Original Paper

Identification of polymorphism of the *CSN2* gene encoding beta-casein in ukrainian black and white breeds of cattle

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Genotyping of 606 animals of the Ukrainian Black-and-White dairy breed was carried out in order to establish the features of genotype formation by beta-casein. It is established that the modern population of this breed has been formed using stud bulls of the Holstein breed, mainly of American and German breeding. It is found that the original breed has determined the features of polymorphism of the beta-casein gene. According to the research results, it is proved that the existing genetic structure of stud bulls of the Holstein and Ukrainian Black-and-White dairy breeds enables the formation of populations homozygous by these characteristics in subsequent generations. Among the breeding stock of the Ukrainian Black-and-White dairy breed, the frequency of the desired A2A2 genotype was 33–53%. It is expected to increase the frequency of occurrence of individuals with the A2A2 genotype by beta-casein in subsequent generations, especially if stud bulls homozygous by A2A2 beta-casein is used.

Keywords: breed, allele, genotype

1 Introduction

Oven recent years, scientists have found that cow's milk usually contains two main types of beta-casein, such as A1 and A2, although there are 13 genetic variants of casein: A1, A2, A3, A4, B, C, D, E, F, H1, H2, I, G. Variant A1 beta-casein is harmful, while A2 beta-casein is a safer choice for human health, especially in infant nutrition and health (Park & Haenlein, 2021; Tim, 2021).

Researchers have found a possible link between milk consumption and certain diseases, such as Type 1 diabetes, cardiovascular diseases, sudden infant death syndrome (SIDS), schizophrenia and autism, gastrointestinal diseases, prostate cancer, and other diseases (Amatya et al., 2021).

The homozygous A2A2 genotype is predominantly found in most Indian cattle (Gir, Hariana, and Kangayam). However, cattle in Western Europe (Denmark, Slovakia) have either the dominant A1 genotype or an equal proportion of A1 and A2 beta-casein variants. Scientists note that A1 and A2 variants of beta-casein are found in the milk of the Holstein and Jersey breeds worldwide. At the same time, the A2 type is more dominant in the Jersey and Holstein breeds, in contrast to A1. In addition, scientists note that there is a significant difference in the frequency of the beta-casein allele of Holstein cattle, depending on geographical origin (Amatya et al., 2021; Antonopoulos et al., 2021; Mumtaz et al., 2021).

The Pakistani Holstein breed population is generally represented by only two genotypes – A1A2 and A2A2, corresponding frequencies of 60 and 40%. The A1A1 genotype does not occur at all. The Greek population of the Holstein breed is characterized by a high frequency of the desired homozygous A2A2 genotype (52.2%). (Mumtaz et al., 2021; Antonopoulos et al., 2021).

The authors cite that the results of studies show the next frequencies of the desired homozygous genotype in the Holstein breed: in Irish (25%), British (26%) and Slovak (4%) (Ivanković et al., 2021; Hanusová et al., 2010).

It has been found that the use of Holstein breed sires to improve local livestock leads to a decrease in the frequency of the A2 allele. Thus, in the local breed Gir

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and Kankrej, the allele frequency are 0.82 and 0.92, respectively. In crossbreeds of the first generation with Holstein sires, the frequency of the A2 allele was, on average, 0.63 (Patel et al., 2019; Gorkhali et al., 2020).

The local Lebedyn cattle (Ukraine) is characterized by a high frequency of the desired homozygous A2A2 genotype and A2 allele. This creates prerequisites for a high frequency of this genotype in the breed structure created on the basis of the Lebedyn breed by its holstinization (Ladyka, Pavlenko & Sklyarenko, 2021; Ladyka et al., 2019; Sklyarenko et al., 2018).

The Ukrainian Black-and-White dairy breed was created using the breeding stock of the local Black-and-White, Simmental and Lebedyn cattle. The Ukrainian Red-and-White dairy breed has a similar history of creation. These breeds were created with the use of Holstein bulls of Western European and North American breeding. Today, both populations in Ukraine are under threat of extinction, due to the uncontrolled use of Holstein sires.

