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GENETIC RELATIONSHIPS AMONG THE DIFFERENT VARIETIES OF LETTUCE (*LACTUCA SATIVA* L.) BY EST-SSR AND MORPHOLOGICAL MARKERS

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ABSTRACT

The investigation of molecular genetic polymorphism of lettuce cultivars by morphological characteristics and EST-SSR (Expressed Sequence Tag Single Sequence Repeats) markers and the determinationof correlation between EST-SSR and morphological characteristics. Morphological description, molecular genetic analysis, statistical analysis. The genetic distances between lettuce cultivars of different varieties were determined based on EST-SSR markers and morphological characteristics. The correlation between morphological characteristics of lettuce cultivars of *Lactuca sativa* L. var. *secalina* and studied EST-SSR markers were determined using the Mantel test. It was found, a total of 37 alleles were obtained with an average of 5.29 alleles by 7 EST-SSR marker in 7 lettuce cultivars. It was determined that the most polymorphic marker is KSL-92, by which 7 alleles were identified (PIC -Polymorphism Information Content 0.98). The lowest PIC value was obtained by KSL-37 marker; and 3 alleles were identified by this marker. As a result of cluster analysis of lettuce cultivars by morphological characteristics and EST-SSR markers, genetic distances between the studied cultivars were determined. It was found that Zorepad, Malakhit, Dublianskyi and Krutianskyi cultivars which belong to the same variety, according to morphological characteristics, formed one cluster group, the most similar were Malakhit and Dublianskyi cultivars with the value of genetic distances of 8.12. The cultivars Krutianskyi and Pohonych, belonging to varieties *Lactuca sativa* L. var. *secalina* and *Lactuca sativa* L. var. *angustana* respectively, were the most distant (genetic distances are 21.79). By EST-SSR markers, as a result of the assessment of genetic distances, it was found that Zorepad and Malakhit, Dublianskyi and Smuhlianka cultivars which were included to one cluster were the most similar. The genetic distances value is 3.16. The most distant cultivar was Skarb which belongs to *Lactuca sativa* L. var. *longifolia* with genetic distances of 3.74 in relation to other studied cultivars. The positive correlation was determined between EST-SSR markers and morphological characteristics as result of Mantel test within the most similar cultivars by morphological characteristics (Zorepad, Malakhit, Dublianskyi and Krutianskyi) (r=0.646). Genetic distances calculated by EST-SSR markers and morphological characteristics reflect the genetic structure of the studied lettuce cultivars. The correlation between EST-SSR markers and morphological characteristics was found. It makes possible to use EST-SSR analysis for breeding in order to select distant genotypes and DUS (Distinctness, Uniformity and Stability) test as an additional method for determining distinctness.

***Keywords*:** lettuce varieties; EST-SSR; morphological characteristics; genetic distances; correlation

Introduction

Lettuce (*Lactuca sativa* L.) is grown worldwide as a vegetable crop (Hu *et al.*, 2005). It is an increasingly popular vegetable because it is a basic ingredient in salads and is rich in vitamin C, E, K, and carotenoids (Wang *et al.*, 2017). Considering the increase of consumer use, the varietal of lettuce cultivars is also expanding. Thus, State register of plant varieties suitable for dissemination in Ukraine includes around 200 lettuce cultivars. The major lettuce varieties are *Lactuca sativa* L. var. *secalina*, *Lactuca sativa* L. var. *capitata*, *Lactuca sativa* L. var. *longifolia* and *Lactuca sativa* L. var. *angustana* (State register of plant varieties suitable for dissemination in Ukraine in 2020, 2020). Hence, with an increase number of cultivars, the variation range of morphological characteristics by which the distinctness, uniformity and stability of new cultivars is determined, gradually narrows. For determination of distinctness within DUS test according to UPOV (Union for the Protection of New Varieties of Plants) models the use of molecular methods, in particular DNA markers, are considered (Jamali *et al.*, 2019).

