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The possibilities of GAIA method application for DUS examination in Ukraine

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Purpose. To determine the applicability of the GAIA method for comparison of reference collection of maize lines based on weights of the difference for morphological characteristics and SSR markers. Methods. Field methods (descriptive plant morphology), molecular methods (PCR, capillary electrophoresis), and statistical methods (principal component analysis, correlation analysis). **Results.** The study examined 57 lines of maize reference collection to determine their differences based on phenotypic and molecular distances using the GAIA method. The comparison of maize lines, considering the difference for morphological characteristics, identified 12 lines classified as "Distinct Plus" compared to other studied maize lines. The obtained data indicate that most of the "Distinct Plus" lines were classified as distinct according to distinctness, uniformity, and stability (DUS) testing. However, three pairs of lines identified as "Distinct Plus" were classified by the DUS expert as similar or very similar. It was determined that the first two principal components explain 23.37% of characteristic variability. Principal component analysis revealed that the high level of variability attributed to the differences of grouping characteristics and traits which are not used for variety grouping during DUS testing. This suggests that to enhance the effectiveness of the GAIA method, it is advisable to increase weights of the difference for qualitative morphological characteristics. Based on the combination of phenotypic and molecular distances, an additional 35 pairs of maize lines were identified with a high degree of distinction, eliminating the need for side-by-side field comparisons in the next growing season. **Conclusions.** The application of the GAIA method for maize line analysis helps reduce the number of side-by-side field comparisons by integrating morphological traits and molecular markers.

Keywords: *weights of the difference for morphological characteristics; SSR markers; principal components; phenotypic and molecular distances; maize.*

Introduction

The use of molecular markers in plant variety evaluation has become routine, providing an additional method of DUS testing. This reduces the number of side-by-side field comparisons and helps to select varieties for the reference collection. It also helps to determine specific traits, such as resistance to diseases or environmental stressors and starch type [1, 2].

The International Union for the Protection of New Varieties of Plants (UPOV) has approved three models for using molecular markers in DUS testing: gene-specific marker use, combining phenotypic and molecular distances to select varieties of reference collection, and using calibrated molecular distances to manage refence variety collections [3].

A number of UPOV member states use molecular markers in the initial stage of DUS testing, combining phenotypic and molecular distances to manage a reference collection [4]. Among these molecular analysis methods, DNA

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markers, particularly SNP (single nucleotide polymorphism) and SSR (simple sequence repeat) markers, are widely used. Although SNPs, particularly when used with KASP (competitive allele-specific PCR) technology, are the most commonly used as they allow maximum automation of the polymerase chain reaction (PCR) and data analysis, SSR markers remain a reliable research method due to their high reproducibility and ability to create a genetic profile of a variety. This profile can be used in future research, particularly to confirm varietal purity in post-control testing or in cases of intellectual property rights infringement at the initial stage of establishing such infringement [5–7].

Although the combination of molecular methods and phenotypic evaluation of varieties is widely used among UPOV member states, recent studies indicate that this approach must be validated for each country, considering the species composition of botanical taxa for DUS testing and the type of molecular markers used [8–10].

Given the increasing number of new plant varieties being examined by the DUS for intellectual property rights, the range of morphological characteristics used to determine compliance with DUS criteria is narrowing. Therefore, it is important to use molecular markers, particularly DNA markers, when examining plant varieties for DUS, to increase the accuracy of the examination and reduce labor costs for side-by-side field comparisons research, as well as reducing the number of varieties in the reference variety collection. One approach to using DNA markers to determine differences between varieties during DUS testing is the GAIA method. This method calculates phenotypic distances between each pair of varieties, which are the sums of the distances between each individual trait according to a particular methodology's table of traits. In combination with molecular markers, comparisons are carried out using molecular distances [11].

The aim of the research is to determine the suitability of the GAIA method for comparing maize lines based on the weighting coefficients of differences in the degree of manifestation of morphological traits and SSR markers.