It is believed that production of milk with the A2A2 genotype based on beta-casein is one of the ways to increase the economic competitiveness of small and medium-sized farms. Therefore, livestock breeders who keep local small-numbered breeds threatened with extinction are interested in obtaining the status of producers of such milk (Ivanković et al., 2021).

Given the above research results, we consider that the issue of studying the polymorphism of the beta-casein gene in the population of the Ukrainian Black-and-White dairy breed, depending on the peculiarities of formation, is relevant.

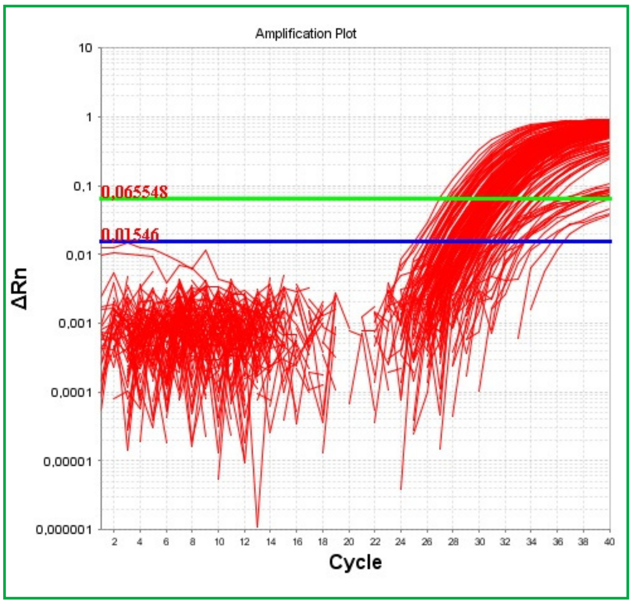
Our study aims to conduct a molecular genetic analysis of the cattle population in the Ukrainian Black-and-White dairy breed to establish the features of genotype formation by beta-casein.

2 Material and methods

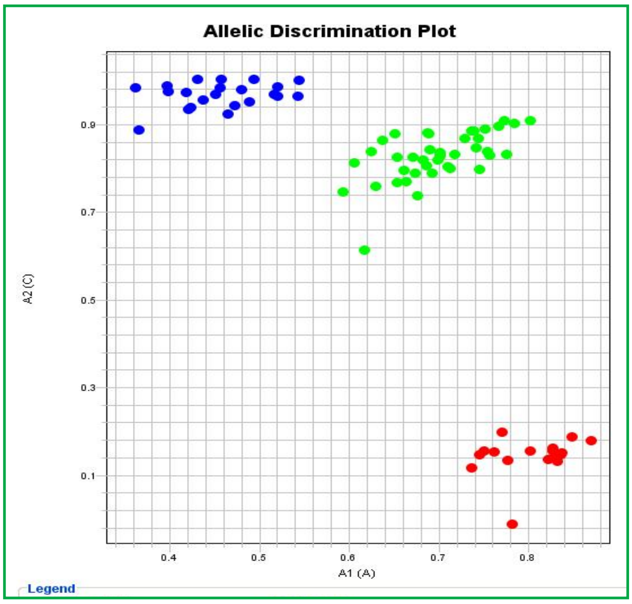
Genotyping was carried out for cattle of the Ukrainian Black-and-White dairy breed of 30 sires (Sumy State-Owned Breeding Center) and 576 cows kept on the farms of the Experimental Farm SOE of the NAAS Institute of Agriculture of the North-East, Mykhailivka BP of Sumy region, Ichnianske and Khliborob of Chernihiv region.

Blood samples were taken under the sterile conditions into 2.7 mL Monovette contains EDTA potassium salt as an anticoagulant ("Sarstedt", Germany) with the following freezing of samples and their storage at -20 °C. The extraction of genomic DNA from whole blood samples was prepared using Monarch®Genomic DNA Purification Kit New England BioLab kits (USA) according to the manufacturer's protocol.

