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DIFFERENTIATION OF MAIZE LINES WITH HIGH CONTENT OF CAROTENOIDS USING PROTEIN AND DNA MARKERS

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ABSTRACT

Wide natural variety of carotenoids, including vitamin A precursors, is characteristic of maize (Zea mays L.), which allows using it to combat vitamin A deficiency in the world. Previous studies have established the effectiveness of the use of functional DNA markers in the selection of maize lines with a high content of carotenoids in grain. However, not only improving grain quality but also creating highly productive hybrids competitive on the grain market is currently important. The purpose of our study was to determine the genetic diversity of maize lines using storage protein and DNA markers, as well as to find correlations of two marker systems with FAO characteristics. On the basis of maize lines selected for high content of carotenoids, the allelic state of six SSR markers (phi022, phi034, phi062, phi073, phi079, phi085), electrophoretic spectra of zein and their electrophoretic mobility have been determined. Cluster analysis of maize lines using electrophoretic spectra of zein yielded eight clusters. It was found that the minimum genetic distance was 4.24 and the maximum 7.48 Cluster analysis by the identified alleles for SSR markers allowed to form seven clusters according to the affinity of the lines. Range of changes in genetic distances was from 1.00. to 3.46 The analysis of genetic distance matrices, using the Mantel test, found a correlation between the marker systems under study (r = 0.184). A correlation between the studied marker systems and their relation to FAO characteristics was established. Therefore, in order to increase selection efficiency of maize, it is advisable to use an integrated approach to the evaluation of breeding genotypes involving protein and DNA markers.

Keywords: *storage proteins; SSR markers; cluster analysis; correlation.*

INTRODUCTION

Maize (*Zea mays* L.) is represented by a sufficiently variable gene pool that allows using of different genotypes in breeding, which contributes to improvement of the hybrids' productivity in terms of crop productivity, disease resistance, duration of

the vegetation season and other agronomic characteristics. Various approaches are used for the corn genetic diversity assessment, which include the assessment of both morphological characteristics and the molecular genetic analysis of genotypes. Previous studies have shown the usage of SSR markers and electrophoretic spectra of maize seeds' reserve proteins for evaluation of maize lines' genetic diversity of domestic breeding and well-known lines (Goncharov *et al*., 2016; Prysiazhniuk *et al*., 2018), as well as usage of DNA markers for the selection of lines with high carotenoids content (Prysiazhniuk *et al*., 2019). Polymorphism of seeds' reserve proteins allows estimation of inbred lines for genetic homogeneity through the component composition of zein spectra, as well as with a high probability indicates the degree of genetic proximity between lines (Pedersen *et al.*, 1982; Feix, and Quayle, 1993). Studies by Sidorova *et al.* (2012, 2015) identified the presence of certain components of zein in maize lines from different maturity groups and identified components, which are distinctive for early-maturing maize with FAO till 299. Such studies are essential in context of searching of optimal parental components for creation of the highly productive hybrids that can be domesticated in different soil-climatic zones.Papers devoted to searching for microsatellite loci related to the agronomic characteristics were carried out by many authors (Lu and Bernardo, 2001; Legesse *et al.*, 2007). Magulama and Sales (2009) and Yang *et al.* (2008) have described usage of phi 057 and phi 112 markers for assessing of maize genotypes with high levels of lysine and tryptophan. Thus, the interest appears for the application efficiency of zein and SSR markers for evaluation of a small sample of lines with specific characteristic such as high carotenoid content, as well as comparing the efficiency of marker systems for lines differentiation. Furthermore, the issue of assessing the correlation connections between SSR markers and maize maturity groups remains to be underinvestigated.

Consequently, the purpose of our study is to determine the genetic diversity of maize lines with high content of carotenoids in grain with usage of storage proteins and DNA markers, as well as to find correlations between two marker systems with FAO indexes.

