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THE COMPARISON ANALYSIS OF SOFTWARE FOR MANTEL TEST BETWEEN DNA MARKERS AND MORPHOLOGICAL TRAITS OF PLANT VARIETIES

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ABSTRACT

An advanced approach to the assessment of varieties for the determination of their differences both within the DUS test and breeding is a combination of morphological traits and DNA markers. An implementation of this approach includes a correlation assessment between genetic distances matrices. To this end, the Mantel test is applied. The purpose of the study was to identify the main advantages and disadvantages of different software products for the Mantel test based on correlation investigation between DNA markers and morphological traits of lettuce varieties and maize lines. As a result of correlation calculation between 8 SSR markers and 36 morphological traits of 100 maize lines by XLSTAT (software for Microsoft Excel) p-value (probability of obtaining test results) was 0.0005. The value of this indicator obtained by PASSaGE software was 0.034. There was a p-value of 0.045, which was calculated by GenAlEx 6.5 in Microsoft Excel (MS Excel). A similar result (0.036) was obtained by software environment R. The p-values, which were calculated between 7 EST-SSR markers and 32 morphological traits for four lettuce varieties by XLSTAT, PASSaGE, GenAlEx, and R, were 0.033, 0.039, 0.038, and 0.035, respectively. In the study, the upper-tailed test served an alternative hypothesis type, the level of significance α was 0.05, the type of correlation was Pearson correlation, and the Monte Carlo method was used for p-value computation. Thus, the obtained p-values allow to reject the null hypothesis (H0) and adopt the alternative hypothesis Ha of correlation ($p < \alpha$). The correlation coefficient for maize lines was 0.05 and for lettuce varieties 0.65. Therefore, XLSTAT and software environment R are the most suitable instruments for correlation assessment between genetic distances.

Keywords: *correlation coefficient, maize, lettuce, Mantel, p-value.*

INTRODUCTION

In molecular genetic analysis, it is often necessary to compare two sets of distance measures. These measures can be based on genetic markers, morphological traits, geographic separation, ecological divergence, etc. (Smouse *et al.*, 1986). Following UPOV (International Union for the protection of new varieties of plants) principles, the description of plant lines and varieties that are subject to the DUS (distinctness, uniformity, and stability) examination is based on marker morphological characteristics. However, since the beginning of the 2000s, some countries have begun to use additional methods for assessing variety differences at the national level (Prysiazhniuk *et al.*, 2020) and test them at the international level. SSR (Simple Sequence Repeats) is a commonly used technique. The main advantages of SSR markers are a high level of polymorphism, widespread genome distribution, codominant type of inheritance, and the presence of validated methods for their use. Among DNA markers, EST-SSR (Expressed Sequence Tag Single Sequence Repeats), which are related to the expression regions, are widely used for the analysis of the genetic diversity of common species and the study of intraspecific diversity (Leshchuk *et al*., 2021). According to UPOV's core principles for the comprehensive analysis of new varieties, there are several approaches to the involvement of DNA markers: the management of a reference collection of varieties, the study of links between DNA markers and economically important traits, and a combination of varieties assessment by morphological and DNA markers. The combination of DNA markers and morphological characteristics includes the study of the correlation between DNA markers and morphological traits. Mantel test is a commonly recognised method for assessing this correlation (Karuri *et al*., 2010; Riday *et al*., 2003; Hong *et al*., 2015; Huseynova *et al*., 2018; Ho *et al*., 2020).

Today many software products may be used for analysing molecular data by Mantel test (Omelka & Hudecova, 2013; Huseynova *et al*., 2018). The most commonly used software for molecular data and population genetic data analysis are add-on XLSTAT MS Excel, PaSSaGE, GenAlex, and software environment R, which differ from each other with particular features such as type input data, methods of results interpretation, type of indexes calculation, etc. Thus, the purpose of this study was to apply XLSTAT, PaSSaGE, GenAlex, and software environment R for correlation determination between DNA markers and morphological traits of different data types by Mantel test.

