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Creation of source for marking and mapping of frost resistance QTL in winter barley

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Purpose. Creation the sets of recombinant-inbred lines for marking and mapping of frost resistance QTL in winter barley. **Methods.** Hybridization, artificial vernalization, growing plants under long-day conditions in phytotron light chambers and on a plot of land under natural daylight duration condition, DNA isolation with using a CTAB-buffer, spectrophotometric determination of DNA concentration, polymerase chain reaction with direct primers, agarose and polyacrylamide gels electrophoresis, method of mathematical statistics chi-square test (χ^2). **Results.** Using the SSD (single seed descent) method with maximum avoidance of selection, a set of 265 recombinant inbred lines F_9 from the cross combination of the varieties 'Khutorok' / 'Grabe' was created, differing in the level of frost resistance, the type of development and other traits. The creation of RIL from the crossing of the varieties 'Akademichnyi' / 'Luran' (170 lines F_4) and 'Timofey' / 'Snihova Koroleva' (145 lines F_5) is still in progress. The polymorphism of parental genotypes for the 14 microsatellite loci of chromosome 5H, including those localized in the region of the *Fr-H1* and *Fr-H2* key genes of the main frost resistance QTL. The allelic differences between parental varieties in several cross combinations were detected for a number of the studied microsatellite loci. The inheritance pattern of polymorphic microsatellite loci alleles in F_1 hybrids from three cross combinations and individual plants from F_2 populations 'Akademichnyi' / 'Luran' was investigated. **Conclusions.** The created RILs are suitable for use in barley genetic studies for identification, labeling, mapping of the main genes and QTL of qualitative and quantitative traits, primarily frost resistance. Polymorphic microsatellite loci were identified, which can be used for further analysis of recombinant-inbred lines, manipulation of genetic diversity, tracing inheritance and studying the effects of their alleles by the level of frost resistance and other traits of barley.

Keywords: *Hordeum vulgare L.; recombinant inbred lines; microsatellite loci; PCR analysis; frost resistance.*

Introduction

Barley is a versatile crop, both in its terms of both its distribution and its uses. The varieties cultivated for production are classified as spring, winter and facultative (alternative or intermediate) according to their developmental type. Winter barley varieties are resistant to negative temperatures and require vernalization. Spring varieties are more sensitive to negative temperatures and do not respond to vernalization [1]. Both winter and spring barley varieties may have different levels of photoperiodic sensitivity. Facultative barley varieties are resistant to negative temperatures like winter varieties, but do not require vernalization like spring varieties [2] and this group of varieties is characterised by a strong response to a reduction in day length [3].

Until the middle of the last century, only spring varieties of barley were grown in Ukraine. The first winter barley variety, 'Odeskyi 17', was developed by P. F. Harkavyi in 1955. However, 'Odeskyi 17' was not a typical winter variety; it was facultative, meaning it could be sown in autumn or spring. Since then, targeted breeding of winter and facultative varieties has been carried out. In the 1990s, winter barley varieties were cultivated only in four southern regions and the Republic of Crimea, covering 300,000 hectares. In recent decades, climate change has contributed to a significant increase in the area under this crop, which now exceeds 1 million hectares [4]. The increase in winter barley acreage has been observed not only in the Steppe zone, but also in the Forest-Steppe and Polissia regions [5].

Frost hardiness of winter spiked crops is a quantitative trait determined by a complex of physiological and biochemical features and anatomical and morphological characteristics. There is a connection between barley frost resistance and the response to vernalization and

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photoperiod [6], light intensity and spectral composition [7], sugar accumulation [8], content of certain amino acids [9], plant height [10], xylem and stomatal cell size [11], and other traits. The contribution of individual indicators to the overall level of frost resistance depends on the biological characteristics of the genotype, the intensity of the manifestation and duration of the stress factor. Thus, the frost resistance of facultative varieties is primarily related to the reaction to the length of the day, and for typical winter varieties, the main determining factor is the duration of the vernalization stage [12]. Under conditions of shortened photoperiod, facultative varieties form a significantly higher level of frost resistance compared to typical winter varieties [13].

