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RAPD-PCR MOLECULAR ANALYSIS OF SPELT REGARDING THE PROTEIN CONTENT

ANALIZA MOLEKULARNA RAPD-PCR PSZENICY ORKISZ POD WZGLĘDEM ZAWARTOŚCI BIAŁKA

Summary. The subjects of the study were 22 genotypes of spelt and three genotypes of bread wheat. The aim of the study was to show the relationship between the genetic distance of the 22 genotypes of winter spelt and three genotypes of bread wheat and protein content in percentages. On the basis of the obtained results, three groups of the genetic diversification of tested objects, depending on the percentage protein content, were differentiated. In the first group, where the similarity between the analysed objects ranged from 11% to 26%, the forms of winter spelt characterised by the lowest protein content were classified. In the second group, in which the probability is contained in the range from 10% to 35%, the forms of higher protein content were classified. The majority of the remaining forms of winter spelt and bread wheat of the medium protein content were classified into the third group of the genetic diversification, and the similarity between them ranges from 5% to 32%.

Key words: genetic distance, percentage content of protein, spelt

Introduction

During the last few years, the rapid development of the molecular biology techniques based on the DNA analysis has been observed. This is why the studies on the development of new, effective and cheaper methods of obtaining molecular markers that serve as research instrument where the genotype analysis is a must, are being performed.

Stripe patterns specific for individual plants may be used in a similar way that certain morphological features (GRZEBELUS and BARAŃSKI 1996).

The molecular markers are inherited according to the Mendel's genetics and are not subjected to the modifying environment effect. Thus, they are useful in research studies and manipulations on the useful characters and allowing for accurate genome analysis.

Molecular markers have also been extensively utilized for the study of genetic diversity and interspecific/intergeneric relationships among a number of species of the tribe Triticeae (BANDOPADHYAY et AL. 2004). The most commonly used techniques of obtaining molecular markers are the methods using the polymerase chain reaction (PCR). The most often used methods are: RAPD (Random Amplified Polymorphic DNA), AP-PCR (Arbitrarily Primed PCR) and DAF (DNA Amplification Fingerprinting). For wheat, the methods based on the polymorphism of the genes coding the protein sequences and microsatellite sequences are preferred (D'OVIDIO 1993).

Spelt (*Triticum aestivum* ssp. *spelta* L.) is important among the hexaploid wheat species. It is an old form of wheat, grown in Europe for thousands of years. Because of the beneficial features connected with the nutritional properties of the grain, it is currently used in many breeding programmes. Spelt grain contains significantly more protein than bread wheat grain (GRELA et AL. 1993, MOUDRÝ 1999). Spelt is also the source of genes that code the ability for high yielding under the unfavourable agrotechnical conditions and low temperature resistance, and has the increased value of certain biochemical ingredients of the grain (ARSENIUK et AL. 1991).

The aim of the study was to evaluate the percentage relationships between the protein content and the genetic distance between spelt and bread wheat determined on the basis of RAPD molecular markers.

Material and methods

The laboratory tests on the protein content were performed in the Department of Agronomy (Poznań University of Life Sciences), Kjeldahl methods by examining the nitrogen content in the sample and multiplied by a factor of 6.25. The molecular analysis was performed in the Department of Genetics and Plant Breeding (Poznań University of Life Sciences).

The plant materials were 22 genotypes of spelt from the gene bank of Institute of Plant Breeding and Acclimatiztion (IHAR) in Radzików near Warsaw, and the gene bank of The Institute of Plant Genetics and Plant Research in Gatersleben, Germany, and three genotypes of bread wheat from Plant Breeding Strzelce.

The following forms of winter spelt were tested:

1) 'Bastard'
2) 'Oberlaender'
3) '0001950 – Radzików'
4) 'Spelt. inz Drogendijk'
5) 'Schwabenkorn'
6) 'Österreischer Burgdorf 1'
7) 'Stickhof'