MATERIAL AND METHODS

Materials for study were 21 lines of maize with high content of carotenoids. Studied maize lines had the following FAO indexes: DK129-4 – 170, DK366 –190, DK2323 – 190 (early-season group); DK959 – 200, DK247 – 210, DK212 – 220, DK267 – 220, DK273 – 220, DK744 – 220, DK276 – 230, DK272 – 240, DK239 – 250, DK742 – 280, DK680 – 280, DK296 – 280 (middle-early group); DK633/266 – 300, DK257 – 3203 (mid-season group); DK411 – 400, DK325 – 400, DK377 – 430, DK633 – 450 (middle-late group). Maize lines were selected based on DNA markers and on total carotenoid content in grain (Prysiazhniuk *et al.*, 2019). The research was performed during 2016-2018 on the basis of the laboratory of biotechnology in the state-owned institution Institute of grain crops of NAAS within the framework of the State Program of Scientific Research 23 "Biotechnology and Genetics in Crop Farming" task 23.00.01.06F "Development

of the Fundamental Basics of Molecular, Genetic and Cell Biotechnologies for the Improvement of Maize Selection" and the department of laboratory tests for the qualifying expertise of plant varieties (Center for Certification Tests) in the Ukrainian Institute of Plant Varieties Expertise. Maize lines polymorphism was investigated based on protein and DNA markers. Electrophoretic spectra of storage proteins were evaluated based on the electrophoretic mobility of zein components (rf) (Prysiazhniuk *et al.*, 2018). For the determination of polymorphism of the six maize lines according to DNA markers six SSR markers (phi022, phi034, phi062, phi073, phi079, phi085) were used (Goncharov *et al.*, 2016). The magnitude of electrophoretic mobility and the size of alleles were determined using TotalLab TL 120 software (trial version). In accordance with the obtained data values of electrophoretic mobility, the frequencies of the identified zein components were calculated according to the formula:

$$F_i = \frac{n_i}{N},$$

where *Fi* – frequency of the *i* component of zein, n_i – number of the *i* component in the sample, *N* – total number of maize lines.

According to SSR markers, allele frequencies and the polymorphic index of the locus (RIS) were determined (Sivolap *et al.*, 1998). Determination of genetic distances between maize lines were estimated with the help of cluster analysis. The unweighted pair-group average method was used as an amalgamation (linkage) rule (Fortin *et al.*, 2002; Everitt *et al.*, 2011). Estimation of the connections of FAO indexes of the studied maize lines with DNA markers was performed using the Pearson linear correlation method, with protein markers, Spearman nonparametric statistical methods. Statistical data was calculated using STATISCA 12 computer program (Trial version) (Johnson and Wichern, 2002; Elliott and Woodward, 2007). The evaluation of the correlation between two marker systems by genetic distances was performed using the Mantel test using the XLSTAT 2018 (Trial version) computer program (Legendre *et al.*, 2010; Diniz-Filho *et al.*, 2013).

RESULTS AND DISCUSSION

As the result of polymorphism study of 21 maize lines according to zein spectra were identified from 12 to 18 components for each line. It was determined that 9 components were unique and identified in one-off event in the studied lines. The electrophoretic mobility (Rf) of these components was between 32 and 102.

According to the obtained distribution, components with Rf 32 and 77 identified in DK129-4 line. It was specified that component with Rf 35 is distinctive for DK2323 line, and component with Rf 102 - for the DK325 line. Also, the unique components were identified in lines DK247 and DK267, Rf 62 and Rf 96, respectively. Components with Rf 70, Rf 78 and Rf 95, which were also found only once, were identified in the DK276 line. In order to assess the similarity of the studied maize lines a cluster analysis carried out according to the spectra of storage proteins in order to determine the genetic distances between the objects of analysis. The results of the hierarchical classification as a phylogenetic tree is represented in Figure 1.

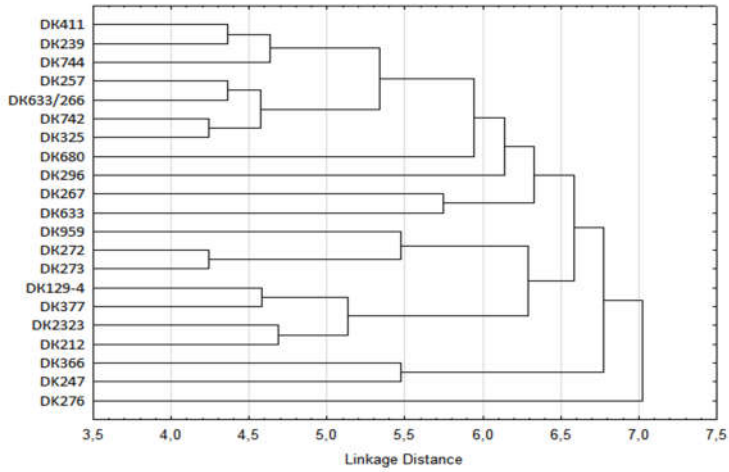

Fig. 1. Distribution of maize lines according to the degree of affinity on the basis of electrophoretic separation of zeines