One of advanced approaches for assessment of cultivars to determine their differences both within DUS test and in breeding is a combination of morphological characteristics and DNA markers. The implementation of this approach involves the calculation of genetic distances between studied cultivars by DNA markers and morphological characteristics as well as the determination of correlation between two types of marker systems for studied cultivars.

Common molecular markers (e.g., AFLP, SSR, SNP) may serve as adequate tools for the rapid genotype screening (Kitner *et al.*, 2015). Among DNA markers, EST-SSR, 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 (Shapturenko *et al.*, 2016).

Nowadays there is research on the development of EST-SSR markers for differentiation of various lettuce types (Simko, 2009; Riar *et al*., 2011; Hong *et al*., 2013; Hong *et al*., 2015; Wang *et al*., 2017), and their use to determine lettuce genetic diversity and relationships with geographic location in studying of wild species (Kitner *et al*., 2015). The genetic relationships between morphological characteristics of lettuce and EST-SSR markers were studied by Hong *et al.* (2013) and Hong *et al.* (2015), there are publications on the description of morphological traits of lettuce (Křístková *et al*., 2008) and the use of SSR markers to assess the genetic diversity of lettuce cultivars (vandeWiel *et al*., 1999; vanTreurenandvanHintum, 2009; El-Esawi, 2015). In this study the genetic relationships between EST-SSR markers and the morphological characteristics of different lettuce varieties and the polymorphism of cultivars within the variety are studied. Therefore, the purpose of the study is to investigate the molecular genetic polymorphism of the lettuce cultivars by morphological characteristics and EST-SSR markers and to determine the correlation between EST-SSR and morphological characteristics.

Materials and Methods

In this study 7 lettuce cultivars of Ukrainian breeding which are belonged to four varieties were investigated (Table 1).

Table 1 : Lettuce cultivars

Variety	Cultivar	Horticultural type
Lactuca sativa L. var. *secalina*	Zorepad	Leaf
	Malakhit	Leaf
	Dublianskyi	Leaf
	Krutianskyi	Leaf
Lactuca sativa L. var. *capitata*	Smuhlianka	Butterhead
Lactuca sativa L. var. *longifolia*	Skarb	Romaine
Lactuca sativa L. var. *angustana*	Pohonych	Stem

DNA extraction and PCR

DNA was extracted from four-day seedling using cationic detergent CTAB (cetyltrimethyl ammonium bromide). Chloroform-isoamyl alcohol and ethanol solution were used in double purification of the mixture. Dissolving DNA was performed in TE buffer (Tkachyk, 2016). The molecular genetic polymorphism of the studied lettuce cultivars was assessed using 7 EST-SSR markers (Hong *et al*., 2015; Leshchuk *et al*., 2019). These EST-SSR markers were chosen for their high diversity values. PCR was carried out according to Hong *et al*., 2015 with thermocycler T-CY (Creacon Technologies B.V., The Netherlands). The obtained amplicons were separated by capillary electrophoresis with Fragment Analyzer (Agilent Technologies, USA) and dsDNA 910 ReagentKit, 35-1,500 bp.The data was proceeded using software PRO Size 2.0.

Morphological analysis

The description of lettuce cultivars morphological characteristics was carried out in 2005-2015 within DUS teston the testing places of Ukrainian Institute for plant Variety Examination (Ukraine) (Plant Variety Right Protection. Bulletin. Issue 3 Vegetablecrops, 2010; Plant Variety Right Protection. Bulletin. Issue 1. Sugar beet, 2010; Plant Variety Right Protection. Bulletin. Issue 2, 2015; Plant Variety Right Protection. Bulletin. Issue 1, 2016; Plant Variety Right Protection. Bulletin. Issue 2, 2017).32 were used among 42 morphological characteristics according to guidelines for the conduct of tests for distinctness, uniformity and stability of lettuce. States of expression were allocated from 1 to 9 notes (Guidelines for the conduct of tests for distinctness, uniformity and stability. Lettuce (*Lactuca sativa* L.).