MATERIAL AND METHODS

To determine the correlations between DNA markers and morphological traits we used 100 lines of maize (Ukrainian and foreign breeding) and 4 varieties of lettuce (Ukrainian breeding).

Maize lines were examined by 8 SSR markers and 36 morphological traits in the years 2018−2019 within the framework of qualification examination (Prysiazhniuk *et al*., 2019). Lettuce varieties were examined by 7 EST-SSR markers and 32 morphological traits. The description of morphological traits of lettuce was performed during its qualification examination in the years 2010-2015 (Leshchuk *et al.*, 2021).

Genetic distances by SSR markers and morphological traits of maize lines were calculated by the MS Excel add-on XLSTAT product using the unweighted pair-group average method. Jaccard distances of lettuce varieties were obtained by IBM® SPSS® Statistics for EST-SSR markers and by add-on XLSTAT for morphological traits (Leshchuk *et al.*, 2021).

The correlation between DNA markers and morphological traits of maize lines and lettuce varieties was determined using XLSTAT, PaSSaGE, GenAlex, and software environment R. For all software products, the following parameters were selected: type of alternative hypotheses: upper one-tailed; significance level α: 5%; p-values computation: Monte Carlo method; type of correlation: Pearson. The number of permutations was chosen according to the capabilities of the software and the number of test samples (Oden & Sokal, 1992; Dutilleul *et al*., 2000). To rank software products for the Mantel test we used the analytical hierarchy process (Saaty, 1980).

RESULTS AND DISCUSSION

Since the Mantel test allows detecting correlations between distance matrices, the first step was to obtain distance matrices by DNA markers and morphological traits.

Mantel test using the add-on XLSTAT MS Excel

The add-on XLSTAT is a flexible data analysis tool based on MS Excel. The add-on XLSTAT allows calculating genetic distances by binary code and digital codes of marker traits using the Agglomerative Hierarchical Clustering (AHC) tool, with the default number of permutations being 10000.

According to the obtained genetic distances of maize lines by SSR markers, 88 out of 100 distinct lines were selected by 8 SSR markers. Four pairs of maize lines (fertile lines and their sterile analogues) were found identical by 36 morphological traits (Prysiazhniuk *et al*., 2020).

Four lettuce varieties were found distinct by 7 EST-SSR markers and 32 morphological traits.

In order to verify the presence of correlations among the studied maize lines pairs and lettuce varieties by morphological traits and DNA markers, the Mantel test (linear Pearson correlation) was performed using the Correlation/Association test. As a result of the analysis of the genetic distances matrices of maize lines and lettuce varieties by DNA markers and morphological traits using the add-on XLSTAT, a scatter plot, histogram and correlation coefficient were obtained. Shown in Fig. 1 is a scatter plot of maize Mantel test results by SSR markers and morphological traits.

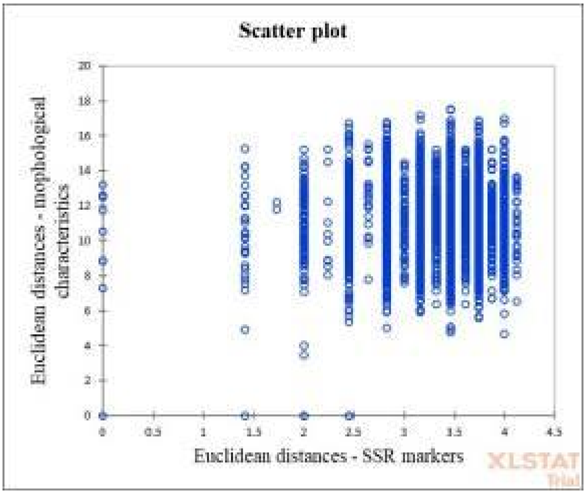


Fig. 1. Relationship between genetic distances of maize lines by SSR markers and morphological traits obtained with XLSTAT

As a result of the Mantel test performed for 100 maize lines, the p-value made up 0.0005 at α=0.05. Therefore, according to the interpretation of the test, an alternative hypothesis Ha about the presence of a positive correlation is accepted. The correlation coefficient r = 0.051.