The breeding strategy for improving the resistance of typical winter barley and alternative types of development to unfavourable temperature conditions requires the development of theoretical foundations for the breeding of this crop [5]. The adaptability of plants to specific conditions can be significantly improved through the targeted manipulation of specific genes, following a detailed study of their effects on specially created, genetically identified material [14]. Such material can be recombinant inbred lines (RILs). Each RIL is a fixed combination of genes for different traits of the parental components of the cross. RILs are a powerful tool for genetic mapping and identifying qualitative trait genes with clear effects, as well as quantitative trait loci (QTL) with minor effects that vary depending on the conditions [15]. Recombinant inbred barley lines are widely used to study the effects of the *Vrn-H1*, *Vrn-H2* and *VRN-H3* genes on flowering time, plant height, biomass and yield [16], as well as the duration of vernalization [17]. They have also been used for mapping loci for quantitative traits such as earing date [18], plant height [19], grain weight and number [20], ear length [21], fusarium ear blight resistance [22], drought tolerance [23], root system traits [24], and for developing genetic maps [25].

The aim of the research is to create sets of recombinant-inbred lines for marking and mapping QTLs of frost resistance in winter barley.

Materials and methods of research

The varieties of winter barley ('Akademichnyi', 'Luran', 'Khutorok') and alternative types of development ('Grabe', 'Timofey', 'Snihova Koroleva'), as well as their F_1, F_2 and $F_{3–8}$ interspecific hybrids, were used as the starting material. The 'Akademichnyi' and 'Snihova Koroleva' varieties were developed at the Plant Breeding and Genetics Institute – National Centre for Seed and Cultivar Research (Ukraine), while the others are of foreign origin, including the 'Luran' and 'Grabe' varieties from the Czech Republic. To prevent intra-varietal polymorphism, individual plants were selected from each crop and used for hybridization to obtain F_1 seeds.

The F_1, F_2 and $F_{3–8}$ seeds were germinated for five days at a temperature of 18 °C with 12 hours of daylight. The green sprouts were then subjected to artificial vernalization at a temperature of 2–4 °C and a day length of 16 hours for 30 days. After vernalization, the sprouts were planted in 5-litre growing vessels containing soil (10 plants per vessel) and cultivated in phytotron chambers at 21–24 °C during the day and 16–18 °C at night, under an extended photoperiod of 16 hours of daylight and 8 hours of darkness, or outdoors under natural conditions. During the growing season, the plants were regularly watered as needed and fertilized with a nitrophoska solution. Disease and pest control was also carried out. Seeds were collected separately from each plant at all stages of RIL creation, from F_1 to F_8.

Recombinant-inbred lines were created by the sequential reseeding of hybrid combinations using the SSD (single-seed descent) method. To achieve this, 150–270 seeds were selected from the F_2 generation of each hybrid population and sown to produce the F_3 generation. One seed was also sown from each F_3 plant to obtain F_4 seeds, and so on. By the $F_{6–8}$ generation, the plants are homozygous for most genes. These plants form the basis of constant lines that can be used for genetic analysis.

Total genomic DNA, suitable for PCR analysis, was extracted from 100 mg of leaves from the original parental genotypes and their progeny, using the Cetavlon method according to protocol [25]. The concentration and quality of the extracted DNA were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), according to the instructions. Samples of the extracted DNA were stored in a refrigerator at +4 °C throughout the study [25].

PCR analysis of DNA samples from barley genotypes was performed at the following microsatellite loci: Bmag812, Bmag0222, Bmag0223, Bmag0323, Bmac0337, Bmag0760, LOXC, GBM1166, GBM1227, GMS061, UMB702, Bmag0357 and Bmag0387 [26, 27]. These microsatellite loci were selected based on information from previous barley studies [26, 27], particularly those localized on the 5H chromosome containing the frost resistance QTL, primarily in the region of the main *Fr-H1* and *Fr-H2* genes. Alleles of the 5H chromosome microsatellites can potentially contribute to frost

resistance in barley genotypes. Where available, information on the quality of PCR products, the level of polymorphism (PIC, or Polymorphic Information Content) and genetic diversity was also taken into account.

PCR amplification was performed using a C1000 Touch thermocycler (Bio-Rad, USA). Commercial PCR reagents/kits (Thermo Fisher Scientific, USA) were used to prepare the PCR mixture: Taq DNA polymerase PCR buffer (10×) [200 mM Tris-HCl (pH 8.4), 500 mM KCl], $MgCl_2$ (50 mM), Dream Taq DNA polymerase (5 U/µl), dNTP mix (10 mM each), Tween-20 (Serva, Germany) and oligonucleotides (Metabion, Germany). The PCR reaction mixture (1×) in a volume of 25 µL contained: 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.01% Tween 20, 0.2 mM each of dNTPs (deoxynucleoside triphosphates), 0.25 µM each of a pair of oligonucleotide primers, 50 ng of DNA and 0.5 units of *Taq* polymerase.

