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Polymorphism in spring and winter rapeseed varieties (*Brassica napus* L.) identified by SSR markers

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Purpose. To assess the genetic diversity of rapeseed varieties using SSR markers in order to create breeding material and use that material in complex *in vitro* selection for drought and salt tolerance. **Methods.** PCR analysis, cluster analysis. **Results.** The results of analysis of rapeseed varieties polymorphism based on molecular-genetic markers is presented. As a result of the analysis of rapeseed varieties, 41 alleles were detected using the studied markers, that is, an average of 10.3 alleles per marker. The number of polymorphic loci identified by four microsatellite markers (Ra3-H09, Na12-A02, FITO-063 and Na10-B07) was 24. The polymorphism level of the studied varieties was 51% on average and varied between 33% (identified by FITO-063) and 87% (identified by Na12-A02). According to the frequency distribution of the obtained alleles, the highest frequency by SSR marker Ra3-H09 had a 117 bp allele identified in three varieties: 'Senator Liuks', 'Danhal' and 'Chornyi Veleten'. It was found that the unique alleles identified by Ra3-H09 were the alleles at a frequency of 0.06 and size of 135 bp (variety 'Aliot') and 156 bp (variety 'Kliff'). FITO-063 marker identified the smallest number of alleles (5) at a frequency distribution ranging from 0.11 to 0.33. The unique alleles identified by FITO-063 marker were the ones at a frequency of 0.1 and size of 258 bp (variety 'Geros') and 273 bp (variety 'Chornyi Veleten'). The maximum number of alleles was obtained using Na12-A02 marker. The distribution showed the highest frequency (0.11) for the 158 bp and 192 bp alleles. Using Na10-B07 marker, three alleles were identified at a frequency of 0.04. These 144, 156 and 194-bp alleles were found in varieties 'Kliff', 'Geros' and 'Nelson'. Cluster analysis revealed four variety clusters: 'Senator Liuks' and 'Danhal', 'NK Technik' and 'NK Petrol', 'Geros' and 'Aliot', 'Kliff' and 'Nelson'. 'Chornyi Veleten' variety did not enter any cluster. The most distant varieties are 'Kliff' and 'Nelson' with a genetic distance value of 3.32. Foreign varieties 'NK Technik' and 'NEC Petrol' with the value of genetic distances between them equal 1.41 appeared to be the most similar by the four studied SSR markers. Other varieties differed by at least one marker. **Conclusions.** Consequently, using the set of four microsatellite markers provides an assessment of rapeseed varietal diversity that can be used in complex *in vitro* selection for drought and salt tolerance.

Keywords: *varietal diversity; rapeseed; microsatellite markers; cluster analysis.*

Introduction

Rapeseed (*Brassica napus* L.) is one of the most important high-yielding industrial crops promising for export and production of rapeseed oil and biodiesel for the domestic market [1–3]. The crop productivity can be increased through creating new hybrids with a better tolerance to environmental changes.

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The constantly growing area of saline lands, which now, according to FAO, exceeds 7% of the world's agricultural land [4–5] along with climate change, requires research aimed at creating hybrids of complex tolerance to adverse abiotic factors.

The efficiency of breeding is largely determined by the genetic diversity of the parent material; consequently, it is necessary to search and introduce new approaches to increasing the genetic heterogeneity of the selection material [6–7]. Such approaches include cell, tissue, plant organ culture and using DNA-markers for identification of the selection material [8–10].

Genotyping technology based on DNA polymorphism is used for both cultural and wild species of the genus *Brassica* L. DNA makers have many advantages. The molecular markers

are not subjected to environmental change, which makes them particularly informative and superior to traditional methods [11].

Various DNA markers have been successfully used to study genetic diversity in Brassica, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), sequence-related amplified polymorphism (SRAP), simple sequence repeat (SSR), single-nucleotide polymorphisms (SNP) [12]. SSR analysis allows distinguishing genera, species and varieties, revealing genetic heterogeneity of breeding material, and controlling genetic material introgression from parents [13–15]. The technology of genotyping based on SSR markers allows identifying genetic diversity of rapeseed varieties and genetic homogeneity of breeding material, selecting parents for crosses and controlling transfer of genetic material from parents to hybrids [7, 16–18].