SNP rs43703011 in the beta-casein gene (*CSN2*) was determined according to our methods using the next primers: upstream 5′-CCCAGACACAGTCTCTAGTCTATCC-3′, downstream 5′-GGTTTGAGTAAGAGGAGGGATGTTT-3′ and the next probe – 5′-VIC-CCCATCCATAACAGCC-MGB-3` and 5′-FAM-CCATCCCTAACAGCC-MGB-3` (Thermo Scientific, USA). Amplification realized using Fast Real-time PCR System (Applied Biosystems, США) in total volume 10 мкл with 2X TaqMan Universal Master Mix (Applied Biosystems, USA), primers, probes, and DNA. Amplification of 84 bp



Figrue 1 Amplification curves of genotype determination by *CSN2* gene polymorphism (rs43703011)



Figrue 2 Allelic discrimination by genotypes polymorphism of the *CSN2* gene (rs43703011)

fragment of *CSN2* consisted of two steps: denaturation (95 °C) during 3 s and annealing and elongation (60 °C) during 30 s. Data were analyzed using 7500 Fast Real-Time PCR Software.

The genotypes of the Holstein breed were determined based on the international website – https://www.cdn. Ca/query/individual.php.

The data analysis was performed in the R statistical environment (www.R-project.org, V.4.0) and STATISTICA 10.

The allele frequency was calculated taking into account the number of homozygotes and heterozygotes found in the corresponding allele using the following formula:

$$P(A) = \frac{2N_1 + N_2}{2n} \qquad (1)$$

where: N_1 and N_2 – number of homozygotes and heterozygotes for the studied allele, respectively; n – sample number

In order to assess the statistical reliability of the discrepancy between the distribution of the obtained results the Pearson criterion was used:

$$X^2 = \frac{\sum(A-T)^2}{T} \qquad (2)$$

where: A – actual number of genotypes; T – theoretical number of genotypes

The actual (available) heterozygosity was determined by direct calculation using the following formula:

$$H_0 = \frac{N_2}{n} \qquad (3)$$

The expected heterozygosity was determined using the following formula:

$$H_E = 1 - \sum_{I=1}^{n} p_i^2 \qquad (4)$$

where: $p_1, p_2 ... p_n$ – frequency of alleles

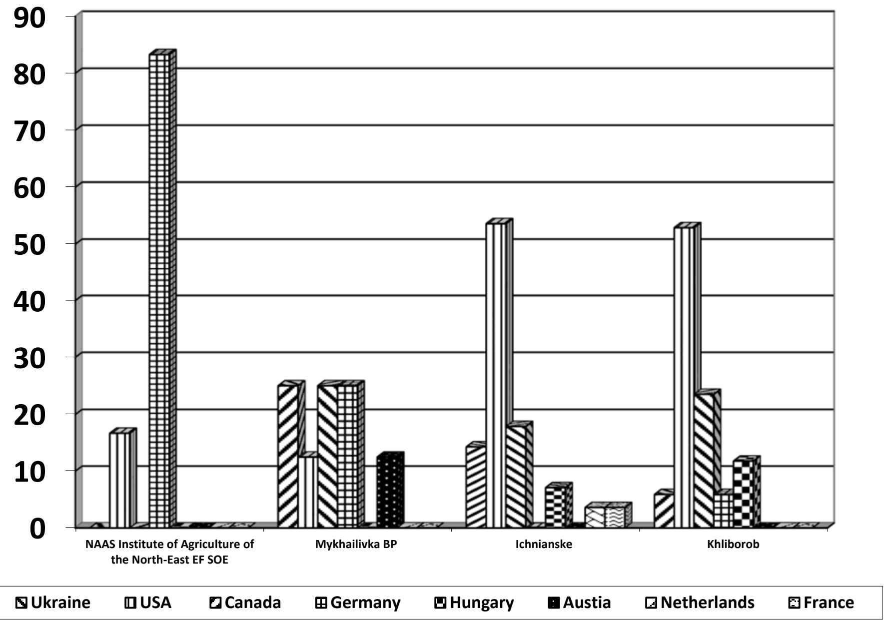
The fixation index was calculated using the following formula:

$$F_{is} = \frac{H_E - H_0}{H_E} \qquad (5)$$

3 Results and discussion

Based on the study of the pedigree characteristics of dairy herds, it was established that on the Experimental Farm of the Institute of Agriculture of the North-East of the National Academy of Agrarian Sciences of Ukraine, most of the bulls used during the last period were of the German breeding and only a small part came from America (Figure 3).