In the analysis of four lettuce varieties, an alternative hypothesis about the presence of a positive correlation between the genetic distances matrices by EST-SSR markers and morphological traits was also accepted. In this case, the p-value was 0.033 at α=0.05. The correlation coefficient was r = 0.646.

Thus, the add-on XLSTAT is a simple and convenient tool for determining the correlation between the genetic distances matrices by DNA markers and morphological traits. A significant advantage is the convenient data entry, the ability to calculate genetic distances directly in the software environment, which eliminates conflicts with the format of data obtained with other programs, a wide range of test options (correlation type, type of alternative hypothesis, etc.), test interpretation explanation and graphical display of results. However, after the expiration of the trial version, the add-on functions of XLSTAT become significantly limited; therefore, it is necessary to purchase the full version for permanent use.

Mantel test using GenAlex

GenAlex (Genetic Analysis in Excel) is designed as a package with an intuitive and consistent interface that allows user to analyse a wide range of population genetic data within a software environment MS Excel (Peakall & Smouse, 2012). The focus of the program on the analysis of population genetics data imposes certain restrictions on the type of data and algorithms for analysis.

In GenAlex, the calculation of genetic distances by binary codes of DNA markers and codes of morphological traits is performed using the tool Distance-Based (Genetic). However, there is only Nei's genetic identity and distance measures using GenAlex. However, the Mantel test can also be performed on ready-made both triangular and symmetric matrices which must be placed on separate MS Excel worksheets with certain input data on the number of populations, their size, etc. according to the instructions. The Mantel test was performed using the tool Distance-Based (Mantel - Paired). The maximum number of permutations (9999) was chosen for the analysis. As a result of the Mantel test, depending on the selected options for result display (Tri distance matrix), a scatter plot with a regression equation was obtained, which illustrates the relationship between the matrices. In addition, p-value (P (rxy-rand> = rxy-data)) and correlation coefficient (Rxy) are specified.

As a result of the analysis, the calculated p-value was 0.038, which does not exceed α (0.05) and allowed to accept the alternative hypothesis Ha about the presence of correlation, the correlation coefficient is 0.646 and does not differ from the value obtained with other software products. For the studied maize lines, the p-value obtained with GenAlex was 0.045 at α=0.05, which indicated the need to reject the null hypothesis H0 and proves the presence of correlation between the genetic distances matrices by SSR markers and morphological traits. The correlation coefficient value (r=0.051) coincides with those calculated using the other software products.

Thus, GenAlex software allows determining correlations between distance matrices by DNA markers and morphological traits. However, its focus on processing population genetics data requires additional preparation of the source data. GenAlex capabilities impose some restrictions on the data format (the ability to calculate genetic distances, a certain order of matrices, and field labels). Besides, the interpretation of the Mantel test is described only in the program manual and there is no choice of the alternative hypothesis. The advantages of using GenAlex for the Mantel test include its implementation based on MS Excel, the availability of detailed step-by-step instructions and demonstration files with examples of calculation, the ability to select the number of permutations, and graphical presentation of results.

Mantel test using PASSaG 2

PASSaGE 2 (Pattern Analysis, Spatial Statistics, and Geographic Exegesis) is a free software package which implements a significant number of statistical methods for biological and other data types, including cluster and correlation analysis. Since PASSaGE 2 does not provide the possibility to calculate genetic distances from binary or digital data, separate MS Excel workbooks for EST-SSR and SSR markers and morphological traits are developed to import the distance matrix. These workbooks contain symmetric matrices with the results of cluster analysis. Mantel test was performed by selecting this type of analysis in the menu for the following parameters: number of permutations 999 for lettuce varieties and 9999 for maize lines. It should be noted that in PASSaGE 2, the correlation is calculated for three types of alternative hypotheses: two-tailed, left-tailed, and right-tailed.