The goal of this research was to assess the genetic diversity of rapeseed varieties using SSR markers in order to create breeding material and use that material in complex in vitro selection for drought and salt tolerance.

Materials and methods

Characteristics of the investigated material

The material for the study was nine varieties of winter and spring rapeseed of Ukrainian ('Senator Liuks', 'Danhal', 'Chornyi Veleten' and 'Aliot') and foreign origin ('NK Technik', 'Necropolis', 'Kliff', 'Geros', 'Nelson') promising for the creation of drought and salt-resistant lines. All the varieties have been listed in the State Register of Varieties Suitable for Distribution in Ukraine from 2001 to 2011 as shown in Table 1.

Table 1

Characteristics of varieties of winter and spring rapeseed

Name of variety	Year of registration	Applicant
Winter varieties		
'Senator Liuks'	2006	National Research Centre Institute of Agriculture Ukrainian Academy of Agrarian Sciences
'NK Technik'	2011	Syngenta Seeds S.A.S.
'NK Petrol'	2011	Syngenta Seeds S.A.S.
'Danhal'	2001	Ivano-Frankivsk Institute of Agroindustrial Production Ukrainian Academy of Agrarian Sciences
'Nelson'	2008	Syngenta Seeds S.A.S.
'Chornyi Veleten'	2002	Institute of Fodder and Agriculture of Podillia Ukrainian Academy of Agrarian Sciences
'Aliot'	2007	National University of Life and Environmental Sciences of Ukraine; Sytnik I. D.; Kolodii Yu. A.
Spring varieties		
'Kliff'	2003	Norddeutsche Pflanzenzucht Hans-Georg Lembke KG
'Geros'	2006	Raps GbR Saatzucht Lundsgaard

The research was carried out at the Ukrainian Institute for Plant Varieties Examination (Kyiv, Ukraine) in collaboration with the National University of Life and Environmental Sciences of Ukraine (Kyiv, Ukraine) in 2017/2018.

Isolation of DNA and PCR

The material was provided by the National University of Life and Environmental Sciences of Ukraine (The Problem Laboratory Of Phytovirology and Biotechnology) where the selection *in vitro* for drought and salt tolerance was carried out. DNA was isolated from the leaves of obtained *in vitro* rapeseed plants using cationic detergent CTAB (cetyltrimethylammonium bromide). Chloroform-isoamyl alcohol and ethanol solution were used in double purification of the mixture [19–21].

PCR (polymerase chain reaction) was used to investigate the molecular and genetic polymorphism of rapeseed varieties. For this purpose, we used four specific primers for four microsatellite loci (MS-loci): Ra3-H09, Na12-A02, FITO-063 and Na10-B07 as shown Table 2.

The primers were chosen based on their ability to differentiate genotypes and PIC [11, 21–24]. The PCR was performed using BioRad IQ5 (USA). The reaction mixture (20 µL) contained 100 ng of total plant DNA, buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% Triton X-100, 2.5 mM $MgCl_2$), 200 µM deoxynucleoside triphosphates mix (dNTPs), 0.2 µM of each primer and 1 unit of Taq polymerase (Thermo Fisher Scientific, USA). The amplification parameters for the examined rapeseed markers were set as follows: initial denaturation (94 °C) 5 min, 35 cycles; denaturation (94 °C) 45 s;

Table 2

Characteristics of SSR-loci primers of rapeseed

Primer	The nucleotide sequence of primers 5'...3'	Motif	Hybridization temperature (°C)	Expected size of amplicones (bp)
Ra3-H09	F – gtggtaacgacggtccattc R – accacgacgaagactcatcc	$(TGG)_3$	53.6	119–129
Na12-A02	F – agccttgttgcttttcaacg R – agtgaatcgatgatctcgcc	$(CT)_{16}$	54.0	161–202
FITO-063	F – gttcagttcccagattcctaa R – tttcctcttccttctctcttc	$(CCG)_{15}$	49.0	267–700
Na10-B07	F – gccttagattagatggtcgcc R – acttcagctccgatttgcc	$(CT)_{29}$	53.0	174–213

annealing (49–54 °C) 45 s; synthesis (72 °C) 1 min; final elongation (72 °C) 10 min.