Materials and Methods

The research was conducted at the Ukrainian Institute for Plant Variety Examination (UIPVE) between 2021 and 2023. One hundred and fourteen maize lines from the common knowledge variety collection were analyzed. Of these, 38 were in the first year of testing, 66 were common knowledge varieties and 10 were lines from the reference variety collection. Fifty-seven lines were selected by the expert for comparative evaluation of the GAIA method and expert evaluation of the differences between the lines, including five reference lines.

Determination of morphological characteristics

The morphological characteristics of the studied lines of maize were determined in the field at the Poltava and Kirovohrad affiliates of the UIPVE, in accordance with the methodology for examining varieties of maize (*Zea mays* L.) for DUS, according to 35 qualitative and quantitative traits [12]. The degree to which qualitative morphological features were manifested was indicated by numerical values from 1 to 9. Quantitative traits were presented as the absolute values of measurements taken at two research points.

Principal component analysis was used to determine the variability of morphological traits using the XLSTAT trial version computer program [13].

Determination of molecular characteristics

To determine the molecular characteristics, PCR analysis of the maize lines was performed using nine SSR markers, taking into account the Polymorphic Index Content (PIC), according to the following protocol: phi064, umc1448, umc1061, bnlg1782, bnlg1129, phi093, phi233376, phi083 and phi96100 [14]. The PCR products were visualised by capillary electrophoresis using an Agilent Fragment Analyzer (USA). The DNF 905 reagent kit (dsDNA 905 Reagent Kit, 1–500 bp, 55 cm matrix length) was used and the analysis was performed according to the manufacturer's protocol.

Based on the obtained data, the presence or absence of a particular allele in the maize lines under study was indicated as 1/0. The R programming language was used to convert the sizes of the alleles obtained into binary code and calculate Roger molecular distances [15].

Determining differences between maize lines using the GAIA method

GAIA software, provided free of charge by GEVES (the official French organization that evaluates new varieties), was used to analyze maize lines for the purpose of determining "Distinct Plus" lines, i.e. lines that exceeded the threshold value of difference in phenotypic distances. To prepare the analysis data in GAIA, a database was created containing downloadable files with the following information: type of crop being tested; test points (two geographical locations); list of lines (application number and line denomination); years of testing; morphological traits (qualitative and quantitative), molecular data (names of SSR markers and sizes of identified alleles); significance matrices of the

difference in the degree of manifestation of each trait (for quantitative traits, the upper and lower limits of the difference in weight between two lines were calculated at 15% and 20% of the average for each experiment); molecular distance types (Roger's distances); the degrees of manifestation of the traits for each line were calculated for qualitative and quantitative traits, as well as for molecular characteristics by SSR markers and molecular distances; session parameters were used to compare varieties with each other and with lines from the reference variety collection. This included threshold values for differences in the total weight of the studied characteristics, such as qualitative and quantitative morphological traits and SSR markers.

To analyze and determine the differences between the studied lines using phenotypic and molecular distances, a session was created with the following parameters: a threshold value of 8, a phenotypic limit of 6, and a genotypic limit of 0.30. According to the analysis algorithm, the difference between the maize lines was first determined by qualitative morphological traits, for which the overall significance of the difference was greater than or equal to the threshold value. Then, the lines that were not "Distinct Plus" were compared by quantitative traits [11].

Research results

Comparisons of maize lines according to established parameters for a combination of qualitative and quantitative morphological traits identified 12 lines as "Distinct Plus": LN26, LN41, LN56, LN54, LN50, LN17, LN51, LN27, LN39, LN11, LN47 and LN46. The largest number of lines (35 and 36 pairs, respectively) had phenotypic distances of 7 and 6 (Fig. 1).