The animals at Mykhailivka Breeding Plant were mostly from Ukraine, Canada and Germany. A slightly smaller part of them was of the USA and Austria breeding. On



Figrue 3 Distribution of sires by country of origin (%)

Ichnianske and Khliborob, the vast majority of sires were of North American breeding (the USA and Canada). A much smaller part of sires came from Ukraine, Germany, Hungary, the Netherlands and France.

These conditions affected the frequency of genotypes of the modern breeding stock of the Ukrainian Black-and-White dairy breed. It was found that the highest frequency of the desired A2A2 genotype was characteristic of cattle of the Mykhailivka Breeding Plant, which exceeded 50%. Almost the same level of frequency of the desired A2A2 genotype was the characteristic of herds of the Experimental Farm, Ichnianske LLC and Khliborob LLC – 33–39%. The herd of Ichnianske Farm was characterized by the frequency of more than 50% of heterozygous genotypes. The lowest frequency of heterozygotes was typical for Mykhailivka Breeding Plant. The breeding stock of livestock of the Experimental Farm and Khliborob LLC had almost the same value of the frequency of heterozygous genotypes – 42–43%. The frequency of homozygous A1A1 genotypes was 14–23% and lowered in farms such as Ichnianske and Khliborob (Table 1).

The results of our research partially coincide with the results obtained by other scientists. The livestock population of the Experimental Farm, which originates from German selection, has almost two times less frequency of A2A2 genotypes compared to the results of other scientists (67%) (Ivanković et al., 2021). In our opinion, this is due to the wide use of breeders of the Holstein breed of North American selection in the period from 1990 to 2005. This population of Holstein cattle is characterized by a low share of the A2A2 genotype (up to 35%) (Ivanković et al., 2021). In the farms of Ichnianske and Khliborob, which mainly used breeders of the Holstein breed of the USA selection and partly from Canadian selection, the share of animals with the A2A2 genotype almost corresponds to the previous results of other scientists (Ivanković et al., 2021). In the Mykhailivka farm, the population was formed mainly under the influence of Ukrainian, Canadian and German selections, has a slightly lower frequency of the A2A2 genotype compared to the German population, and higher than the Canadian (Ivanković et al., 2021) and Ukrainian (Figure 5).

The frequency of the A2 allele was the highest at Mykhailivka BP, and the lowest – on the Experimental Farm of the Institute of Agriculture of the North-East of the National Academy of Agrarian Sciences. At the same time, we note that the frequency of the desired A2 allele was higher compared to the literature data (0.46–0.55) (Amatya et al., 2021; Antonopoulos et al., 2021; Mumtaz et al., 2021). In our opinion, this is due to the original breeds used on the farms – Lebedyn, Simmental, Black-and-White of the European breeding.

The degree of conformity of the observed distribution of genotypes with the expected values was determined using of criterion χ^2. It is found that the observed distribution of the number of different phenotypes

Table 1 Frequency of alleles and genotypes by locus of the beta-casein gene in cows of the Ukrainian Black-and-White dairy breed

Distribution*	Genotype						Allele (pcs)		χ^2
	A1A1		A1A2		A2A2		A1	A2	
	n	%	*n*	%	*n*	%			
NAAS Institute of Agriculture of the North-East EF SOE									
Observed	23	23	42	42	35	35[a***]	0.44	0.56	2.18
Expected	19.4	19	49.3	49	31.3	31			
Mykhailivka BP									
Observed	13	20	17	27	34	53	0.34	0.66	10.47
Expected	7	11	29	45	28	44			
Ichnianske LLC									
Observed	28	13	111	54	67	33[a***, b*]	0.41	0.59	2.86
Expected	33.8	17	99.3	48	72.9	35			
Khliborob LLC									
Observed	36	17	89	43	81	39[a***]	0.39	0.61	1.77
Expected	31.5	15	98.0	48	76.5	37			

