AGGGGTCTTG-3'	-
OPA-08	5'-GTGACGTAGG-3'	-
OPA-25	5'-GACAGACAGA-3'	+
OPB-06	5'-TGCTCTGCCC-3'	-
OPB-07	5'-GGTGACGCAG-3'	+

Genetic variation within samples of *Aegilops sp*. Three RAPD primers, out of six tested, have been selected (OPA02, OPA25 i OPB07) to determine the polymorphism of different species of *Aegilops* genus (tab. 2). A total of 25 polymorphic bands were generated, ranging from 500 to 3000 bp. The highest number of polymorphic bands (10 bands)was achieved with primer OPA02. The result is in accordance to previously reported resultswhere RAPD primer OPA-02 was detected as U-genome specific marker (Cenkci et al., 2008). All the tested samples of *Aegilops* sp.,have a common U genome - *Aegilops biuncialis* (UM), *Aegilops kotschy* (SU), *Aegilops columnaris* (UM), *Aegilops triaristata/Ae. Neglecta* (UM), A*egilops ovata/Ae. geniculate* (MU), *Aegilops umbellulata* (U), fig. 2.

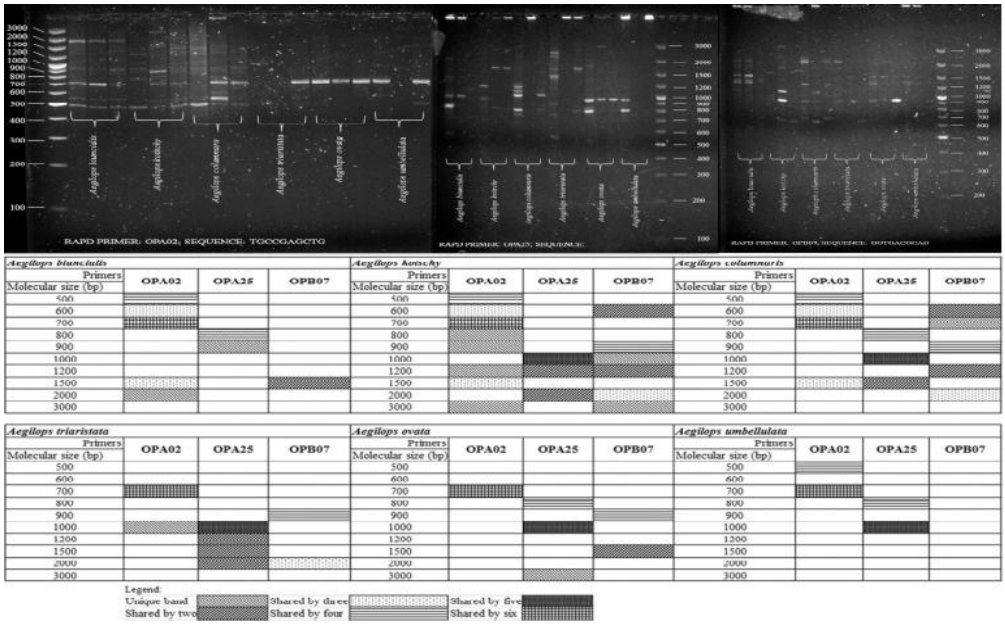

Figure 2. Amplification patterns with the selected RAPD primers (upper photographs) and DNA fingerprints of *Aegilopssp.* genotypes.

Number and percentage of polymorphic loci, effective number of alleles, expected heterozygosity and Shannon's information index were used to estimate genetic variation. *Aegilops kotschy* had the highest values for all tested parameters, exhibiting the highest level of variation, whereas variety *Aegilops biuncialis*exhibited the lowest (tab. 3).

Table 3. Estimates of genetic variation in *Aegilops* sp. using RAPD markers

Species	P (No.)	P (%)	Ne±SD	He±SD	I±SD
Aegilops biuncialis	2	7. 69	1. 044±0. 159	0. 028±0. 098	0. 042±0. 148
Aegilops kotschy	15	57. 69	1. 308±0. 314	0. 195±0. 180	0. 299±0. 268
Aegilops columnaris	9	43. 62	1. 249±0. 383	0. 140±0. 204	0. 204±0. 293
Aegilops triaristata	7	26. 92	1. 180±0. 306	0. 107±0. 181	0. 158±0. 266
Aegilops ovata	3	11. 54	1. 070±0. 214	0. 042±0. 122	0. 063±0. 181
Aegilops umbellulata	4	15. 38	1. 132±0. 270	0. 082±0. 157	0. 124±0. 235
P (No.): number of polymorphic loci; P (%): the percentage of polymorphic loci; Ne: effective number of alleles; He: expected heterozygosity; I: Shannon's information index; SD:standard deviation					

CONCLUSION

A small number of samples from germplasm collection were tested using gliadin and hordein loci variation, as well as RAPD primers. Samples of wheat landraces tested using *Gli* loci variation shoved some unique alleles commonly presented in genotypes of ancient landraces. The sample of particular interest is spring wheat collected in Zabrdje (sample 1), having allelic variation *Gli-B1s*, and *Gli-A2z* that is very rare and cannot be seen in Europe and Caucasus, even, but in Central Asia. Barley samples, screened by HRD loci variation, appeared to be mixtures of landraces and modern barley varieties. *Aegilops* sp.,showed distinct patterns tested by RAPD primers. The results confirmed that RAPD markers, could be of value in germplasm collections management for identification and measurement of variation.

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