Based on the results of the comparisons, it was determined that most of the lines found to be "Distinct Plus" were also identified as "Distinct" in the DUS testing results. However, the expert classified pairs of lines LN56 and LN53, LN54 and LN57 as similar, and lines LN40 and LN27 as very similar; the difference between these could only be determined using additional molecular methods. Conversely, lines LN50 and LN39 were not assigned to any group as they were found to be different according to the DUS testing results. However, the expert did not identify a sufficiently different pair among the tested lines [16]. Obtaining such a result may be due to the peculiarities of the GAIA method and depend on the different significance of the differences in traits that are taken into account by the expert when grouping maize lines and are not taken into account during the analysis using the GAIA method. The DUS expert determines the significance of the difference in the degree of trait expression based on professional knowledge and experience using the 'try-and-verify' approach [11]. As this is the first time the method has been tested in Ukraine, it is assumed that the difference in the degree of manifestation of the grouping features recommended by the methodology [12] is maximum. The significance of differences in other qualitative traits was determined by the reliability with which each trait was manifested under certain environmental conditions, taking genetic variability into account.

To determine the variability of the morphological traits of the studied lines, and to use the results to improve the determination of the significance of differences in quality traits, the principal components method was employed. The results of the principal components analy-

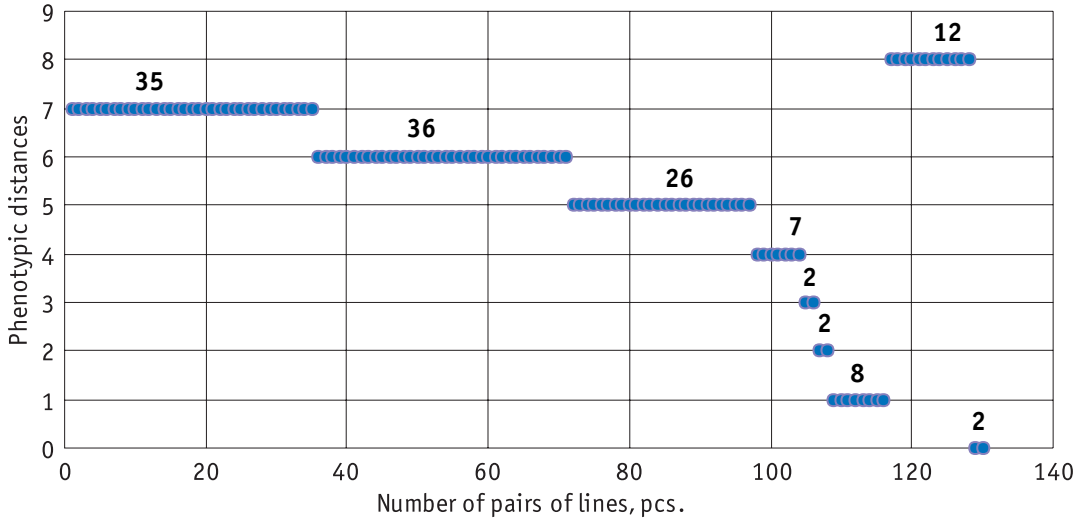

Fig. 1. Phenotypic distances between maze lines

sis of maize morphological traits revealed that only 12 out of 35 components were significant at a level greater than 1.0, accounting for approximately 75.14% of the variability among the studied traits (see Table 1).

Table 1

Significance of the principal components for the morphological traits of the maize lines

Principal components (PC)	Eigenvalues	Variability, %	Cumulative, %
PC1	4.578	13.081	13.081
PC2	3.601	10.289	23.370
PC3	2.611	7.460	30.830
PC4	2.339	6.682	37.512
PC5	2.271	6.488	44.000
PC6	2.028	5.794	49.794
PC7	1.927	5.507	55.301
PC8	1.599	4.569	59.870
PC9	1.519	4.340	64.210
PC10	1.391	3.974	68.184
PC11	1.318	3.765	71.949
PC12	1.117	3.191	75.140

It was determined that PC1 accounted for 13.081% of the variation in maize morphological traits in this study, PC2 for 10.289%, and PC3 for 7.460%. PC1 is associated with trait 13 (panicle: position of lateral branches in space), and PC2 is associated with trait 17 (stem: anthocyanin color of aerial roots) (Table 2).