Data analysis

To characterize the genetic structure of the studied cultivars the allele frequencies and PIC were calculated (Prysiazhniuk *et al*., 2019). The genetic distances between studied cultivars were calculated with cluster analysis using Un weighted Pair-Group Average (UPGA) with Euclidean distances. STATISTICA 12 software (trial version) was used for data proceeding (Everitt *et al*., 2011). The correlation between EST-SSR markers and morphological characteristics by genetic distances of studied cultivars was estimated with Mantel test by software environment for statistical computing R (Legendre and Fortin, 2010; Crabot *et al*., 2017). Mantel test is nonparametric form of analysis of the relationship between two dissimilarity matrices computed from two sets of multivariate data concerning the same n individuals or sampling units (Legendre *et al*., 2015).

Results and Discussion

As a results of PCR analysis and capillary electrophoresis of lettuce cultivars by 7 EST-SSR markers a total of 37 alleles were obtained with an average of 5.29 alleles (Figure 1).

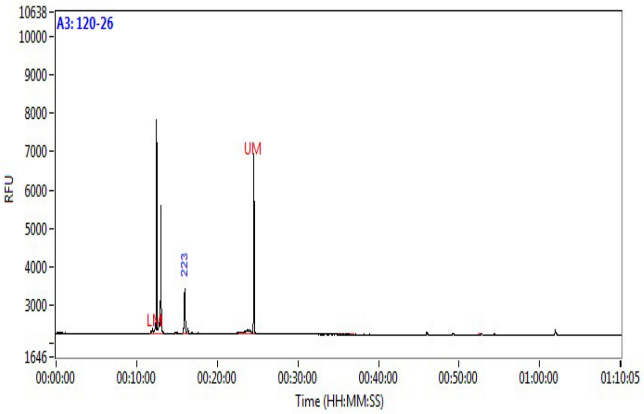

Fig. 1 : Amplified EST-SSR fragments of lettuce cultivar at KSL-26 marker: 120 – Zorepad cultivar; LM – lower marker (35 bp); UM – upper marker (6000 bp)

The most polymorphic among studied markers was KSL-92marker by which 7 alleles were identified. The minimum number of alleles (3 alleles) was detected by KSL-37marker. By other studied markers 5-6 alleles were identified for each marker. The alleles sizes were ranged from 193 to 311 bp, allele frequencies were 0.14-0.57.

It was determined that the highest frequency (0.57 and 0.43) had alleles 193 bp by KSL-37 marker and allele 204 bp by KSL-173 marker, respectively. For other studied markers, the frequencies of identified alleles varied within 0.14-0.29. The average PIC value was 0.91, the highest value (0.98) was obtained by markers KSL-173, KSL-26, KSL-32, KSL-92 and KSL-271, the lowest value (0.57) was calculated by KSL-37 marker (Leshchuk *et al*., 2020).

SSR markers are mainly used to differentiate lettuce cultivars of different varieties due to their high information content, spread throughout genome, and high reproducibility (vandeWiel *et al*., 1999; RauscherandSimko, 2013; Oliya *et al*., 2018; Zhou *et al*., 2019; Rui *et al*., 2020). However, EST-SSR, which have the same advantages as SSR, are more conservative due to their location in the coding regions of genome and are used to compare related varieties. Thus, the microsatellite markers for lettuce were developed by van de Wiel *et al*. (1999) using enriched genomic libraries. The authors demonstrated the efficiency of using a marker set of 28 SSR for six cultivars of *Lactuca sativa* L. However, the developed markers were not so effective by application to related species.

The same approach to the development of SSR markers for *Lactuca sativa* L. was applied by Rauscher and Simko (2013). The authors used developed 97 SSR markers to study 36 samples of the genus *Lactuca.* It was shown that an average of 5.5 alleles.Wang *et al*. (2017) described the use of developed 15 EST-SSR markers to assess the genetic diversity of 66 lettuce samples. As a result of research, the authors obtained from 3 to 11 alleles, with an average of 6.2 alleles per locus (Wang *et al*., 2017). In this study, we have used a smaller number of samples and markers, so the average number of alleles per locus was 5.29.