Visualization of amplifications and cluster analysis

After amplification, the reaction products were visualized by electrophoresis in a 2% agarose gel in 0.5 × TBE (tris-borate buffer solution) [25]. DNA electrophoresis was carried out for 1 hour at an electric field intensity of 5 V/cm.

After the completion the electrophoresis, based on the obtained data a matrix was constructed, where the presence / absence of a certain amplicon was designated as 1/0, respectively.

The method of hierarchical clustering with Euclidean distance and STATISTICA 12.0 (Trial version) were exploited to analyse the obtained research data [26–27].

Results and discussion

Determination of polymorphism in rapeseed varieties by SSR markers

Alleles of the expected size were obtained by PCR on four SSR markers with specific primers. The amplicons were obtained by markers Na12-A02 (the most polymorphic loci) and FITO-063 (the lowest level polymorphism) and are shown Figures 1 a, b.

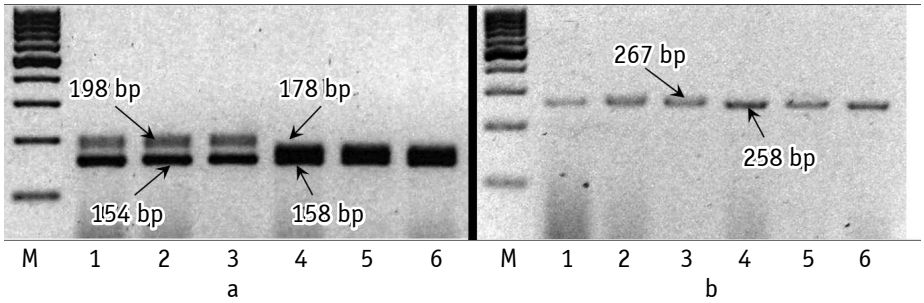


Fig. 1. Electropherogram of DNA amplification products of different rapeseed varieties: M – molecular weight marker 100 bp DNA Ladder O'GeneRuler (Thermo Scientific); **(a)** based on Na12-A02 marker: 1–3 genotypes of 'Kliff' variety, 4–6 genotypes of 'Geros' variety; **(b)** based on FITO-063 marker: 1–3 genotypes of 'Kliff' variety, 4–6 genotypes of 'Geros' variety

As a result of PCR the Kliff' variety by primer Na12-A02 marker, amplicons of 198 bp and 154 bp were found. The amplification products of 'Geros' variety were 178 bp and 158 bp. In general two alleles of different size were identified for all studied varieties by Na12-A02 marker. Using FITO-063 marker five alleles were obtained for nine varieties. Figure 1 b illustrates that the allele 267 bp was identified in 'Kliff' variety. Allele 258 bp was found 'Geros' variety by FITO-063 marker. It should be mentioned that the allele 267 bp was identified also in 'Aliot' and 'Nelson' varieties.

Resulting from analysis of the rapeseed varieties by the investigated markers 41 alleles were detected, i.e. on the average of 10.3 alleles per marker as shown Table 3.