According to the obtained data, the most influential trait in explaining the variability of PC11 is trait 38 (ear: color of the top of the grain, with a value of 0.522), while trait 7 (stem: degree of zig-zag) is associated with PC12. Notably, PC1 and PC2, which together explain 23.37% of the variability among the traits, were unaffected by the manifestation of grouping traits. However, in PC3, the grouping traits were the most decisive, namely trait 8 (tassel: time of anthesis) and trait 24.1 (plant length: quantitative trait).

In [17], maize lines were studied for agromorphological traits in order to identify patterns of variation in morphology. The results obtained by the authors showed that PC1 explained 54.794% of the variation in traits and was associated with plant length and ear length. PC2 was responsible for variation in 1000-seed weight, while PC3 was responsible for the

Table 2

Eigenvectors of principal components for morphological traits of maize lines

Trait	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
1	0.010	0.012	−0.076	−0.227	**0.446**	−0.003	−0.102	−0.022	−0.087	−0.165	0.207	−0.218
2	−0.105	0.231	0.130	−0.076	−0.202	0.042	0.235	−0.058	0.133	−0.083	0.133	−0.162
3	0.021	0.065	−0.049	**0.358**	−0.087	−0.216	0.153	0.051	−0.159	−0.022	0.188	−0.311
4	0.173	0.041	−0.037	0.294	−0.006	−0.062	0.250	−0.030	0.154	0.048	−0.310	**0.307**
5	0.246	0.113	0.215	0.286	0.026	−0.027	−0.082	−0.217	−0.094	−0.227	0.138	0.121
6	0.222	−0.078	0.300	0.086	0.088	0.068	0.101	−0.061	0.244	0.269	0.120	0.129
7	−0.058	−0.135	0.214	−0.121	0.205	−0.067	0.208	−0.028	−0.056	−0.139	−0.106	**0.541**
8**	0.015	0.196	**0.318**	−0.200	−0.205	−0.077	−0.165	−0.113	−0.282	0.021	−0.079	0.054
9**	0.092	0.208	0.166	−0.027	0.093	−0.295	0.205	0.042	0.061	−0.172	−0.321	−0.212
10**	0.224	0.232	−0.124	0.072	−0.052	0.127	0.070	0.084	0.016	−0.248	0.119	−0.013
11**	0.063	0.278	0.164	−0.281	−0.083	0.117	−0.092	0.177	0.067	−0.053	−0.196	0.203
12	0.284	0.010	0.068	0.143	0.171	0.143	0.089	0.078	−0.304	−0.175	−0.028	−0.054
13	**0.344***	−0.018	0.094	0.168	0.119	0.133	−0.026	−0.011	−0.138	−0.044	0.006	0.014
14**	0.070	−0.072	0.145	−0.195	0.084	**0.419**	0.060	0.005	0.137	−0.213	0.040	−0.064
15**	0.155	0.263	0.024	−0.117	−0.229	−0.094	−0.168	−0.188	−0.284	−0.096	0.168	0.144
16**	0.194	0.227	−0.083	−0.073	−0.030	0.082	−0.122	0.100	**0.381**	0.207	−0.025	−0.085
17	0.073	**0.379**	0.027	0.043	0.105	−0.057	−0.030	0.125	0.040	−0.005	−0.182	−0.180
18	−0.014	−0.193	−0.105	0.109	−0.028	0.048	0.274	**0.490**	−0.169	−0.111	−0.035	0.105
19	−0.145	0.180	0.059	0.062	0.017	0.223	0.128	0.131	−0.287	**0.449**	0.057	0.003
20	−0.218	0.143	−0.009	0.157	0.177	**0.375**	0.001	−0.093	−0.216	0.096	−0.038	0.005
21	0.191	−0.264	0.155	−0.150	0.096	−0.055	−0.178	−0.252	−0.003	0.047	−0.185	−0.187
22	0.284	−0.192	−0.298	−0.015	0.027	−0.149	−0.126	0.029	0.012	0.141	−0.076	−0.015
23	0.207	−0.174	0.174	0.182	−0.092	−0.011	−0.259	0.194	−0.094	0.022	0.188	0.056
24.1**	0.038	−0.152	**0.345**	−0.205	0.028	0.035	0.146	0.191	−0.203	0.281	−0.040	−0.205
25	−0.057	0.145	−0.088	0.116	−0.228	**0.361**	0.110	−0.126	0.061	−0.046	−0.018	0.038
26	0.100	0.083	−0.252	−0.280	−0.123	−0.132	−0.002	**0.325**	−0.153	−0.165	−0.079	0.148
27	0.293	0.002	−0.020	−0.095	0.055	0.269	−0.059	0.269	0.159	−0.029	0.155	−0.080
28**	0.198	0.113	0.014	0.032	0.046	−0.121	**0.307**	−0.201	0.123	0.189	0.000	−0.034
29**	0.106	−0.074	−0.210	−0.270	−0.041	0.046	**0.316**	−0.291	−0.109	0.013	**0.306**	0.128
30**	−0.218	0.050	−0.063	0.119	**0.303**	0.071	−0.157	−0.088	0.183	−0.242	0.052	0.102
31**	0.138	−0.043	−0.095	−0.223	0.096	0.051	**0.401**	−0.158	−0.076	−0.097	−0.072	−0.191
36**	0.166	−0.149	−0.173	−0.052	−0.393	0.079	0.008	−0.117	0.016	0.050	0.054	0.016
38**	−0.021	0.092	0.209	−0.081	0.063	−0.230	0.107	0.221	0.242	0.055	**0.522**	0.151
39**	−0.196	−0.153	0.244	0.066	−0.205	−0.115	0.123	0.051	0.067	−0.297	0.124	−0.174
41**	−0.009	−0.257	0.199	0.036	−0.316	0.217	−0.002	0.015	0.141	−0.208	−0.177	−0.128