Hong *et al*. (2015) studied 92 lettuce cultivars of various horticultural types: leaf, butter head and romaine by 58 EST-SSR markers (Hong *et al*., 2015). A total number of alleles which was obtained by authors was 176, with an average of 3 alleles. For the markers used by Hong *et al.* (2015), the PIC ranged from 0.505 to 0.743. The most polymorphic marker was KSL-26, the lowest PIC was obtained by the authors with KSL-271 marker. In this study, the most polymorphic marker was KSL-92 with PIC 0.98. Moreover, it should be noted that the sizes of the alleles obtained by this marker differed. Hong *et al*. (2015) obtained alleles of 188-196 bp for the studied lettuce cultivars. In this study, the allele sizes ranged from 193 to 311 bp. Alleles larger than 300 bp were identified by marker KSL-92in cultivars of varieties *Lactuca sativa* L. var. *capitata*, *Lactuca sativa* L. var. *longifolia* and *Lactuca sativa* L. var. *angustana*. This distribution of alleles can be explained by the origin of the studied cultivars; in this study, only cultivars of Ukrainian breeding were involved.

Therefore, rather uniform distribution of allele frequencies and PIC values obtained for studied EST-SSR markers indicate the possibility of their use for assessing the genetic diversity of lettuce cultivars of different varieties.

To assess the genetic diversity of lettuce cultivars by EST-SSR markers, the cluster analysis was performed and the genetic distances between cultivars were determined (Figure 2).

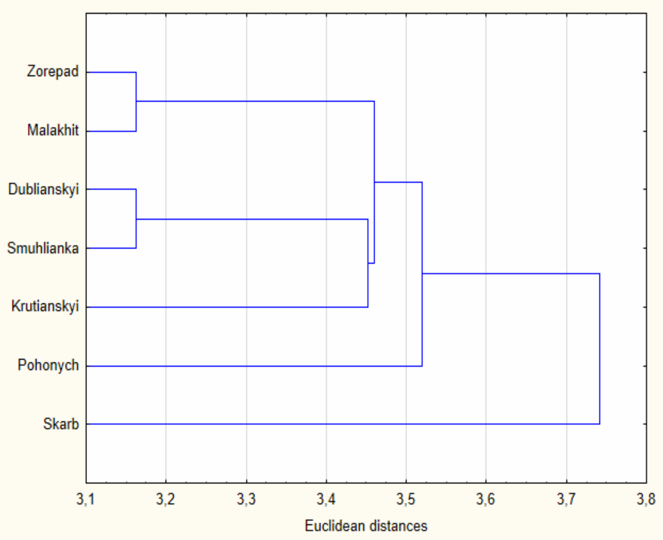

Fig. 2 : The dendrogram of cluster analysis lettuce cultivars by EST-SSRmarkers

As a result of cluster analysis, it was determined that the studied cultivars were divided into three clusters. By 7 EST-SSR markers the most similar were Zorepad and Malakhit, Dublianskyi and Smuhlianka cultivars. The genetic distances value is 3.16.According to the obtained distribution, Krutianskyi, Pohonych and Skarb cultivars consisted another cluster, which was separated from the clusters formed by Zorepad and Malakhit, Dublianskyi and Smuhlianka cultivars.

The most distant cultivar was Skarb with genetic distances of 3.74 in relation to other studied cultivars. Krutianskyi cultivar is adjacent to the cluster formed by Dublianskyi and Malakhit cultivars, the value of genetic distances is 3.16-3.74.Separately from other cultivars, there is also Pohonych cultivar with genetic distances of 3.46-3.74. In accordance with obtained distribution, Zorepad and Malakhit cultivars belong to the same variety *Lactuca sativa* L. var. *secalina* and have a high degree of genetic affinity by 7 studied EST-SSR markers. Skarb and Pohonych cultivars, which belong to varieties *Lactuca sativa* L. var. *longifolia* and *Lactuca sativa* L. var. *angustana*, respectively, are genetic distant from other studied cultivars, which is confirmed by high values of genetic distances.

The degree of genetic similarity by morphological characteristics between studied cultivars was carried out by cluster analysis using their notes of expression. It was shown that two clusters were formed by morphological characteristics. The results of hierarchical clustering are shown in Figure 3.