As a result of the analysis, a significant correlation was found between the genetic distances matrices by EST-SSR markers and morphological traits of lettuce varieties (correlation coefficient r = 0.64587). The need to reject the null hypothesis H0 is evidenced by the result of calculating p-value by the upper one-tailed test (p-value is 0.03900 at α = 0.05).

For maize lines, the upper one-tailed test result indicates the need to accept an alternative hypothesis about the presence of correlation between genetic distances matrices by SSR markers and morphological traits (p-value is 0.03400 at α = 0.05), with the correlation coefficient being 0.0512. Thus, as a result of the evaluation of the correlation between the genetic distances matrices by DNA markers and morphological traits of maize lines and lettuce varieties using PASSaGE 2, it was found that there is a direct positive correlation between the studied matrices, and the obtained correlation coefficients that coincide with the values calculated with the use of the other software products.

One of the significant disadvantages of work in PASSaGE 2 for the Mantel test is that all data, including input, must have a matrix format, which causes some difficulties in formatting the results obtained from other statistical software. In general, PASSaGE 2 can be assessed as easy-to-use software, if we do not consider the input procedure. There are parameters of randomization (permutation test) and the possibility of partial correlation, but there is no flexibility in the settings of the analysis, the output of graphical information, and the indication of the type of correlation. Among positives is a good help system with an explanation of the methods used.

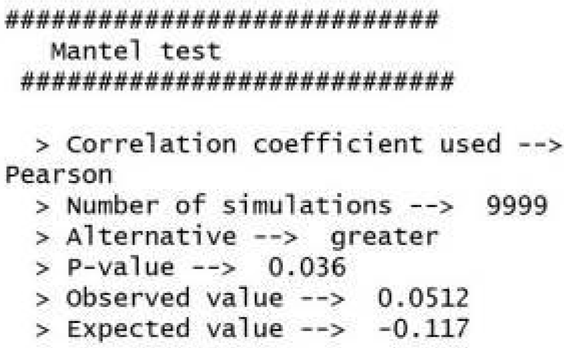
Mantel test using software environment R+EcoGenetics

Software environment R is a cross-platform open-source software environment for statistical calculations. The EcoGenetics package has the most opportunities for analysis. It is designed to simplify and speed up data analysis procedures in the fields of ecology and genetics. Also, it has functions for the analysis of spatial autocorrelation and interactive visual data environment.

Software environment R allows calculating genetic distances both by the binary code of DNA markers and digital codes of morphological traits. In addition, this software environment allows importing data from many possible formats, including MS Excel worksheets.

To assess the correlations between DNA markers and morphological traits of lettuce varieties and maize lines using software environment R as a source data we used symmetric genetic distances matrices. In the Mantel test, we used an *eco.mantel function.* Before using the function we connected the EcoGenetics library. The following *eco.mantel* function arguments were used to calculate the correlation: **method =«pearson», nsim = 9999, alternative = «greater»**. The displayed results are shown in Listing 1.

Listing 1. Mantel test results by SSR markers and morphological traits of maize lines



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##############################
   Mantel test
 ##############################