Each microsatellite region was amplified using specific primer pairs of oligonucleotide primers. The sequences of these primers and the PCR amplification conditions were described previously [26, 27].

The products of the PCR amplification reaction were separated by electrophoresis using 4% agarose and 10% PAGE gels in 1× TCE. To visualize the PCR-amplified DNA products in the agarose gels, an aqueous solution of ethidium bromide, an intercalating dye, was used at a final concentration of 0.5 µg/ml. This forms a stable compound with the DNA fragments that appear as bands when the gel is irradiated with UV light. To visualize the PCR products in the PAGE gel, a silver staining procedure was performed [25].

The Gel Doc XR+ visualisation system (Bio-Rad, USA) was used to document the electrophoretic distribution of PCR products in gels. The digitized gels were processed using ImageLab software, which is designed for basic and advanced image analysis. The size of the PCR products was determined relative to the 50 bp and/or 100 bp DNA Ladder molecular weight markers (Fermentas/Thermo Fisher Scientific, USA), which contain fragments of known sizes in bp covering the range of microsatellite locus allele sizes (100–400 bp).

The correspondence between the data obtained on the allele cleavage of polymorphic microsatellite (MS) loci in the F_2 population and the theoretically expected data was calculated using the chi-squared (χ^2) statistical method.

Research results

The success of genetic analysis depends on the genetic polymorphism of the trait under consideration [15]. Therefore, winter barley varieties 'Akademichnyi', 'Luran', 'Khutorok' and facultative barley varieties 'Grabe', 'Timofey', 'Snihova Koroleva', which differed significantly in terms of frost resistance, were used as parental components for the initial crossing to create RILs [28]. 'Akademichnyi' has a high level of frost resistance, 'Khutorok' and 'Timofey' have an above-average level, 'Grabe' and 'Snihova Koroleva' have a below-average level, and 'Luran' has a low level. Since winter and facultative barley varieties form frost resistance through different physiological mechanisms [13], three crossing combinations were used to create RILs: 'Akademichnyi' / 'Luran' (winter / winter), 'Timofey' / 'Snihova Koroleva' (facultative / facultative) and 'Hutorok' / 'Grabe' (winter / facultative). In the first two combinations of crossings, the effects of alleles of genes/loci for resistance to low temperatures can be determined against the background of winter or alternative development, respectively. In the third combination, the differences in the varieties' developmental types are taken into account.

As a result of implementing the above scheme (see Materials and Methods), a set of 265 recombinant-inbred F_9 lines from the cross 'Khutorok' / 'Grabe' was created, and the creation of RILs from the crosses 'Akademichnii' / 'Luran' (170 F_4 lines) and 'Timofey' / 'Snihova Koroleva' (145 F_5 lines) is ongoing. The main condition for creating RILs is to minimize the effect of selection for the trait under study as much as possible [29]. To avoid the effects of negative temperature on the reproduction of RIL generations, $F_{1–4}$ plants of the 'Khutorok' / 'Grabe' cross combination were grown in light chambers in the phytotron greenhouse (two generations per year). Plants $F_{5–9}$ of the 'Akademichnyi' / 'Luran' cross combination and $F_{1–5}$ of the 'Timofey' / 'Snihova Koroleva' cross combination were grown on the vegetation site during spring planting with spring sprouts.

In parallel with creating the RIL sets, we evaluated the polymorphism of the parental genotypes at 14 microsatellite loci (Bmag812, Bmag0222, Bmag0223, Bmag0323, Bmac0337, Bmag0760, LOXC, GBM1166, GBM1227, GMS061, UMB702, Bmag0357, Bmag0387 and Bmag0113a). These loci are located on chromosome 5H [26, 27], including in the area of the frost resistance QTL, particularly the main *Fr-H1* and *Fr-H2* genes [30]. The presence of polymorphism enables us to estimate the contribution of allelic differences at the studied microsatellite loci to the population of recombinant-inbred barley lines' overall frost resistance.