Table 3

Analysis of the level of polymorphism with SSR markers

Primer	Total alleles	Polymorphic alleles	Polymorphism level* (%)
Ra3-H09	11	5	45
Na12-A02	16	14	87
FITO-063	5	2	33
Na10-B07	9	3	40

*Polymorphism level = (number of polymorphic bands/ number of total bands) × 100%

The total number of polymorphic loci was 24 and varied from 2 to 14 by markers. The polymorphism level for the varieties under study was 51%. The highest level of polymorphism (87%) was marked by Na12-A02 marker and the lowest (33%) by FITO-063. A slightly lower level of polymorphism (40%) was obtained by Li and co-authors [11] in the study of 16 rape-

seed varieties. Hasan et al. [16], 2006 used combinations of 30 SSR-primers, which included the primer groups Ra3 and Na12, to estimate genetic diversity of rapeseed varieties. They obtained 51 polymorphic loci of (out of 220) for 96 genotypes. Polymorphic loci demonstrated unique allele sizes for all 96 genotypes. Tommasini et al. [30] obtained a high level of polymorphism (68%) for the primer Na12-A02 2003. The researchers suggested the use of a set of 15 SSR-primers for use in the DUS-test.

We determined the frequencies of identified alleles for each marker under study as shown Figures 2 a, b; 3 a, b.

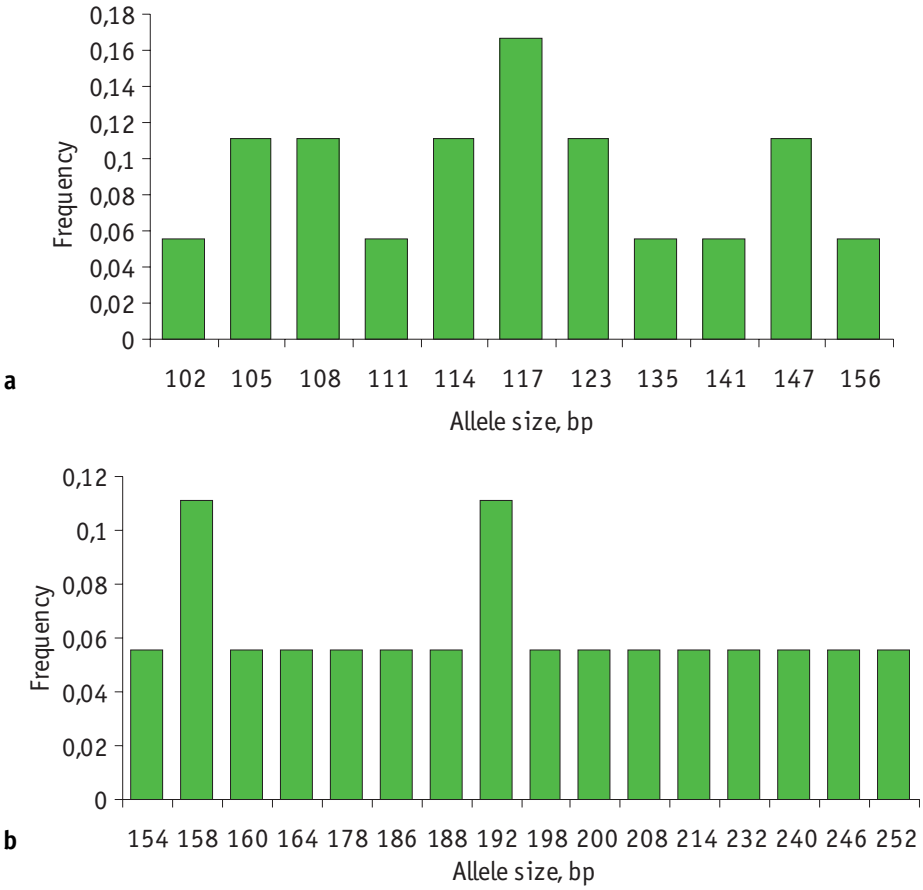
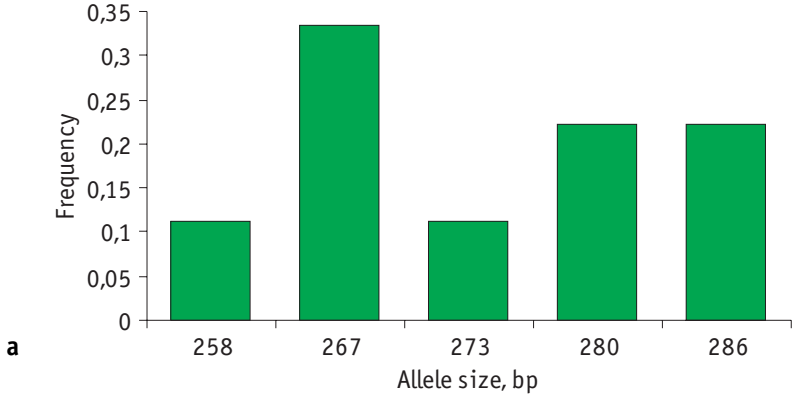