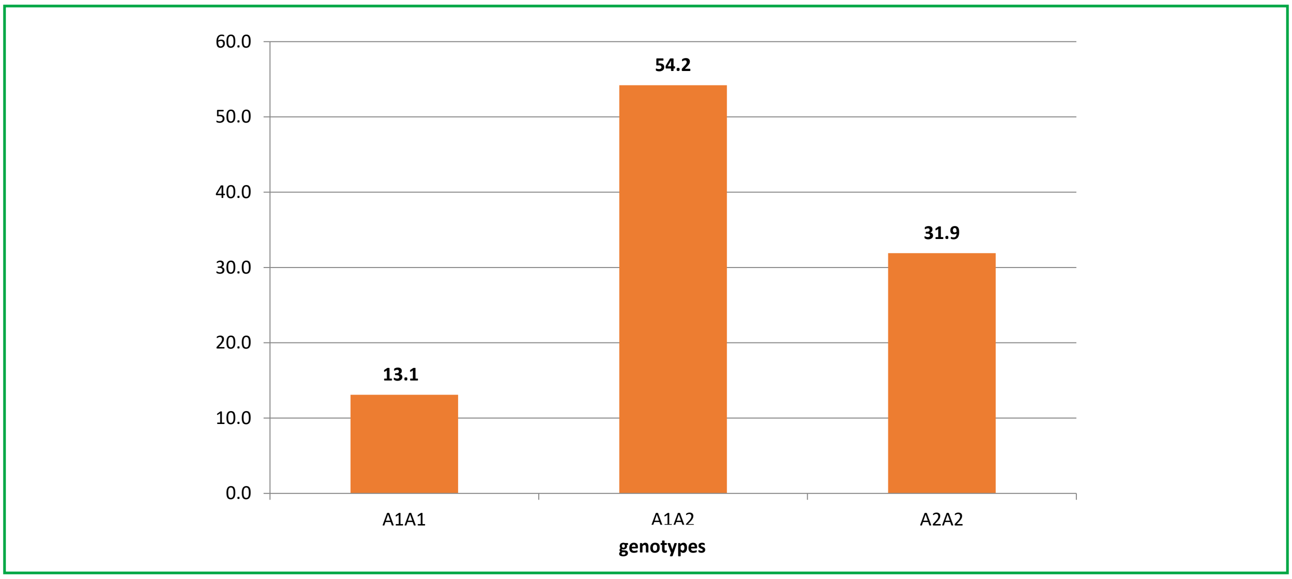
a – the difference between the frequencies of genotypes in the population of Mykhailivka BP and the populations of other farms; b – the difference between the frequencies of genotypes in the population of Mykhailivka BP and the populations of other farms; P – level of significance according to Fisher's test: * – P <0.05; *** – P <0.001

Table 2 Genetic variability of Ukrainian Black-and-White dairy cows by beta-casein locus in the Experimental Farm of the Institute of Agriculture of the North-East of the National Academy of Agrarian Sciences

Indicators	EF SOE		Mykhailivka BP		Ichnianske LLC		Khliborob LLC	
	indicators*		indicators*		indicators*		indicators*	
	observed	expected	observed	expected	observed	expected	observed	expected
Heterozygotes (heads)	42	49.3	17	29	111	99.3	89	98
Homozygotes (heads)	58	50.8	47	35	95	106.7	117	108
Hetero/homozygote coefficient	0.72	0.97	0.36	0.83	1.16	0.93	0.76	0.90
Heterozygosity test	-0.25	–	-0.44	–	0.24	–	-0.15	–
Homozygosity degree, Ca (%)	50.7	–	55.4	–	51.8	–	52.4	–
Polymorphism level, Na	1.97	–	1.80	–	1.93	–	1.91	–
Excess coefficient D	-0.15	–	-0.41	–	0.17	–	-0.09	–
Proportion of homozygotes (%)	58	–	73	–	46	–	56.8	–

corresponds to the theoretically expected one with a high degree of reliability (*P* <0.01) on the three farms – the Experimental Farm of the Institute of Agriculture of the North-East of the National Academy of Agrarian Sciences, Ichnianske LLC and Khliborob LLC. A dairy herd of Mykhailivka Breeding Plant does not correspond to the theoretically expected distribution of this feature.