Of the microsatellite loci studied, nine out of 14 were found to be non-polymorphic. A 280 bp

allele was detected at the UMB702 locus in all six genotypes studied, as well as a 165 bp allele at the GBM1166 locus, a 123 bp allele at the Bmag0387 locus, a 145 bp allele at the Bmag0357 locus and 300/310 bp alleles at the GBM1227 locus. 190 bp and 153 bp alleles were detected at the Bmag812 and Bmag0113a loci, respectively. A 130 bp allele was detected at the Bmag0337 locus, and a 182 bp allele at the LOXC locus.

At the same time, microsatellite analysis of loci Bmag0223, Bmag0760, Bmag0222, GMS061 and Bmag0323 revealed polymorphisms in one or more crossing combinations between parental varieties. These polymorphic regions provide an opportunity to manipulate genetic diversity. For instance, allelic polymorphism between the parents was detected at the microsatellite locus Bmag0223 in the initial genotypes of the 'Akademichnyi' / 'Luran' combination. The parental genotype 'Akademichnyi' had a 160 bp allele, while the second parental genotype of this combination, 'Luran', had a 150 bp allele (Fig. 1B).

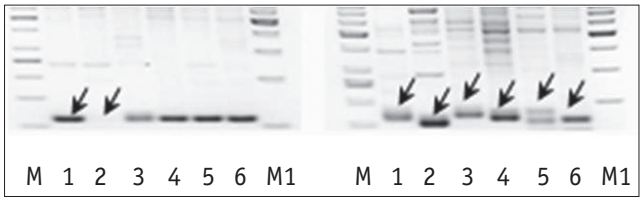

Fig. 1. Electrophoregram of DNA amplification products of the original barley parental genotypes at the microsatellite loci of chromosome 5H:

A – Bmag0760; B – Bmag0223; 1–6 – varieties: 'Akademichnyi' (1), 'Luran' (2), 'Snihova Koroleva' (3), 'Timofey' (4), 'Khutorok' (5), 'Grabe' (6); M – 50 bp DNA Ladder molecular weight marker, M1 – 100 bp DNA Ladder molecular weight marker

At the Bmag0760 locus, the varieties 'Akademichnyi' and 'Luran' had 110 bp and null alleles, respectively (Fig. 1A). At locus Bmag0222, 'Akademichnyi' exhibited genotypes with two different allelic variants, measuring 155 bp and 160 bp respectively. Only the 155 bp allele was detected in 'Luran'. Both varieties were also present at the GMS061 locus, with 145 bp and 140 bp alleles, respectively. At the Bmag0323 locus, the allelic difference between the parental genotypes of the 'Akademichnyi' / 'Luran' combination was not detected either. Both varieties were heterogeneous, carrying two 148 bp and two 165 bp alleles.

PCR analysis of two initial genotypes, 'Timofey' and 'Snihova Koroleva', revealed allelic polymorphism with the microsatellite marker Bmag0223 (160 and 170 bp alleles, respectively). Both parents exhibited heterogeneity at the Bmag0323 locus, with alleles of 148 bp and 160 bp. Alleles of 150 bp at the Bmag0222 locus, 110 bp at the Bmag0760 locus, and 145 bp at the GMS061 locus were also identified in both parental genotypes.

For the initial genotypes of the 'Khutorok' / 'Grabe' combination with the Bmag0223 microsatellite marker, a 127 bp allele was detected in 'Khutorok', and two allelic variants of 127 and 160 bp were detected in 'Grabe'. No allelic differences were found between the parental genotypes 'Khutorok' and 'Grabe' at other microsatellite loci. Both genotypes have the same allelic variants: 160 bp at the Bmag0222 locus; 110 bp at the Bmag0760 locus; 145 bp at the GMS061 locus; and 155/148 bp at the Bmag0323 locus.

The DNA of F_1 hybrids and individual plants from the F_2 populations of certain crossing combinations was analyzed at the above polymorphic MS loci. Thus, at the Bmag0223 locus, the presence of a combination of alleles of parental genotypes of 160/170 bp was observed in the F_1 hybrid plants 'Timofey' / 'Snihova Koroleva' (Table 1), as would be expected with codominant inheritance of a microsatellite marker. A similar pattern was observed for F_1 hybrids resulting from the cross between 'Akademichnyi' / 'Luran' with this microsatellite marker. Two alleles of 150/160 bp from both initial genotypes were detected in each of the studied F_1 plants.