8) ‘Ostro’
9) ‘Weisser W. Gran. aus Hohenheim’
10) ‘Schweizer Alt Gold’
11) ‘Bauländer’
12) ‘Vögelers Dinkel Weiss’
13) ‘Zeiners Weiβer Schlegeldinkel’ (*T. spelta* L. var. *album* Alef.)
14) ‘Blauer Wi. Kolbendinkel’ (*T. spelta* L. var. *alefeldii* Körn.)
15) HTRI 4613/75 SKL (*T. spelta* L. var. *album* Alef.)
16) HTRI 9631/75 SKL (*T. spelta* L. var. *alefeldii* Körn.)
17) HTRI 4629/75 SKL (*T. spelta* L. var. *alefeldii* Körn.)
18) ‘Weiβer Wi. – Grannendinkel aus Hohenheim’
19) HTRI 4473/93 SKL (*T. spelta* L. var. *arduini* Mazz)
20) ‘Schwarzer Beh. Wi. – Kolbendinkel aus Nördlingen’
21) HTRI 16981/95 SKL (*T. spelta* L. var. *duhamelianum* Mazz)
22) ‘Waggershauser Hohenh. Weiβer Kolben’ (*T. spelta* L. var. *albispictum* = *album*)

and bread wheat (*Triticum aestivum* ssp. *vulgare*):

23) ‘Symfonia’
24) ‘Nutka’
25) ‘Sukces’

The genomic DNA of the parental components and half-breeds of maize, rye and triticale were isolated using the modified THOMSON and HENRY (1995) procedure. The leaf discs of the surface of 2 mm^2 were taken from 10 plants. The plant tissue was then treated using 200 μl of the TPS buffer of the following composition: 100 mM Tris HCl (pH 9.5), 1 M KCl, 10 mM EDTA. The samples were incubated in a water bath at the temperature of 95°C for 15 min. The leaf tissue fragments were taken out of the test tubes after incubation and they were placed at the temperature of –20°C for longer storage. The reagents used for the isolation were produced by Sigma.

The measurements of the concentration using the Gene Quant II fluorometer with quartz-capillary tubes were performed directly after the DNA extraction. The DNA concentration resulting from the isolation was diluted to the concentration of 25 ng/μl. The concentration was determined using the following formula:

$$V_1 = \frac{C_2 \times V_2}{C_1}$$

where:

C_1 – concentration of the sample after isolation,
C_2 – concentration to be obtained,
V_1 – DNA amount of the known concentration necessary for dilution,
V_2 – constant conventional sample volume, e.g. 50 μl.

The polymerase chain reaction was performed in the volume of 12.5 μl of the mixture of the following composition: DI water – 9.75 μl, 1 M Tris HCl (pH 8.3) – 0.125 μl,

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25 mM $MgCl_2$ – 1.0 µl, BSA – 0.0625 µl, 2 mM dNTP – 0.625 µl, starter – 5 pmol/µl – 0.25 µl, polymerase Taq-5 U/µl – 0.1875 µl, DNA extract – 25 ng/µl – 0.5 µl.

The DNA amplification was performed using the T3 Biometra thermocycler (Polygen). Polymerase Taq used in the mixture was bought in MBI-Fermentas – ABO, the remaining reagents were bought in Sigma.

The electrophoresis was performed in the 1.5% agarose gel of the following composition: 1.5 g of agarose, 100 ml of TBE 1× buffer and 1.0 µl of etidin bromide. Electrophoresis was performed in the TBE 1× buffer under the voltage of 100 V and intensity of 200 mA. TBE 5 M buffer (1 l) composition: 10.8 g of Tris base, 5.5 g of boric acid, 4 ml of 0.5 M EDTA (pH 8.0). 1 Kb Ladder was bought in Gibco BRL and the remaining reagents were bought in Sigma.

The documentation of the results of RAPD includes pictures of the obtained electrophoretic images and their processing using the UVIMAP software in the model of Nei and Li (1979):

$$GS = 2n_{xy} / (n_x + n_y)$$

where:

$2n_{xy}$ – number of stripes in both genotypes,
n_x and n_y – number of stripes characteristic for individual genotypes.

The GS value is the similarity index between the two tested genotypes. For each half-breed, the genetic distance between the parental components was calculated using the following formula:

$$D = 1 - GS$$

Results

Of the 230 tested oligonucleotide starters, 20 generated a high polymorphism (196 polymorphic stripes), which allowed for the determination of the genetic distance between the tested forms of spelt and bread wheat. The starters and their nucleotide sequences coding the polymorphism are given in Table 1.

The value of the genetic distance between the analysed objects is in the range of 0% to 65%, which is pictured by the UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram (Fig. 1) plotted on the basis of the similarity index.