Fig. 2. Distribution of allele frequencies detected by markers (a) Ra3-H09 and (b) Na12-A02

The distribution showed that the highest frequency by SSR marker Ra3-H09 had a 117 bp allele identified in three varieties: 'Senator Liuks', 'Danhal' and 'Chornyi Veleten'. The unique alleles at a frequency of 0.06 were found in 'Aliot' variety (135 bp) and 'Kliff' variety (156 bp). The biggest number of alleles was obtained using Na12-A02 marker. The distribution showed the highest frequency (0.11) for the 158 bp and 192 bp alleles. An allele of 158 bp was detected in 'Geros' and 'Nelson' varieties and another allele of 192 bp was identified in 'NK Technik' and 'NK Petrol'. Other detected alleles had a frequency of 0.06.



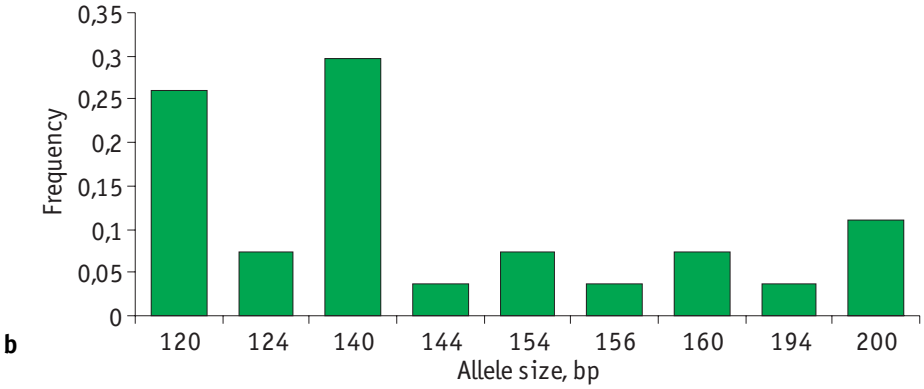


Fig. 3. Distribution of allele frequencies detected by markers (a) FITO-063 and (b) Na10-B07

FITO-063 marker identified the smallest number of alleles (5) at a frequency distribution ranging from 0.11 to 0.33. The 258 bp and 273 bp alleles at a frequency of 0.11 were unique. The specified alleles were identified in 'Geros' and 'Chornyi Veleten' varieties, respectively. Na10-B07 marker detected three alleles at a frequency of 0.04: 144, 156 and 194 bp, in 'Kliff', 'Geros' and 'Nelson' varieties, respectively.

Thus, it was found that 'Geros', 'Kliff', 'Aliot' and 'Chornyi Veleten' had the unique alleles, while 'Nelson' differed by Na10-B07 marker. It was found that the studied rapeseed varieties differed by at least one marker.

Cluster analysis of rapeseed varieties

To find out the similarity of rapeseed varieties, we carried out cluster analysis of the matrix of the presence/absence of identified alleles. The method of unweighted pair-group average was used to divide the genotypes under investigation into clusters. The average value of genetic proximity between the members of a cluster and a candidate for inclusion in the cluster was the criterion for determining the degree of similarity [28–29]. Shown in Figure 4 are the clustering results, presented as a phylogenetic tree.