* Eigenvectors of the principal components with an absolute value greater than 0.3 are marked in red;
**Grouping features according to the methodology [12].

number of ear rows, ear length, and plant length. However, the authors did not demonstrate variability in the qualitative morphological features used for DUS testing. They explained this by stating that only three principal components had weights greater than one. Similar studies were conducted by the authors [18–20]. These studies aimed to identify the principal components of variation in valuable economic traits, such as yield, 1000-seed weight, ear length, number of grains per row and number of rows of grains. The authors generally studied morphological traits affecting maize maturity and yield, such as the number of days to flowering and silking in 50% of plants, no of grains per the ear and ear quantity without focusing on morphological traits that determine the difference between lines.

However, if we consider other studies in which the authors focused on the variability of morphological traits that determine differences between varieties during DUS testing, this variability is due to the manifestation of qualitative and quantitative traits, as well as traits that are not recommended for grouping varieties. Work [21] shows that PC1 exhibits the greatest variability (23.09%), associated with leaf shape (0.963), spot intensity on petals (0.963), and the boll surface (0.963). The first characteristic is used for grouping in DUS testing, while the second and third are not. PC2 accounted for 9.66% of the variability and included traits such as the pubescence of the leaf hairiness (0.604), the growth habit (0.579), the seed fuzz density (0.394) and the flower petal color (0.273). Of these traits, only flower petal color is a grouping trait [22].

Similar results were obtained by the authors of [23], who conducted a comparative analysis of morphological assessments for DUS in the field and of DNA genotyping using SNP markers in cucumbers. The authors determined through principal component analysis that the greatest variability was due to the fruit length-to-diameter ratio and the presence or absence of warts. However, these traits were not useful for grouping during DUS testing. However, traits such as fruit length, the plant sex expression, and the ground color of fruit skin at market stage also showed a high percentage of variability and are grouping traits [24]. Our research also yielded similar results from the analysis of the principal components based on the morphological characteristics of maize studied during the DUS testing. This indicates that, in order to apply the GAIA method to determine the significance of differences in trait manifestation, attention must be paid to adjusting the weighting values for traits that caused a high percentage of variability in the principal component analysis.