In the result of cluster analysis, 8 clusters were obtained, which were formed by the maize lines according to the spectra of storage proteins. According to the received distribution, the most similar were lines included in the same cluster: DK742 and DK325, DK272 and DK273 with a genetic distance between them 4.24. It was determined that lines with the smallest distance between them was different according to 5-6 components. Thus, in DK742 line, components with Rf 55, 68, 83 and 101 were discovered, and in DK325 line components with Rf 66, 82, 88, and 102 were identified. It was also found that in the other pair of the most similar lines DK272 and DK273 determined components with Rf 46, 66, 72, 76, 80, 89 and Rf 47, 73, 81 respectively, which differed them from each other.

The most distant in relation to other lines were DK296 and DK276. It was determined that the values of genetic distances between DK276 and DK633/266 lines and DK272 varied from 6.0 to 7.48, respectively.

The research established that zeines are inherited by electrophoretic units: components with average electrophoretic mobility, components that are grouped into a block of polypeptides with the largest and smallest electrophoretic mobility, and components that are independently inherited (Zayakina and Sozinov, 1993; Zayakina and Sozinov, 1997; Zayakina *et al*., 1998). According to the Spearman statistical analysis, a correlation between components with average electrophoretic mobility and with its maximum and minimum values was determined. Thus, the correlation coefficient between components with Rf 56 and Rf 86 was 0.68. The statistically significant correlation coefficients between Rf 62 and Rf 84, Rf 48 and Rf 74, Rf 72 and Rf 80 were also determined and were 0.69, 0.70 and 0.71, respectively. It was determined that the correlation coefficient between components with Rf 70 and Rf 78 was 1.00, which indicates a close correlation.

Our research also confirmed the existence of correlation between components with maximum and minimum electrophoretic mobility. Thus, the existence of a close

connection (1.00) between components with Rf 32 and Rf 77 was determined. It was found that the correlation coefficients between Rf 42 and Rf 99 were 0.56, between Rf 40 and Rf 100 – 0.61, and correlation between components with Rf 39 and Rf 92 – 0.52 and between Rf 49 and Rf 91 the correlation coefficient was 0.74. Studies that identified the complex organization of zein encoding genes were performed by Hagen and Rubenstein (1981) by analysis of restriction fragments and southern blot hybridization, and confirmed by Zayakina and Sozinov (1993) by means of electrophoregram analysis of prolamins. Therefore, according to our research, a very strong, strong and significant correlation between zein components with different electrophoretic mobility was determined.

According to the results of our work, polymorphism of maize lines according to SSR markers was also determined. The electrophoregrams of the studied maize lines according to the markers phi034 and phi085 are shown in Figures 2 and 3.

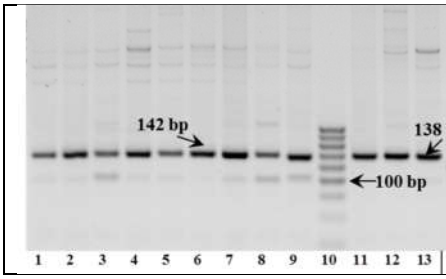

Fig. 2. Electrophoresis of amplification products of maize DNA with a marker phi034: 1 – DK411; 2 – DK257; 3 – DK742; 4 – DK744; 5 – DK325; 6 DK633/266; 7 – DK680; 8 – DK296; 9 – DK267; 10 – molecular weight marker 20 bp DNA Ladder O'GeneRuler (Thermo Scientific, USA); 11 – DK633; 12 – DK366; 13 – DK247; 14 – DK276; 15 – DK959