The tested spelt genotypes differed in the tested qualitative features of the grain. The lineages with the highest total protein content were HTRI 4473 and HTRI 9631, and with the lowest – genotypes ‘0001950 – Radzików’, ‘Österreischer Burgdorf 1’ and ‘Spelt. inz Drogendijk’ (Table 2).

The analysis of the UPGMA dendrogram allows for the distinction of three groups of genetic diversification of the tested objects. The objects were classified into groups depending on, among other things, the percentage content of protein. In the first group, where the similarity between the analysed objects is from 11% between the forms ‘Bastard’ and ‘Stickhof’, to 26% between ‘Bastard’ and ‘Spelt. inz Drogendijk’ (Fig. 1),

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Table 1. Nucleotic sequences of primers detecting polymorphism
Tabela 1. Startery i ich sekwencje nukleotydowe tworzące polimorfizm

No Nr	Primer's No Nr startera	Sequences 5'-3' of primers Sekwencje 5'-3' starterów
1	OPA 04	AATCGGGCTG
2	OPA 07	GAAACGGGTG
3	OPA 09	GGGTAACGCC
4	OPA 12	TCGGCGATAG
5	OPB 10	CTGCTGGGAC
6	OPB 11	GTAGACCCGT
7	OPB 18	CCACAGCAGT
8	OPC 11	AAAGCTGCGG
9	OPC 15	GACGGATCAG
10	OPC 19	GTTGCCAGCC
11	OPC 20	ACTTCGCCAC
12	OPD 09	CTCTGGAGAC
13	OPD 15	CATCCGTAAG
14	OPD 18	GAGAGCCAAC
15	OPF 15	CCAGTACTCC
16	OPF 20	GGTCTAGAGG
17	OPG 12	CAGCTCACGA
18	OPI 04	CCGCCTAGTC
19	OPI 11	ACATGCCGTG
20	OPI 19	AATGCGGGAG

the forms of winter spelt of the lowest protein content were included (Table 3). In the second group, where the similarity ranges from 10% between HTRI 4613/75 SKL and 'Schwarzer Beh. Wi. – Kolbendinkel aus Nördlingen' to 35% between HTRI 4473/93 SKL and winter spelt 'Waggershauser Hohenh. Weiβer Kolben' (Fig. 1), the forms of the highest protein content were included (Table 3). The majority of the remaining forms of the winter spelt and bread wheat with medium protein content (Table 3) were included in the third group of the genetic diversification. The genetic similarity between them ranges from 5% between the bread wheat 'Nutka' and 'Sukces' to 32% between 'Schwabenkorn' and 'Vögelers Dinkel Weiss'.

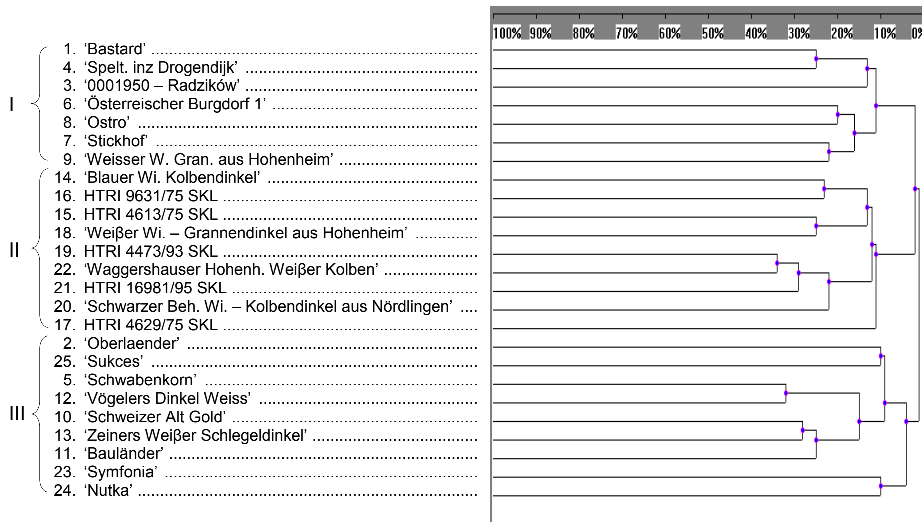

Fig. 1. Dendrogram showing similarity between spelt and bread wheat
Rys. 1. Dendrogram przedstawiający podobieństwo genetyczne pomiędzy orkiszem pszennym i pszenicą zwyczajną