  > Correlation coefficient used -->
Pearson
  > Number of simulations -->  9999
  > Alternative -->  greater
  > P-value -->  0.036
  > Observed value -->  0.0512
  > Expected value -->  -0.117
```

As a result of the calculations, the correlation coefficient (r) is equal to 0.0512 and does not differ from the value obtained by using other software products, and p-value of 0.036 at α = 0.05 indicates the need to accept an alternative hypothesis Ha about the positive correlation between matrices and morphological traits of maize lines.

For lettuce varieties, a direct positive correlation was found between genetic distances matrices by EST-SSR markers and morphological traits. The correlation coefficient (r) is 0.646. The obtained p-value of 0.035 at α = 0.05 indicates the rejection of the null hypothesis H0 and the adoption of an alternative hypothesis about the presence of the correlation Ha.

Even though the use of software environment R together with EcoGenetics package provides the widest possibilities and variability of analysis, the statistical environment is difficult to use. In particular, because of the use of commands rather than a graphical environment and the need to convert data into different formats for the relevant statistical analysis methods. The advantages of using R include cross-platform, the ability to integrate into Excel (RExcel,) and creating software scripts for analysis, which can be used in similar research. The advantages also include a built-in help and tips system, a lot of free literature on the implementation of statistical methods in the software environment R, graphical capacity for displaying research results, and the installation of support packages (libraries) with the necessary functions.

Thus, the obtained correlation coefficients for maize lines and lettuce varieties turned out to be the same with the use of different software products, which was achieved by setting the same parameters for calculation. It should be noted that the p-value calculated in the studied programs varied. This can be explained by the peculiarity of the Mantel test, which is to calculate the coefficients of significance using random permutations of the columns or rows of the studied matrices. Thus, even when performing calculations using the same program, obtained p-values are slightly different (Dutilleul *et al.*, 2000).

Thus, for both a quite large and a small number of samples, a direct positive correlation was found. Although a large sampling was studied for maize, the correlation coefficient between SSR markers and morphological traits was lower than the correlation obtained for lettuce. This can be explained by the fact that the EST-SSRs used to analyse lettuce varieties are located in the coding region of the gene and may be closely related to genes that are responsible for a particular morphological trait. Importantly, the studied varieties of lettuce belong to the same species. As a result, they have a sufficient number of the same manifestation degrees of morphological traits, even though varieties were found different by EST-SSR markers (Leshchuk *et al*., 2021).

To evaluate the studied software products, the analytical hierarchy process was used, which allows to structure the problem of choosing alternatives and criteria for their selection, to rank alternatives, and to mathematically substantiate the obtained results.

In the analytical hierarchy process, a decision-making hierarchy is developed: the comparison hierarchy contains one hierarchical level with four criteria and four software alternatives (XLSTAT, GenAlex, PaSSaGE, and R + EcoGenetics) (Fig. 2). The criteria chosen are: the cost of the software package, the possibilities of statistical analysis, the complexity of working with the package («entry threshold» and help service), and data processing, which includes the complexity of data preparation and information processing speed.

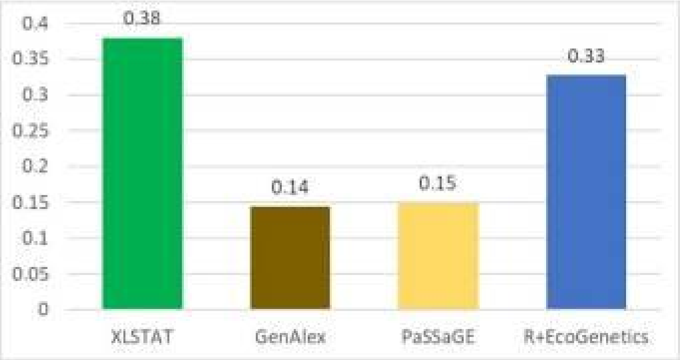

Fig. 2. Ranking of the software obtained with the analytical hierarchy process

As a result of the calculation, the add-on XLSTAT package was found to have the highest rate (0.38) among the studied programs. R + EcoGenetics is ranked in second place (0.33). GenAlex and PaSSaGE have almost the same rating (0.14 and 0.15, respectively). According to the obtained data, the most convenient and suitable for the Mantel test is the add-on XLSTAT. R + EcoGenetics is somewhat inferior to XLSTAT in terms of ease of use, but has more features and is free. Although GenAlex and PaSSaGE allow determining the correlation using the Mantel test, they have limitations on data entry and flexibility of adjusting test parameters.