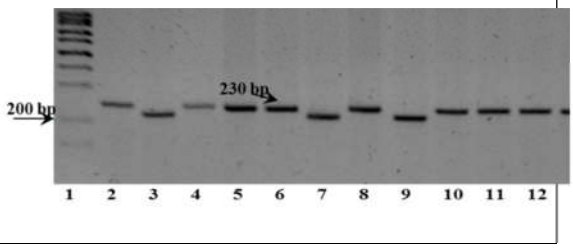

Fig. 3. Electrophoresis of maize DNA amplification products with marker phi085: 1 – molecular weight marker 100 bp DNA Ladder O'GeneRuler (Thermo Scientific, USA); 2 – DK296; 3 – DK267; 4 – DK633; 5 – DK366; 6 – DK247; 7 – DK276; 8 – DK959; 9 – DK272; 10 – DK273; 11 – DK129-4; 12 – DK377; 13 – DK2323; 14 – DK239

As can be seen from Figure 2, alleles with a size 118, 138, 142 and 147 bp was identified according to the phi034 marker. The frequencies of investigated alleles were 0.10-0.48, PIC – 0.66. The phi085 marker identified six alleles, the size of which ranged from 213 to 252 bp (Fig. 3). It was determined that allele with the size 252 bp identified in the DK212 line was specified as unique for studied lines according to phi085 marker.

The most common allele was 230 bp, which identified in 8 out of 21 studied lines. The frequency of the identified alleles according to the phi085 marker is 0.05-0.38, the PIC is 0.83. All alleles, which were determined in the studied lines and the PIC values, represented in Table 1.

Table 1. Alleles identified by SSR markers and their PIC

Name of markers	Alleles size, bp	Alleles frequency	PIC
phi034	118; 138; 142; 147	0.10-0.48	0.66
phi062	143; 148; 155	0.05-0.90	0.18
phi073	174; 178; 182; 187	0.14-0.48	0.70
phi079	171; 176; 183; 189	0.10-0.62	0.59
phi022	125; 130; 155; 160	0.10-0.62	0.60
phi085	213; 220; 230; 236; 241; 252	0.05-0.38	0.83

The size of alleles according to the phi085 marker in our studies coincided with the study results of North American maize germplasm investigation published in Maize Genetics and Genomics DataBase (MaizeGDB). Smith *et al.* (1997) conducted research with 58 inbred lines and 4 maize hybrids according to 131 SSR markers with the purpose to identify lines and evaluate genetic distances between them. With markers phi022 and phi062, authors obtained two alleles for each marker; the PIC was 0.46 and 0.48, respectively. In our studies, these markers identified from 3 to 4 alleles, with a PIC value 0.60 and 0.18 for markers phi022 and phi062, respectively. In the result of Sharma *et al*. (2010) studies, by means of phi062 marker four alleles were received, and the PIC was 0.18, which also coincides with our results. In order to determine the ability of investigated SSR markers to differentiate the maize lines, they performed a cluster analysis according to the presence/absence of particular size of alleles (Fig. 4).

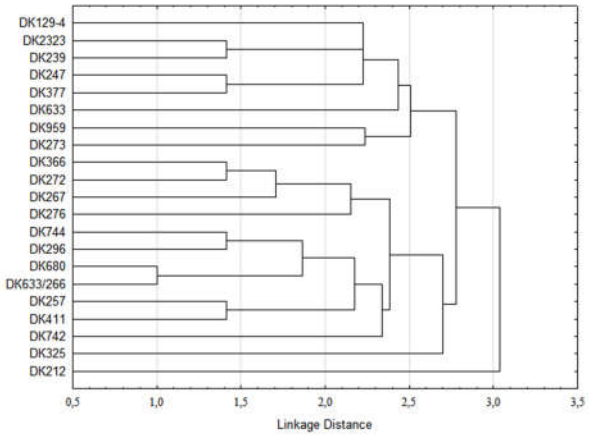

Fig. 4. Distribution of maize lines according to the degree of affinity based on SSR markers

In consequence of the maize lines distribution according to SSR markers, seven clusters were obtained according to the genetic distances between the lines. It was found that the most similar were the lines included in one cluster DK680 and DK633/266, the value of genetic distances between them was 1.0. The most distant lines that formed the same cluster were lines DK959 and DK273 (genetic distances 2.24). According to the obtained data, the most distant line was DK212. The value of genetic distance towards to other studied lines was from 2.83 to 3.46. Accordingly, the most distinguished lines were DK212 and DK2323, genetic distances between which were the strongest. The DK325 line was also not included in any cluster and was at a distance of 2.45-3.16. Consequently, it can be concluded from the obtained data that a marker system of six SSR markers is effective for the differentiation of 21 studied maize lines.