Table 2. Protein content in spelt and bread wheat genotypes (%)
Tabela 2. Zawartość białka w genotypach orkiszu pszennego i pszenicy zwyczajnej (%)

No Nr	Genotype Genotyp	Years – Lata	
		2002	2003
1	2	3	4
1	'Bastard'	14.2	18.4
2	'Oberlaender'	13.7	19.3
3	'0001950 – Radzików'	13.6	17.7
4	'Spelt. inz Drogendijk'	13.3	18.4
5	'Schwabenkorn'	–	–
6	'Österreischer Burgdorf 1'	14.4	17.0
7	'Stickhof'	14.1	18.1
8	'Ostro'	14.3	19.2
9	'Weisser W. Gran. aus Hohenheim'	14.5	18.5
10	'Schweizer Alt Gold'	13.8	18.2
11	'Bauländer'	–	–
12	'Vögelers Dinkel Weiss'	15.0	18.5
13	'Zeiners Weiβer Schlegeldinkel' (*T. spelta* L. var. *album* Alef.)	14.7	17.9

Table 2 – cont. / Tabela 2 – cd.

1	2	3	4
14	'Blauer Wi. Kolbendinkel' (*T. spelta* L. var. *alefeldii* Körn.)	15.6	19.3
15	HTRI 4613/75 SKL (*T. spelta* L. var. *album* Alef.)	14.9	18.6
16	HTRI 9631/75 SKL (*T. spelta* L. var. *alefeldii* Körn.)	16.7	20.4
17	HTRI 4629/75 SKL (*T. spelta* L. var. *alefeldii* Körn.)	16.3	18.5
18	'Weiβer Wi. – Grannendinkel aus Hohenheim'	14.1	18.3
19	HTRI 4473/93 SKL (*T. spelta* L. var. *arduini* Mazz)	17.8	21.5
20	'Schwarzer Beh. Wi. – Kolbendinkel aus Nördlingen'	16.8	18.5
21	HTRI 16981/95 SKL (*T. spelta* L. var. *duhamelianum* Mazz)	15.6	18.4
22	'Waggershauser Hohenh. Weiβer Kolben' (*T. spelta* L. var. *albispictum = album*)	16.4	19.6
23	'Symfonia'	–	–
24	'Nutka'	–	–
25	'Sukces'	–	–
$LSD_{\alpha = 0.05}$ – $NIR_{\alpha = 0,05}$		1.82	2.12

Discussion

In Europe spelt has been known and grown for approximately 3000 years. In comparison with the grain of bread wheat, the grain of this species contains much more protein and is rich in gluten and many minerals. This fact is well known (BAUMGÄRTEL-BLASCHKE 1992, GRELA et AL. 1993, MOUDRÝ 1999, OLIVEIRA 2001, DVORÁČEK et AL. 2002). STALLKNECHT et AL. (1996) tested 164 forms of spelt and proved the high variability of the protein content in grain (18-40%) being a result of the genotype and the effect of localisation (agro-technology) and environmental factors. The environmental factors clearly modify the quality of the grain, including the protein content (OLIVEIRA 2001). Under the conditions of low nitrogen fertilisation, the decrease in the protein content is stronger for bread wheat than for spelt. Moreover, spelt contains large amounts of the essential nutrients: cellulose, non-saturated fatty acids, carbohydrates, vitamins and bioelements. Ripe spelt contains large amounts of siliceous acid which supports the activity of the brain and concentration. In the 35-year long studies, Doctor Gotfried Hertzka and a naturotherapist, Wighard Strehlow, proved that the systematic eating of spelt regenerates the body and enables recovery of health (WOŹNIAK 2004).

Thanks to these properties, spelt could become a source of valuable genes for the breeding of varieties with improved nutritional and healthy properties (WAGA et AL. 2002). In the studies by WAGA et AL. (2002) it was proved that it is a valuable source of the genes responsible for the nutritional value. This corn is especially useful for ecological agriculture and for crops in the areas with increased ecological requirements (KLING and UTZ 1991, EUSTERSCHULTE and KANT 1995).