According to the GAIA analysis algorithm, after a cycle of comparisons based on qualitative and quantitative morphological traits, line pairs that were not “Distinct Plus” but overcame the established phenotypic limit of 6 (35 line pairs) were compared in the next cycle of comparisons based on molecular distances.

The genotypic boundary shows that, when choosing a comparison based on qualitative and quantitative, or qualitative/quantitative and molecular distances, phenotypic and molecular distances will be combined for a pair of lines whose molecular distances are greater than or equal to 0.30. In other words, when combining qualitative and quantitative or qualitative/quantitative and molecular distances, both analyses will only be taken into account for pairs of lines that meet the following conditions simultaneously: a phenotypic limit greater than or equal to 6 and a molecular distance greater than or equal to 0.30.

As a result of comparing maize lines based on qualitative and quantitative traits and molecular distances, 12 “Distinct Plus” lines were obtained that differ from the comparison group only in terms of qualitative and quantitative traits. A similar example is described in UPOV TGP/8, where electrophoresis results confirm the presence of “Distinct Plus” varieties for qualitative and quantitative traits. However, the molecular distance approach identified additional pairs of “Distinct Plus” lines based on established phenotypic and genotypic boundaries. Based on these comparisons, four zones were identified on the results display graph in which pairs of maize lines were distributed according to their phenotypic and molecular distances (see Fig. 2).

In accordance with the defined conditions for combining morphological and molecular distances, zone 4 is important when deciding whether to compare lines side-by-side in the field.

Zone 4 contains maize lines that exceed the established limits in terms of both phenotypic and molecular distances (phenotypic limit of 6 and genotypic limit of 0.30). Therefore, these pairs of lines can be considered “Distinct Plus” and do not require side-by-side testing in future research years. According to the obtained data, Zone 4 included 35 pairs of maize lines under study (see Fig. 3).

Two pairs of lines in Zone 1 deserve special attention: LN23 and LN55, and LN30 and

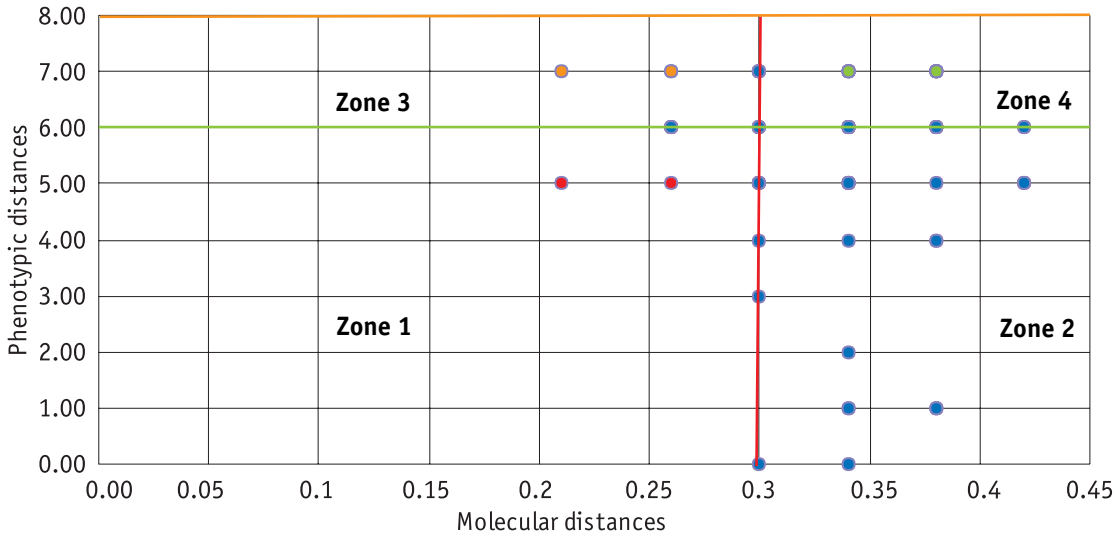

Fig. 2. Results of comparisons of maize lines by qualitative, quantitative traits and molecular distances in graphical form