However, it should be noted that comparing the results of cluster analysis of maize lines by means of protein markers and DNA markers, the difference between clusters was noted. It was determined that genetically close lines according to the spectra of storage proteins have a significant genetic distance according to SSR markers. Thus, the closest lines by the protein markers DK272 and DK273 were sufficiently distant from SSR markers, genetic distances value was 2.83. The same situation was observed with another pair DK742 and DK325 lines, which were close in zein spectra, with genetic distances for SSR markers 2.45.

To determine the relationship between genetic distances for protein and DNA markers, Mantel correlation analysis was performed (linear correlation Pearson) (Fig. 5).

As a result of the analysis, measures of calculated significance level p-value and the correlation coefficient r (AB) for the theoretical significance level α=0.05 were determined, which, according to the interpretation of the test, allowed to accept one of the analysis hypotheses about the presence (Ha) or the absence of correlation (H0).

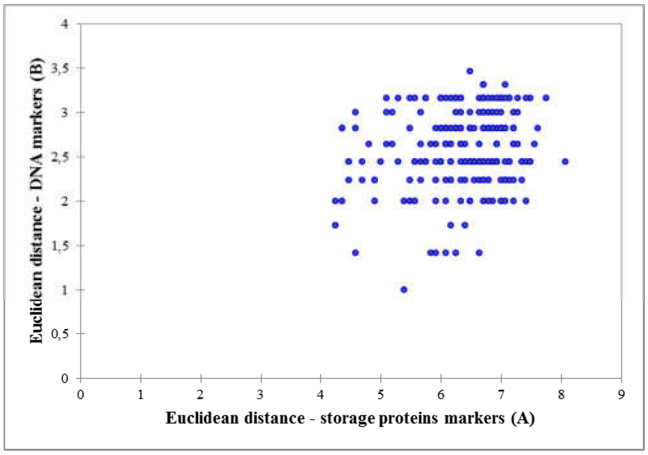

Fig. 5. Relationship between genetic distances of the maize lines according to protein and DNA markers

It is known that the hypothesis H0 about the absence of correlation is accepted on the assumption that $p>\alpha$. As a result of our research, calculated p-value (0.009) was lower than the significance level $\alpha=0.05$, therefore, it is necessary to accept alternative hypothesis Ha about the presence of correlation (Diniz-Filho *et al.*, 2013). The correlation coefficient r(AB) is 0.18. Consequently, as a result of the analysis, the presence of a weak correlation between marker systems for the determination of the maize lines' polymorphism according to the spectra of storage proteins and DNA markers was determined.

It is known that zeins are encoded by families of closely related structural genes located in two chromosomes of maize (Soave *et al.*, 1981; Soave and Salamini, 1982). Based on individual clones hybridization with genomic DNA, it has been proven that zeins are coded by 150 genes (Zayakina *et al.*, 1998), which are located on chromosomes 4 and 7 (Wilson *et al.*, 1989). Based on the study of linkage between zein coding genes by applying isoelectric point electrophoresis of zeins in polyacrylamide gel and isoelectric point electrophoresis in agarose gel. Two clusters are located on a short shoulder of 4th chromosomes and one cluster on 7th chromosomes.

According to data presented by Smith *et al.* (1997) microsatellites, which we used in our work, are localized on different chromosomes. In particular, it is reported that microsatellite reiterations phi079 and phi034 are located on chromosomes 4 and 7, respectively. However, it should also be noted that other markers are localized on distinct chromosomes: phi073 - on chromosomes 3, phi085 on 5, phi022 on 9 and phi062 on 10 chromosomes. Considering that only microsatellite markers phi079 and phi034 have the same spatial localization with zein encoding genes, it can be assumed that this fact is due to the presence of a weak correlation between marker systems.