One hundred and seventeen plants of the F_2 population 'Akademichnyi' / 'Luran' were identified according to the alleles of the microsatellite locus Bmag0223. The results of the analysis revealed that a 160 bp microsatellite allele of the Bmag0223 locus was present in 32 individual plants, a trait inherited from the 'Akademichnyi' parent (Fig. 2). A microsatellite

Table 1

Differences of parental varieties (P_1, P_2) and genotypes of F_1 hybrids at microsatellite loci Bmag0223, Bmag0760 and GMS061

Locus	Crossbreeding combination (P_1 / P_2)	P_1	P_2	F_1
Bmag 0223	'Timofey' / 'Snihova Koroleva'	160	170	160/170
Bmag 0223	'Akademichnyi' / 'Luran'	160	150	150/160
Bmag0760	'Akademichnyi' / 'Luran'	110	n. a.	110
GMS061	'Akademichnyi' / 'Luran'	140/145	140/145	140

allele of 150 bp was detected in the other 25 samples studied, which corresponds to the original parental variety 'Luran'. The heterozygous state of the locus and the presence of two fragments, one of 150 bp and one of 160 bp, which are found in both original parental genotypes, were detected in 60 out of 117 analysed plants. When the two parental varieties differ in two alleles of the same locus and codominant inheritance is observed in the F_2 generation, a 1:2:1 split should be observed. Thus, in F_2 'Akademichnyi' / 'Luran', in the DNA analysis of 117 individual plants, 29.25 homozygous plants were theoretically expected to have the 'Akademichnyi' allele in their genotype, 29.25 plants to have the 'Luran' allele, and 58.5 heterozygous plants to carry the alleles of both parents. The actual cleavage at the Bmag0223 locus corresponded to the theoretically expected ratio. The χ^2 criterion was 0.99 with $\chi^2_{0.05} = 5.99$ for df = 2. The codominant nature of allele inheritance in F_1 hybrids and the correspondence of the actual cleavage to the theoretically expected ratio of genotypes of the F_2 population makes it possible to use the Bmag0223 marker locus in the analysis of RILs in further studies.

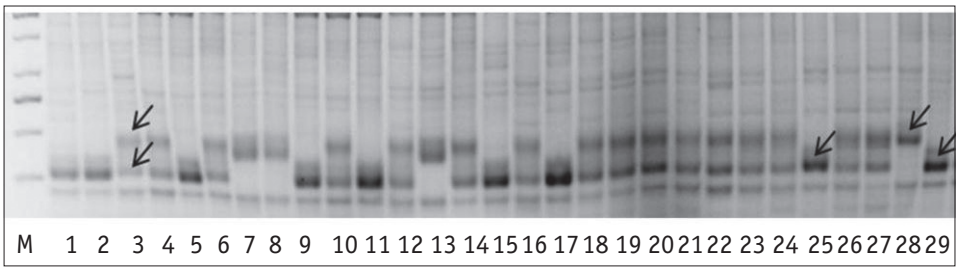

Fig. 2. Electrophoregram of DNA amplification products of F2 'Akademichnyi' / 'Luran' plants at the Bmag0223 microsatellite locus:
1–27 – individual F_2 plants; 28 – 'Akademichnyi'; 29 – 'Luran'; Arrows indicate genotype variants. M – 50 bp DNA Ladder molecular weight marker

According to PCR analysis of F_1 hybrids of this crossing combination at the Bmag0760 locus, only the 110 bp allele inherent in the 'Akademichnyi' variety was detected in all plants, since the null allele (absence of a PCR fragment) was noted for the 'Luran' variety at this locus. To avoid false results, further use of Bmag0760 should include checks for the presence of the null allele.

According to the results of the PCR analysis, the 145 bp allele was detected in the F_1 hybrids resulting from the cross 'Akademichnyi'/ 'Luran', as well as in all the plants in the F_2 population, using the GMS061 microsatellite marker (Fig. 3). This indicates that individual plants of parental genotypes with the same allele were involved in the primary cross, taking into account the genetic heterogeneity detected in the original varieties at this locus. As the same allele was detected in all F_2 plants, further use of this marker to analyze RILs for allele effects is not possible or informative.