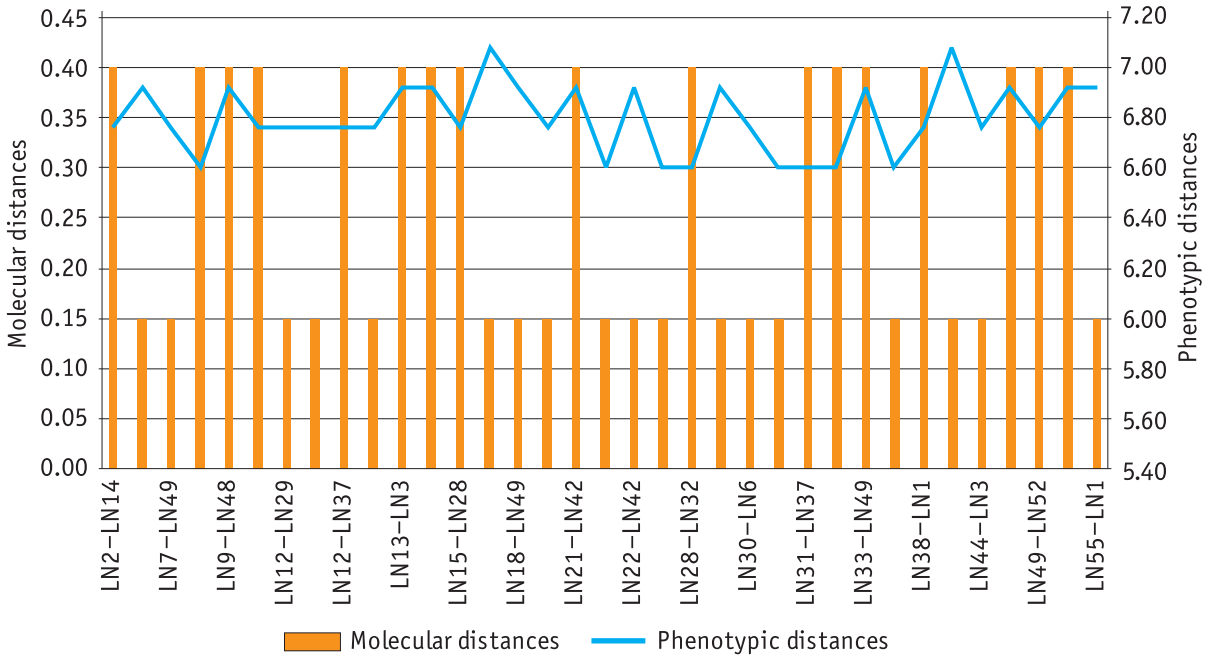

Fig. 3. Maize lines included in zone 4 by total phenotypic and molecular distances

LN37. According to the values obtained for total significance by phenotypic and molecular distances, these lines were the most similar. It is worth noting that the line pairs identified as the most similar based on a comparison of phenotypic and molecular traits differ from the conditional distribution of the studied lines, as determined by an expert based on the results of the DUS testing [16]. For instance, line LN30 was identified as being highly similar to line LN29, whereas line LN23 was classified as being similar to line LN25.

Paper [9] presents the results of an integrated approach to evaluating new alfalfa varieties, combining genotyping by sequencing with morphological traits using the GAIA method. The authors demonstrate the effectiveness of combining molecular analysis and morphological traits to determine the significance of differences in trait expression. As the GAIA method is one of the UPOV-approved approaches for identifying differences between varieties during DUS testing and is employed in routine testing of new varieties, it is also being considered for studying significantly derived varieties [25]. The effectiveness of combining phenotypic and molecular distances to distinguish durum wheat varieties was also demonstrated in [26].