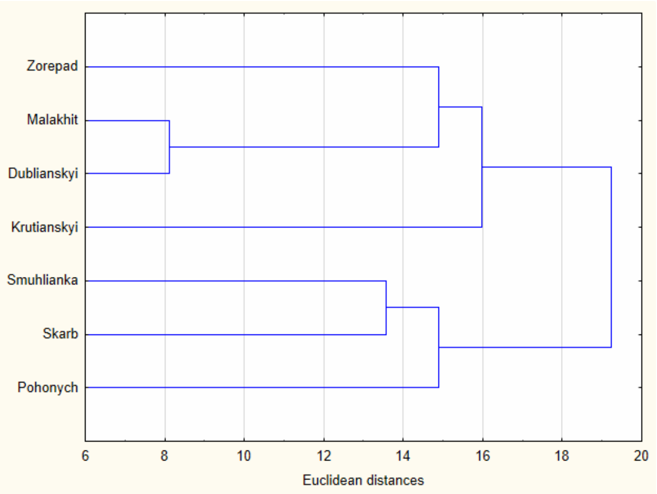

Fig. 3 : The dendrogram of cluster analysis lettuce cultivars by morphological characteristics

According to the results of analysis, it was found that Malakhit and Dublianskyi cultivars were the most similar by morphological characteristics, the value of genetic distances is 8.12.Zorepad and Krutianskyi cultivars are adjacent to them with genetic distances 14.25-15.52 and 15.52-15.72, respectively. The one cluster included Smuhlianka and Skarb cultivars, the value of genetic distances is 13.56. Pohonych cultivar is adjacent to these cultivars with genetic distances of 15.56 and 14.21.It should be noted that Pohonych cultivar is the most distant in relation to Zorepad, Malakhit, Dublianskyi and Krutianskyi cultivars.The value of genetic distances by morphological characteristics is 18.60-21.79.Moreover, the most distant cultivars were Krutianskyi and Pohonych (genetic distances are 21.79), which are belonged to varieties *Lactuca sativa* L. var. *secalina* and *Lactuca sativa* L. var. *angustana* respectively. In accordance with the obtained distribution, it can be seen that lettuce cultivars belonging to *Lactuca sativa* L. var. *secalina* formed one cluster group, and cultivars which are representatives of other varieties are included to another.

It should be noted that according to EST-SSR markers, Dublianskyi and Malakhit cultivars, which are in the same cluster and have a low value of genetic distances, are representatives of two varieties *Lactuca sativa* L. var. *secalina* and *Lactuca sativa* L. var. *capitata* respectively. This result can be explained by the fact that as EST-SSR markers are developed from the coding regions of genome, they are likely more conserved in related species and varieties than are SSR (Simko, 2009; Wang *et al*., 2017). In the case of cultivar analysis by morphological characteristics, it is necessary to highlight that the morphological description of cultivars provides for the determination of expression notes of certain characteristic, for example, head size, which obviously does not form in leaf types.

The possibility of using EST-SSR markers to differentiate lettuce of several horticultural types was studied by Simko (2009). The author investigated 96 samples of lettuce. According to the results of cluster analysis obtained by Simko (2009), the developed markers made it possible to form clusters which included lettuce samples of the same horticultural type. However, the author also found that one cluster can contain samples of different lettuce types: head and leaf, iceberg and Batavia lettuces. Such cases were also observed in this study using EST-SSR markers, when lettuce cultivars of varieties *Lactuca sativa* L. var. *secalina* and *Lactuca sativa* L. var. *capitata* were included in one cluster.

Rauscher and Simko (2013) determined the degree of genetic similarity between different lettuce horticultural types and species by SSR markers. The authors studied 36 lettuce samples by 97 SSR markers. According to results obtained by authors, the most similar were lettuce cultivars belonging to head type and romaine, also cultivars which are representatives of the same type did not form one cluster (Rauscher та Simko, 2013). The authors noted that genotyping of lettuce cultivars using SSR markers is associated with more errors than with involvement of EST-SSR.