It is known that the distribution of maize maturity groups according to FAO is based on the estimation of the leaves number per plant, the length of the vegetation period and the sum of effective temperatures (Andriuscenko and Kryvyckyi, 2007; Bavec and Bavec, 2002). Excluding the component of the regional placement (the sum of effective temperatures), characteristics such as the number of leaves and the length of the vegetation period are genetically determined. As a result of the research, it has been determined that there is a moderate correlation between the FAO indexes of the studied lines and the presence of zein components with different electrophoretic mobility. It has been established that the component of zein with Rf 50 is a characteristic for the maize lines of middle-early, mid-season, and middle-late maturity groups (FAO>250), the correlation coefficient is 0.44. The correlation between the presence of a component with Rf 47 in maize lines, FAO of which was less than 230, i.e. in the middle-early and mid-season lines (correlation coefficient 0.48), was also determined. For components with Rf 34 and Rf 57 inverted correlation with FAO indicator was estimated. Consequently, the correlation coefficient -0.68 shows that lines with FAO less than 230 (middle-early and mid-season groups) the Rf 34 component is absent. Its presence is noted only in the middle-early and middle-late lines. The presence of a component with Rf 57

is noted for lines with FAO 190-210 and 280-320 (correlation coefficient -0.44). Sidorova *et al.* (2012) obtained similar data, according to the results of research the component with Rf 57 was identified in early-season maize groups. Thus, it has been established that a positive correlation between FAO and zein components with particular electrophoretic mobility indicates that with increasing of FAO the frequency of the corresponding component in particular mature group of maize increases.

The conducted studies allowed revealing correlation on the level of 95% between FAO index of the maize lines and presence of particular allele for several studied SSR markers. Consequently, moderate correlation between FAO indexes and identified alleles with phi034 marker was determined. It was found that the presence of allele with a size 138 bp is distinctive for the early-season and middle-early maturity group, and 142 bp allele is identified predominantly in the middle-early, mid-season and middle-late groups. The correlation coefficient was 0.45. The phi085 marker has a moderate inverse correlation (correlation coefficient -0.32), which shows that with increasing of 230 bp allele frequency, the FAO index decreases. That is noted that the allele of the specified size is distinctive for the overwhelming majority for lines with FAO<220, which belong to the middle-early and early-season groups. Weak correlation was identified for phi062, phi079 and phi022 markers, the correlation coefficients were 0.20, 0.18 and 0.17, respectively. No correlation dependencies were discovered for the phi073 marker. This indicates the lack of regularities of the particular allele's presence according to these markers and the belonging of the studied lines to the particular maturity groups.

It should be noted that the mechanism of leaves' initiation and the duration of the vegetation period have a polygenic structure of the coding and regulating genes, the effect of which is related to the activity of auxins and cytokinins (Wang *et al.*, 1999; Sinha, 1999; Werner *et al.*, 2001; Juarez *et al.*, 2004; Ezhova and Vu, 2008; Alter *et al.*, 2016; Li *et al.*, 2016). On this basis, without further research, it is not possible to determine the nature of the correlation between FAO indexes and molecular genetic markers. However, the presence of such dependencies allows indirectly predict a group of lines' maturity or obtained on its basis hybrid combinations, based on particular identified zein components or alleles, which were identified by the investigated DNA markers. Thus, obtained results show the effectiveness of using molecular genetic markers for the evaluation not only the genetic diversity of maize lines, but also their application in comprehensive assessment of the economic characteristics of lines and selection material.

CONCLUSIONS

As a result of the studies, polymorphism of 21 of the maize lines according to the DNA markers and seeds' storage proteins was determined. It was identified that the most similar according to zein spectra were DK742 and DK325, DK272 and DK273 lines, genetic distances between them was 4.24. It is noted that the most similar lines differed by at least five components. The correlation between zein components with different electrophoretic mobility was determined, which

indirectly confirms the complex organization of zein coding genes. It was shown that the distribution of lines according to SSR markers differed from the distribution obtained by electrophoretic spectra of zein. It was found that the most similar lines according to SSR markers were lines DK680 and DK633/266, genetic distances between them was 1.0. The polymorphism level of the investigated marker system varied from 0.18 (phi062) to 0.83 (phi085) and averaged on the level 0.59. It was determined that there was correlation between two marker systems. The obtained correlation coefficient (r(AB)=0.18) indicates a weak correlation between the genetic distances of the studied lines according to protein and SSR markers. Correlation relationship between FAO indexes of the studied lines and presence of particular zein components or alleles according to SSR markers have been determined. Obtained data regarding usage of molecular genetic markers indicates the possibility of their usage for the maize lines evaluation in order to determine the most favorable combinations for breeding. Correlation bonds, which were identified for studied parameters, can be used in breeding work to predict the future characteristics of the obtained lines and hybrids.

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