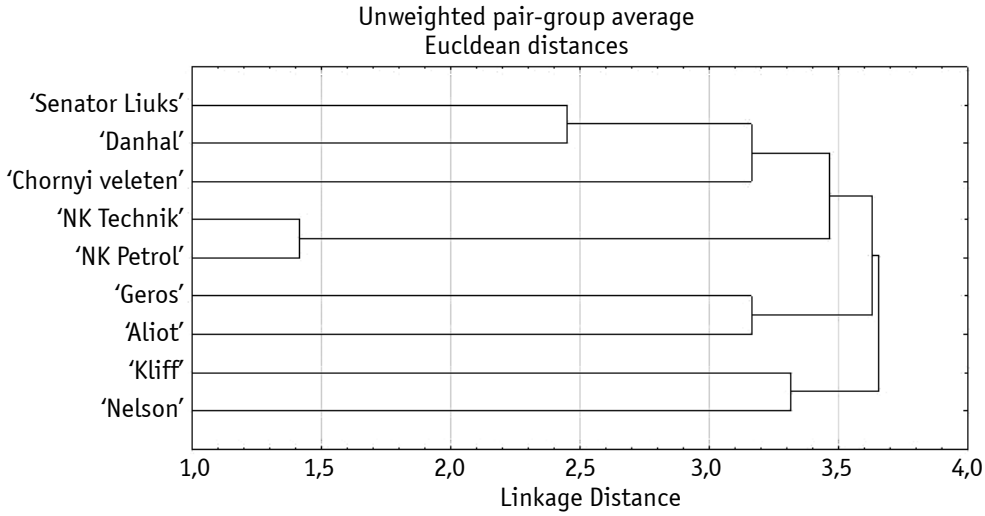


Fig. 4. Cluster analysis of rapeseed varieties by SSR markers

As a result of clustering, four variety clusters emerged: 'Senator Liuks' and 'Danhal', 'NK Technik' and 'NEC Petrol', 'Geros' and 'Aliot', 'Kliff' and 'Nelson'. 'Chornyi Veleten' formed a separate cluster next to 'Senator Liuks' and 'Danhal' varieties. The value of its genetic distances in relation to other varieties was 3.46. Foreign varieties 'NK Technik' and 'NEC Petrol' with the value of genetic distances between them equal 1.41 appeared to be the most similar by the four studied SSR markers. The most distant varieties included in one cluster were 'Kliff 'and 'Nelson' with a value of 3.32. The highest value of genetic distance (4.00) was recorded between the 'Geros' and 'Kliff' varieties. However, despite the fact these varieties belong to the same type (spring varieties), they considerably differ from each other. However, it should be emphasized that these varieties are the most distant from winter varieties and the value of their genetic distance was 3.74.

In a research conducted by Tommasini et al. [30], 10 rapeseed varieties were differentiated in terms of type (winter/spring) using a set of 15 markers. The authors found that the SSR markers had the potential to differentiate the varieties of *Brassica napus* L. and can be used to determine the homogeneity and differences at the initial stage of the DUS-test for the candidate varieties. However, the researchers point out that it is necessary to study and evaluate a larger number of assays and markers to develop a system for their differentiation, not only for testing purpose but also for evaluating the source materials in the breeding process.

We observed a certain differentiation of varieties according to the type of development (winter/spring). However, it should be noted that two clusters were formed by varieties of spring and winter type. The genetic distances between the 'Geros' and 'Aliot', 'Kliff' and 'Nelson' varieties were 3.16 and 3.32, respectively. Since available research data on the presence of any correlation between the marker and genes responsible for the type of plant development is not sufficient, it can be assumed that the presence or absence of certain allele by the markers under study may be related to the historical aspects of rapeseed breeding. Li et al. [11] differentiated 26 rape varieties using 11 SSR markers and found it more effective to use SSR markers compared to AFLP.