Table 3. Genotypes groups of spelt and bread wheat according to protein content
Tabela 3. Grupy genotypów orkiszu pszennego i pszenicy zwyczajnej według zawartości białka

Genotypes with the greatest protein content (X + 1 SD) Genotypy o największej zawartości białka (X + 1 SD)	Genotypes with the smallest protein content (X – 1 SD) Genotypy o najmniejszej zawartości białka (X – 1 SD)	The other genotypes Pozostałe genotypy
16. HTRI 9631/75 SKL (*T. spelta* L. var. *alefeldii* Körn.) 17. HTRI 4629/75 SKL (*T. spelta* L. var. *alefeldii* Körn.) 19. HTRI 4473/93 SKL (*T. spelta* L. var. *arduini* Mazz) 20. ‘Schwarzer Beh. Wi. – Kolben-dinkel aus Nördlingen’ 22. ‘Waggershauser Hohenh. Weiβer Kolben’ (*T. spelta* L. var. *albispictum* = *album*)	3. ‘0001950 – Radzików’ 4. ‘Spelt. inz Drogendijk’	1. ‘Bastard’ 2. ‘Oberlaender’ 5. ‘Schwabenkorn’ 6. ‘Österreischer Burgdorf 1’ 7. ‘Stickhof’ 8. ‘Ostro’ 9. ‘Weisser W. Gran. aus Hohenheim’ 10. ‘Schweizer Alt Gold’ 11. ‘Bauländer’ 12. ‘Vögelers Dinkel Weiss’ 13. ‘Zeiners Weiβer Schlegeldinkel’ (*T. spelta* L. var. *album* Alef.) 14. ‘Blauer Wi. Kolbendinkel’ (*T. spelta* L. var. *alefeldii* Körn.) 15. HTRI 4613/75 SKL (*T. spelta* L. var. *album* Alef.) 18. ‚Weiβer Wi. – Grannendinkel aus Hohenheim‘ 21. HTRI 16981/95 SKL (*T. spelta* L. var. *duhamelianum* Mazz) 23. ‘Symfonia’ 24. ‘Nutka’ 25. ‘Sukces’

The most important aim of plant cultivation is to improve the existing varieties of the cultivable species to increase their disease and pest resistance, yield, quality and other features important from the breeding point of view. One of the first stages of breeding is the selection of the parental forms from the available gene pool of the existing varieties. Until now, to properly assess the gene resources in terms of productivity, quality and susceptibility to biotic and abiotic stressors, expensive and time-consuming methods depending on mulityear hybridisation and phenotypical selection, or the analysis of the isoenzymatic profiles, have been used (KARP et AL. 1998).

Often, the long-lasting and complex breeding cycle may be significantly shortened by performing the selection using DNA markers. For this purpose, the existing close linkage between the marker and locus responsible for inheritance of the useful character is used. The method is known as MAS (Marker Assisted Selection) and is widely used in the breeding of tree-like plants, and resistance breeding. For example, when the

marker linked with the gene coding the purple colour of the fruits of the sweet cherry is known, the selection may be performed in this direction few years before the fructifying period.

SZAFRAN et AL. (1997) using the RAPD technique, tried to find the genetic markers for the resistance to *Peronospora* in cucumber. In the study using six selected starters, amplified DNA fragments polymorphic for the individuals of the tested populations were obtained. The analysis of the patterns of the DNA fragments obtained using these starters proved the occurrence of the PCR products characteristic for individuals with a high level of resistance, and sensitive individuals. However, to consider the marker as informative (linked with the character) it has to be in the distance smaller than 15 cM from the gene responsible for the character. Thus, the obtained results are considered as an introductory study, the continuation of which will be to identify the found DNA fragments as RAPD markers and their assignation to the character of resistance to *Peronospora*.

WILLIAMSON et AL. (1994) searched for the AP-PCR markers linked to the resistance to namatodes in tomato. They have transformed one of the polymorphic amplification products into the SCAR marker differentiating plants as resistant and sensitive. The specific starter caused a DNA fragment multiplication in all plants, but after the digestion of the product using a Taq I restrictase, the stripe pattern of the resistant plants was different than for sensitive plants. The marker turned out to be significantly stronger, linked with the resistance to nematodes, than the isoenzymatic marker used up to now (GRZEBELUS and BARAŃSKI 1996).

For the determination of the genetic similarity of winter spelt and bread wheat the RAPD markers were used.