It should be noted that the use of the add-on XLSTAT is quite common in determining the correlations of microbial communities of plant and soil, as well as for DNA markers. Thus, Kumar *et al*. (2014) studied the correlation between ISSR and RAPD markers in the analysis of genetic divergence of *Justicia adhatoda* L. The study of Ding *et al.* (2015) is dedicated to the determination of the correlation between EST-SSR markers and the geographical distribution of *Phoebe* *chekiangensis*. Add-on XLSTAT was also applied for determination of the correlation between sampling points belonging to the same plant within the study of the vertical distribution of the ectomycorrhizal community in the topsoil of Norway spruce stands (Scattolin *et al*., 2008).

The use of GenAlex to determine the correlations between the genetic distance matrices of DNA markers and geographical coordinates is also common. In the study of Huang *et al*. (2019), the Mantel tests implemented in GenAlEx were performed to analyse the effects of geographical distance on genetic structure. Similar studies have been conducted by Nantoume *et al*. (2013). They studied the correlation between the geographical distances matrix and the genetic distance matrix using the Mantel test in GenAlex. Gupta *et al*. (2018) used the Mantel test to test the goodness of fit between RAPD and ISSR markers by GenAlEx within accessions of Cymbopogons studing. Rebaa *et al*. (2017) studied variability in Tunisian populations of faba bean. They conducted the Mantel test using the GenAlEx program for correlation between genetic, geographical, and morphological distances.

Ghalmi *et al*. (2010) used PASSaGE for morphological and molecular diversity within Algerian cowpea by Mantel test. Mushtaq *et al*. (2016) compared agro-morphological and SSR data of maize inbred lines. The analysis was performed by calculating the correlation between the agro-morphological and SSR similarity matrices through the Mantel test using PASSaGE software. The PASSaGE was used by Yadav *et al*. (2013) for the Mantel test to assess genetic diversity in Indian rice germplasm.

Extensive capabilities of the software environment R in combination with various packages involve its use for processing molecular genetic data. Thus, Nagamatsu *et al*. (2021) studied strawberry fruit shape and QTL detection by genome-wide association. They used software environment R with EcoGenetics package for significant associations determination between genotype and phenotype of strawberry. Hoppe *et al*. (2016) studied the species assemblages and their geographical distribution correlation by distances matrices comparison using R + EcoGenetics. The EcoGenetics package was also used by Bessega *et al*. (2017) in the study of semi-arid *Prosopis flexuosa* (Leguminosae) species.

Thus, it is shown that the studied software products have a wide range of possibilities for determining the correlation between distance matrices, and they are used in various fields of ecology, biology, and genetics. Our research shows the possibility of using these products to assess the correlations between DNA markers and morphological traits of plant varieties, describes the main stages in the calculation of genetic distances and conducting the Mantel test, identifies the main advantages and disadvantages of the products using the analytical hierarchy process.

CONCLUSIONS

In the study of 4 lettuce varieties by 7 EST-SSR markers and morphological traits and 100 maize lines by 8 SSR markers and morphological traits, correlations between the genetic distances matrices of traits were determined with the aid of the Mantel test. In the course of the research, we studied four different statistical data processing software products that allow calculating the correlation coefficient and the level of significance based on random permutations of distance matrices: XLSTAT, PaSSaGE, GenAlex, and software environment R. It was found that there is a direct positive correlation between genetic distances matrices of lettuce by EST-SSR markers and maize by SSR markers and morphological traits.

The ranking of the software products with the use of analytical hierarchy process shows that the most suitable for determining the correlation between DNA markers and morphological traits based on the selected evaluation criteria is XLSTAT (0.38). R + EcoGenetics (0.33) is ranked in second place in terms of ability to perform tasks. Such an approach allowed evaluate the software products in the form of mathematical information, which minimizes subjective judgments. Thus, the possibilities of different software products for determining the relationship between DNA markers and morphological traits shown in the work open up prospects for studying links between DNA markers and economically valuable traits, and a combination of varieties assessment by morphological and DNA markers.

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