According to the research results, the differentiation of varieties according to their origin was revealed: the varieties of Ukrainian breeding ('Senator Liuks', 'Danhal' and 'Chornyi Veleten') were included in one group of clusters while foreign varieties ('NK Technik', 'Necropolis', 'Geros', 'Kliff' and 'Nelson') in the other one. However, we noted that 'Aliot' variety (Ukrainian) entered the same cluster together with a foreign spring variety. The value of genetic distances between them is 3.16. Such distribution can be explained by the involvement of genetic plasmas of high-yielding foreign varieties into the breeding process aimed at obtaining varieties with improved agronomic and economic features [31–32].

Conclusions

The research data on the allele status of microsatellite loci in winter and spring rapeseed varieties of Ukrainian and foreign origin show the polymorphism by the SSR markers Ra3-H09, Na12-A02, FITO-063 and Na10-B07. The level of polymorphism averaged 51%. The number of polymorphic alleles was 24, which allowed us to differentiate genotypes using the studied markers. The determined genetic distances indicate that the 'Kliff' and 'Nelson' varieties are the most distant from other studied rapeseed varieties with a value of 3.32. In addition, 'Chornyi Veleten' is attributed to a separate group. The rapeseed varieties differ by at least one marker from each other, indicating the possibility of using a set of markers for their identification. This approach will allow to evaluate the breeding material for selection.

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Кляченко О. Л.[1], Присяжнюк Л. М.[2*], Шофолова Н. В.[1], Піскова О. В.[2] Поліморфізм сортів ріпаку озимого та ярого (*Brassica napus* L.) за SSR маркерами. *Plant Varieties Studying and Protection*. 2018. Т. 14, № 4. С. 366–374. https://doi.org/10.21498/2518-1017.14.4.2018.151898

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Мета. Оцінити генетичне різноманіття сортів ріпаку за допомогою SSR-маркерів для створення селекційного матеріалу із застосуванням його в комплексній селекції *in vitro* на посухо- та солестійкість. **Методи.** ПЛР аналіз, кластерний аналіз. **Результати.** Наведено результати досліджень поліморфізму сортів ріпаку на основі молекулярно-генетичних маркерів. У результати аналізу сортів ріпаку за досліджуваними маркерами виявили 41 алель, тобто в середньому 10,3 алелі на маркер. Кількість поліморфних локусів за чотирма мікросателітними маркерами (Ra3-H09, Na12-A02, FITO-063 та Na10-B07) складала 24 локуси. Рівень поліморфізму для досліджуваних сортів у середньому становив 51%: найвищий рівень (87%) відмічений для маркера Na12-A02, найнижчий (33%) – FITO-063. Відповідно до отриманого розподілу найбільшою частотою за SSR маркером Ra3-H09 вирізнялась алель розміром 117 п.н., яку ідентифіковано у трьох сортів: 'Сенатор Люкс', 'Дангал' та 'Чорний велетень'. Виявлено, що за маркером Ra3-H09 унікальним для досліджуваних сортів виявились алелі з частотою 0,06 та розмірами 135 п.н. у сорту 'Аліот' та 156 п.н. у сорту 'Кліфф'. За маркером FITO-063 ідентифіковано найменшу кількість алелів (5), при цьому розподіл частот варіював від 0,11 до 0,33. Унікальними алелями за маркером FITO-063 виявились алелі розміром 258 та 273 п.н. з частотою 0,11 у сортів 'Герос' та 'Чорний велетень' відповідно. Найбільшу кількість алелів було отримано за допомогою маркеру Na12-A02. Відповідно до отриманого розподілу найбільше значення частоти (0,11) мали алелі розміром 158 та 192 п.н. За маркером Na10-B07 з частотою 0,04 було ідентифіковано три алеля. Вказані алелі розмірами 144, 156 та 194 п.н. виявили у сортів 'Кліфф', 'Герос' та 'Нельсон'. У результаті кластерного аналізу отримано чотири кластери: 'Сенатор Люкс' та 'Дангал', 'НК Технік' та 'НК Петрол', 'Герос' та 'Аліот', 'Кліфф' та 'Нельсон'. Відмічено, що сорт 'Чорний велетень' не належить до жодного кластеру. Встановлено, що найбільш віддаленими виявились сорти 'Кліфф' та 'Нельсон' із значенням генетичних дистанцій 3.32. Найбільш подібними за чотирма досліджуваними SSR маркерами виявились сорти іноземної селекції 'НК Технік' та 'НК Петрол' зі значенням генетичних дистанцій між ними 1.41. Інші сорти мають відмінності за щонайменше одним маркером. **Висновки.** Застосування системи із чотирьох мікросателітних маркерів забезпечує оцінку сортового різноманіття ріпаку для комплексної селекції *in vitro* на посухо- та солестійкість.