The method is based on the PCR technique commonly used *in vitro* for the amplification of the DNA fragments. As opposed to the classical PCR, in RAPD a single arbitrary starter of the length of several to dozen or so nucleotides, containing 50-80% of the G and C bases, is used. The starters hybridise in many sites of the tested genome and cause the amplification of several to several dozen various fragments (PRZYKŁADY... 2001).

The most common problem when using the RAPD technique is poor repeatability of the obtained results caused by the possibility of the sensitivity of the method to the alternations of the conditions of amplification (PRZYKŁADY... 2001). However, these markers are successfully used in many studies, e.g. studies on the polymorphism between wild and cultivable forms of plants (XU and GAI 2003) for the analysis of the diversity between the varieties of the cultivable plants (LIU et AL. 1998, ŠUŠTAR-VOZLIČ and JAVORNIK 1998, FACCIOLI et AL. 2000). On the basis of the performed analyses, it was proved that the following starters: OPA 04, OPA 07, OPA 09, OPA 12 and OPB 10 distinguished all tested genotypes. Thus, the used starters may be used for testing the genetic similarity of winter spelt and bread wheat. The value of the genetic distance between the analysed objects ranges from 0% to 65%.

In Poland there is an increasing interest in breeding spelt which is especially connected to the high content of readily available proteins. In the authors' own studies, 22 genotypes of winter spelt and three genotypes of bread wheat were subjected to the RAPD-PCR molecular analysis. Three groups of the genetic diversification in relation to the percentage protein content were distinguished. In the first group, where the simi-

larity between the analysed objects ranged from 11% to 26%; the forms of winter spelt characterised by the lowest protein content were classified. In the second group, in which the probability is contained in the range from 10% to 35%, the forms of higher protein content were classified (Table 3). The majority of the remaining forms of winter spelt and bread wheat of the medium protein content (Table 3) were classified into the third group of genetic diversification, and the similarity between them ranged from 5% to 32%. The lineages with the highest total protein content were HTRI 4473 and HTRI 9631, and with the lowest – genotypes '0001950 – Radzików', 'Österreischer Burgdorf 1' and 'Spelt. inz Drogendijk' (Table 2). The HTRI 4473 and HTRI 9631 lineages may prove to be a valuable gene source for breeding studies on wheat. They are characterised by high baking value as the RMT ratio.

The baking value of the flour does not depend on the protein content but most of all on the quality and composition of gluten. In the studies by ACHREMOWICZ et AL. (1999) the baking value of the flour of spelt was similar to bread wheat, but the addition of the spelt flour improves the taste and prolongs the freshness of the wheat bread (OSTROWSKA 1993).

Conclusion

For the RAPD molecular markers, the number of generated polymorphic stripes allowing for the determination of the genetic distance between the analysed forms was 196. The value of the genetic distance between the analysed objects ranged from 0% to 65%. The analysis of the UPGMA dendrogram enabled the distinction of three groups of genetic diversification of the tested objects. The objects were classified into groups depending on, among other things, the percentage content of protein.

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ANALIZA MOLEKULARNA RAPD-PCR PSZENICY ORKISZ POD WZGLĘDEM ZAWARTOŚCI BIAŁKA

Streszczenie. Przedmiotem badań były 22 genotypy pszenicy orkisz oraz trzy genotypy pszenicy zwyczajnej. Celem pracy była próba wykazania zależności pomiędzy dystansem genetycznym 22 genotypów pszenicy ozimej orkisz oraz trzech genotypów pszenicy zwyczajnej a procentową zawartością białka. Na podstawie uzyskanych wyników wyróżniono trzy grupy zróżnicowania genetycznego badanych obiektów w zależności od procentowej zawartości białka. W pierwszej grupie, gdzie podobieństwo pomiędzy analizowanymi obiektami wynosiło od 11% do 26%, znalazły się formy pszenicy ozimej orkisz charakteryzujące się najmniejszą ilością białka. W grupie drugiej, w której podobieństwo mieściło się w przedziale od 10% do 35%, znalazły się formy z największą ilością białka. Pozostałe formy pszenicy ozimej orkisz oraz pszenicy zwyczajnej, charakteryzujące się średnią zawartością białka, w większości znalazły się w trzeciej grupie zróżnicowania genetycznego, a podobieństwo genetyczne pomiędzy nimi wyniosło od 5% do 32%.

Słowa kluczowe: dystans genetyczny, procentowa zawartość białka, orkisz

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