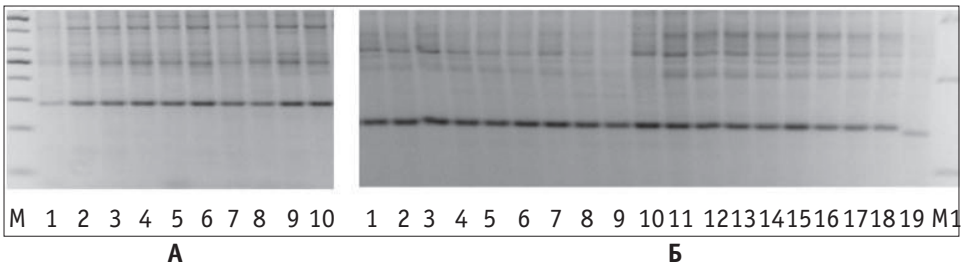

Fig. 3. Electrophoregram of PCR amplification products of DNA from F_1 and F_2 hybrids of the crossing combination 'Akademichnyi' / 'Luran' at the GMS061 locus:
A 1–10 – F_1 plants, M – 50 bp DNA ladder molecular weight marker; B 1–17 – individual F_2 plants, 18 – 'Luran'; 19 – 'Akademichnyi'; M 1 – 100 bp DNA ladder molecular weight marker

Conclusions

A set of 265 recombinant inbred F_9 lines was created by crossing the 'Khutorok' and 'Grabe' varieties. The creation of RILs from crosses between 'Akademichnyi' and 'Luran' (170 F_4 lines) and 'Timofey' and 'Snihova Koroleva' (145 F_5 lines) is ongoing. Allelic differences were revealed at five microsatellite loci (Bmag0223, Bmag0760, Bmag0222, GMS061 and Bmag0323) between the parental varieties in one or more crossing combinations. The codominant nature

of allele inheritance in F_1 hybrids, and the fact that the actual cleavage corresponds to the theoretically expected ratio of genotypes in the F_2 population, means that the Bmag0223 marker locus can be used to identify RIL genotypes obtained from crossing the 'Khutorok' and 'Grabe' varieties, or the 'Akademichnyi' and 'Luran' varieties, and to study the effects of alleles at this locus on frost resistance. However, the presence of intra-varietal variation at the Bmag0222 and Bmag0223 loci, or a null allele at the Bmag0760 locus, in the parental genotypes complicates the evaluation and interpretation of the results, and precludes the use of such markers to identify RIL genotypes created using the 'Akademichnyi', 'Grabe' and 'Luran' varieties.

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Мета. Створити набори рекомбінантно-інбредних ліній для маркування та картування QTL морозостійкості озимого ячменю. **Методи.** Гібридизація, штучна яровизація, вирощування рослин в умовах подовженого дня світлих камер фітотрона та на вегетаційному майданчику за природної тривалості дня, виділення ДНК за допомогою ЦТАБ-буфера, спектрофотометричне визначення концентрації ДНК, полімеразна ланцюгова реакція з направленими праймерами, електрофорез в агарозному та поліакриламідному гелях, метод математичної статистики хі-квадрат (χ^2). **Результати.** Методом НОН (SSD) – нащадки однієї насінини (single seed descent) – з максимальним уникненням дії добору створено набір з 265 рекомбінантно-інбредних ліній F_9 комбінації схрещування сортів 'Хуторок' / 'Grabe', що різняться за рівнем морозостійкості, типом розвитку та іншими ознаками. Триває створення рекомбінантно-інбредних ліній від схрещування сортів 'Академічний' / 'Luran' (170 ліній F_4) і 'Тимофей' / 'Снігова королева' (145 ліній F_5). Оцінено поліморфізм батьківських генотипів за 14 мікросателітними локусами хромосоми 5H, зокрема локалізованих в області ключових генів QTL морозостійкості *Fr-H1* і *Fr-H2*. Виявлено алельні відмінності між батьківськими сортами в декількох комбінаціях схрещування за низкою проаналізованих мікросателітних локусів. Досліджено характер успадкування алелів поліморфних МС-локусів у гібридів F_1 трьох комбінацій схрещування та рослин популяцій F_2 'Академічний' / 'Luran'. **Висновки.** Створені рекомбінантно-інбредні лінії придатні для використання у генетичних дослідженнях з метою ідентифікації, маркування, картування головних генів та QTL якісних і кількісних ознак ячменю, передусім морозостійкості. Визначено поліморфні мікросателітні локуси, що можуть бути застосовані для подальшого аналізу популяції рекомбінантно-інбредних ліній, маніпулювання генетичним різноманіттям, простеження успадкування, визначення ефектів їхніх алелів або генів, тісно зчеплених з ними за рівнем морозостійкості, та інших ознак ячменю.

Ключові слова: *Hordeum vulgare L.; рекомбінантно-інбредні лінії; мікросателітні локуси; ПЛР-аналіз; морозостійкість.*

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