As a result of an assessment of genetic similarity of lettuce cultivars by Hong *et al.* (2015), the authors showed that one cluster, according to Jaccard distances, included lettuce cultivars of both one horticultural type (red leaf lettuce) and lettuce cultivars of different types: romaine, head, green leaf lettuce(Hong *et al*., 2015). However, according to morphological assessment of cultivars by 29 characteristics, the authors found 3 pairs of cultivars with 100% similarity. In our study, 32 morphological characteristics were used, which made it possible to identify similar cultivars within the same variety.

To determine the correlation relationships between studied EST-SSR markers and morphological characteristics within the most similar cultivars by morphological characteristics (Zorepad, Malakhit, Dublianskyi and Krutianskyi), Mantel test (linear correlation by Pearson) was used (Figure 4).

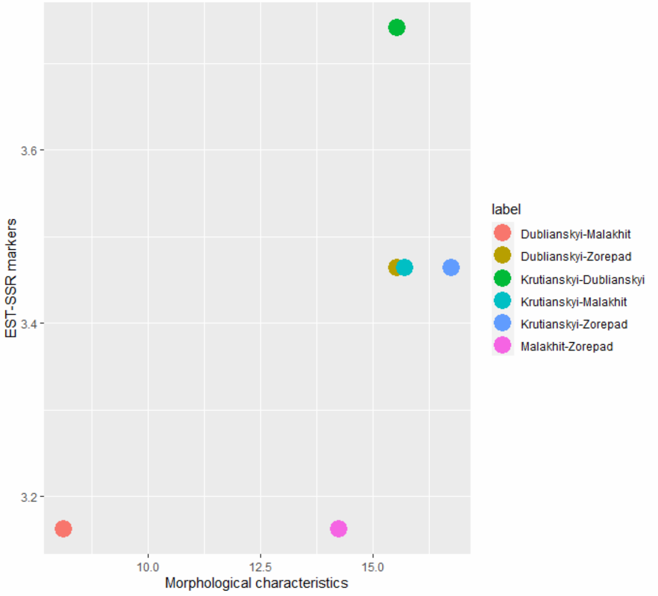

Fig. 4 : The relationships between genetic distances by EST-SSR markers and morphological characteristics of lettuce cultivars

As a result of the analysis, the p-value and r(AB) value at the significance level α=0.05 were found, which, according to the interpretation of the test results, allows to accept the hypothesis of the presence of (Ha) or absence (H0) of correlation (Legendre *et al*., 2015). It is known that the hypothesis of H0 (absence of correlation) is assumed under the condition of p>α. As a result of this study, the calculated p-value (0.029) was lower than the significance level α=0.05, therefore, we should reject the hypothesis H0 and adopt the alternative hypothesis Ha regarding the correlation. The correlation coefficient is 0.646. Thus, the obtained data indicate the presence of correlation between EST-SSR markers and morphological characteristics.

The positive correlation between 29 morphological traits and 58 EST-SSR for lettuce cultivars which were morphologically identical was found by Hong *et al.* (2015).The correlation coefficient obtained by the authors was 0.82. For our study, the correlation coefficient was slightly lower, which may be due toa smaller number of cultivars and markers, and also whereas Zorepad, Malakhit, Dublianskyi and Krutianskyi cultivars, although they belong to the same variety *Lactuca sativa* L. var. *secalina*, however, had morphological differences. Such results can be explained by the use of highly polymorphic markers, derived from EST representing coding regions, and precise genotyping through DNA sequencing system for cultivar identification (Hong *et al.*, 2015).

Thus, it was found that the studied EST-SSR markers are highly polymorphic and allow to differ lettuce cultivars of various varieties. The correlation was found between EST-SSR and morphological characteristics, which indicates the possibility of using EST-SSR to identify the most distant genotypes at the initial stages of breeding and variety examination. EST-SSR markers could be useful for the clarification of inter-specific relationships, genetic diversity, origin and evolution of different varieties of lettuce, choosing the most similar cultivars and an alternative method for DUS test in lettuce.

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