Ключові слова: *сортове різноманіття; ріпак; мікросателітні маркери; кластерний аналіз.*

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Цель. Оценить генетическое разнообразие сортов рапса с помощью SSR маркеров для создания селекционного материала с применением его в комплексной селекции *in vitro* на засухо- и солеустойчивость. **Методы.** ПЦР анализ, кластерный анализ. **Результаты.** Приведены результаты исследований полиморфизма сортов рапса на основе молекулярно-генетических маркеров. В результате анализа сортов рапса по испытуемым маркерам обнаружили 41 аллель, то есть в среднем 10,3 аллелей на маркер. Количество полиморфных локусов по четырем микросателитным маркерам (Ra3-H09, Na12-A02, FITO-063 и Na10-B07) составляло 24 локуса. Уровень полиморфизма изучаемых сортов в среднем составляет 51%: самый высокий уровень (87%) отмечен для маркера Na12-A02, самый низкий (33%) – FITO-063. В соответствии с полученным распределением наибольшей частотой по SSR маркеру Ra3-H09 отличалась аллель размером 117 п.н., которую идентифицировано у трех сортов: 'Сенатор Люкс', 'Дангал' и 'Черный велетень'. Выявлено, что по маркеру Ra3-H09 уникальным для изучаемых сортов оказались аллели с частотой 0,06 и размерами 135 п.н. у сорта 'Алиот' и 156 п.н. у сорта 'Клифф'. По маркеру FITO-063 идентифицировано наименьшее количество аллелей (5), при этом распределение частот варьировало от 0,11 до 0,33. Уникальными аллелями по маркеру FITO-063 оказались аллели размером 258 и 273 п.н. с частотой 0,11 у сортов 'Герос' и 'Черный велетень' соответственно. Наибольшее количество аллелей было получено с помощью маркера Na12-A02. В соответствии с полученным распределением наибольшее значение частоты (0,11) было у аллелей размером 158 и 192 п.н. По маркеру Na10-B07 с частотой 0,04 было идентифицировано три аллеля. Указанные аллели размерами 144, 156 и 194 п.н. обнаружили у сортов 'Клифф', 'Герос' и 'Нельсон'. В результате кластерного анализа получено четыре кластера: 'Сенатор Люкс' и 'Дангал', 'НК Техник' и 'НК Петрол', 'Герос' и 'Алиот', 'Клифф' и 'Нельсон'. Отмечено, что сорт 'Черный велетень' не принадлежит ни к одному кластеру. Установлено, что наиболее удаленными оказались сорта 'Клифф' и 'Нельсон' со значением генетических дистанций 3.32. Наиболее подобными по четырем исследуемыми SSR маркерами оказались сорта иностранной селекции 'НК Техник' и 'НК Петрол' со значением генетических дистанций между ними 1.41. Другие сорта имеют различия по крайней мере по одному маркеру. **Выводы.** Таким образом, применение системы из четырех микросателлитных маркеров обеспечивает оценку сортового разнообразия рапса для комплексной селекции *in vitro* на засухо- и солеустойчивость.

Ключевые слова: *сортовое разнообразие; рапс; микросателлитные маркеры; кластерный анализ.*

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