Our research indicates that the GAIA method can be used in DUS testing to determine

differences in the combination of phenotypic and molecular distances in maize lines in Ukraine. However, while many UPOV member states use molecular markers as part of DUS testing, in Ukraine there are currently certain legal restrictions on the use of molecular methods, which create additional difficulties for detecting differences in new varieties given the significant increase in number of common knowledge varieties. Additionally, for botanical taxa represented by hybrids, UPOV defines testing approaches for hybrids and their parental components. When combining phenotypic and molecular distances, for example, the total significance of the difference is determined based on the weights of the parental components rather than by comparing hybrid to hybrid [11]. Currently, this approach is limited in Ukraine, as according to the legislation, the parental components of a hybrid undergoing DUS testing are not made available for research purposes [28]. This, in turn, leads to problems with outdated methods being used, creating additional obstacles to international cooperation in plant variety rights protection and maintaining the current level of DUS testing.

Thus, the research shows that it is currently possible to use modern research methods, particularly molecular markers, to minimize the risk of disclosing genetic information about the varieties under study by comparing only statistically processed data.

Conclusions

The study of 57 lines of maize revealed that, based on the significance of the difference in the manifestation of morphological traits using the GAIA method, 12 "Distinct Plus" lines were identified that do not require side-by-side comparisons in the field during the next vegetation cycle.

Based on the data obtained from the principal component analysis, it was determined that, to improve the identification of significant differences in qualitative morphological traits, the range of traits for which maximum significance values are set should be expanded beyond grouping traits.

An additional 35 pairs of maize lines were identified as sufficiently different based on the results of combining phenotypic and molecular distances, and these lines do not require side-by-side comparisons. The use of molecular markers has been identified as a powerful modern auxiliary tool for DUS testing that allows reliable results to be obtained using methods approved and used by UPOV member states.

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Присяжнюк Л. М., Стариченко Є. М., Таганцова М. М., Шитікова Ю. В., Гринів С. М., Стадніченко О. А. Можливості застосування методу GAIA для кваліфікаційної експертизи на ВОС в Україні. *Plant Varieties Studying and Protection*. 2025. Т. 21, № 1. С. 52–59. https://doi.org/10.21498/2518-1017.21.1.2025.327502

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Мета. Визначити придатність методу GAIA для порівняння ліній кукурудзи звичайної на основі вагових коефіцієнтів відмінності ступенів прояву морфологічних ознак та SSR маркерів. **Методи.** Польові (описова морфологія рослин), молекулярні (ПЛР, капілярний електрофорез), статистичні (метод головних компонент, кореляційний аналіз). **Результати.** Досліджено 57 ліній кукурудзи звичайної колекції загальновідомих сортів для встановлення відмінності на основі фенотипових і молекулярних дистанцій методом GAIA. 12 ліній виявилися «Відмінними плюс» до інших досліджуваних, що визначено за результатами їх порівняння з урахуванням вагомості різниці ступенів прояву морфологічних ознак. Згідно з отриманими даними серед «Відмінних плюс» ліній більшість класифіковано як відмінні за результатами експертизи на відмінність, однорідність і стабільність (ВОС), втім три пари експертом зараховано до групи подібних і дуже подібних. З'ясовано, що перші дві головні компоненти пояснюють 23,37% варіабельності ознак. За результатами аналізу головних компонент встановлено, що високий рівень варіабельності зумовлений відмінностями за групувальними ознаками та ознаками, які не використовують для групування сортів під час експертизи на ВОС. Це свідчить про те, що для підвищення ефективності застосування методу GAIA доцільно збільшити вагомість відмінності різниці ступенів прояву цих ознак. Внаслідок поєднання фенотипових і молекулярних дистанцій визначено додатково 35 пар ліній кукурудзи, що мають високий ступінь відмінності та не потребують прямих порівнянь у польових умовах в наступному вегетаційному циклі. **Висновки.** Встановлено, що застосування методу GAIA для дослідження нових ліній кукурудзи допомагає зменшити кількість прямих порівнянь у польових умовах за поєднання морфологічних ознак і молекулярних маркерів.

Ключові слова: *вагомість різниці ступенів прояву ознак; SSR маркери; головні компоненти; фенотипові та молекулярні дистанції; кукурудза звичайна.*

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