Using the genetic and statistical methods of analysis, by determining the values of such genetic constants as the degree of homozygosity (Ca), and the level of polymorphism (Na), we have tried to assess the prospects for increasing the frequency of the desired A2A2 genotype in herds. The degree of homozygosity, which indicated the gene consolidation, was relatively the same in all the studied herds and was in the range of 50.7–55.4. It was higher in Mykhailivka Breeding Plant, and the lowest – on the Experimental Farm of the Institute of Agriculture of the North-East of the National Academy of Agrarian Sciences. The level of polymorphism (the number of effective active alleles – Na), on the contrary, was higher on the Experimental Farm of the Institute of Agriculture of the North-East of the NAAS, and the lowest in Mykhailivka Breeding Plant. The heterozygosity test (HT), which reflected the deviation of frequencies of heterozygous genotypes from the theoretically expected proportion of heterozygotes according to the Hardy-Weinberg Equilibrium, was negative in the studied herds, indicating a smaller proportion of observed heterozygotes compared to the proportion of theoretical heterozygotes. The only exception was the Ichnianske LLC herd. As for the excess coefficient (D), which characterizes the ratio of observed heterozygosity



Figrue 4 Structure of the Holstein breed sires allowed to reproduce in Ukraine by the beta-casein genotype (532 sires) (%)
Source: https://www.cdn.Ca/query/individual.php

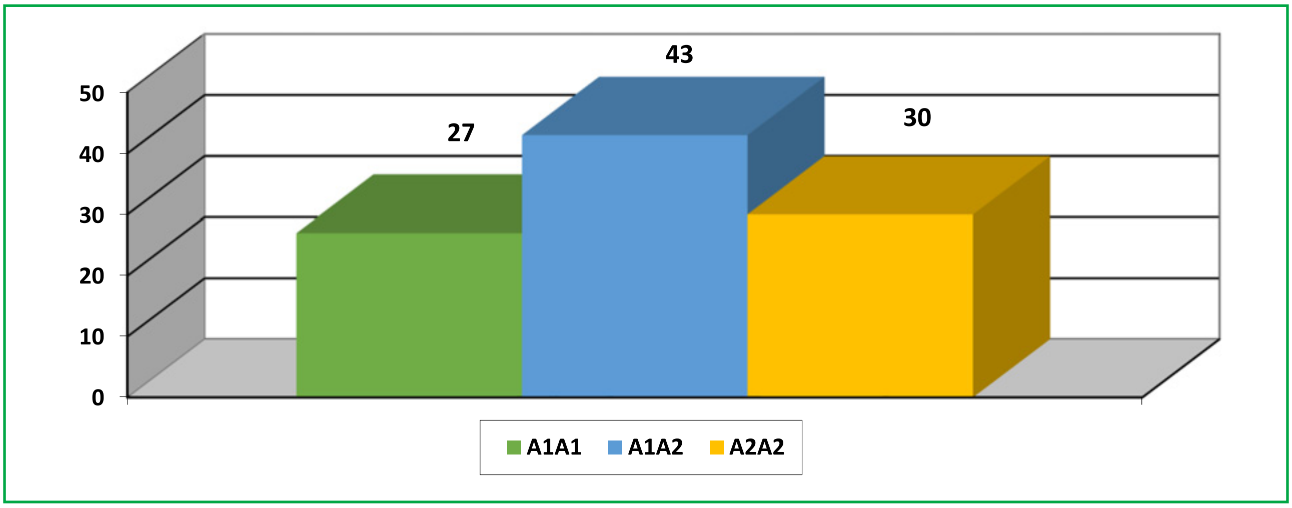


Figure 5 Frequency of beta-casein genotypes in sires of the Ukrainian Black-and-White dairy breed (30 sires)

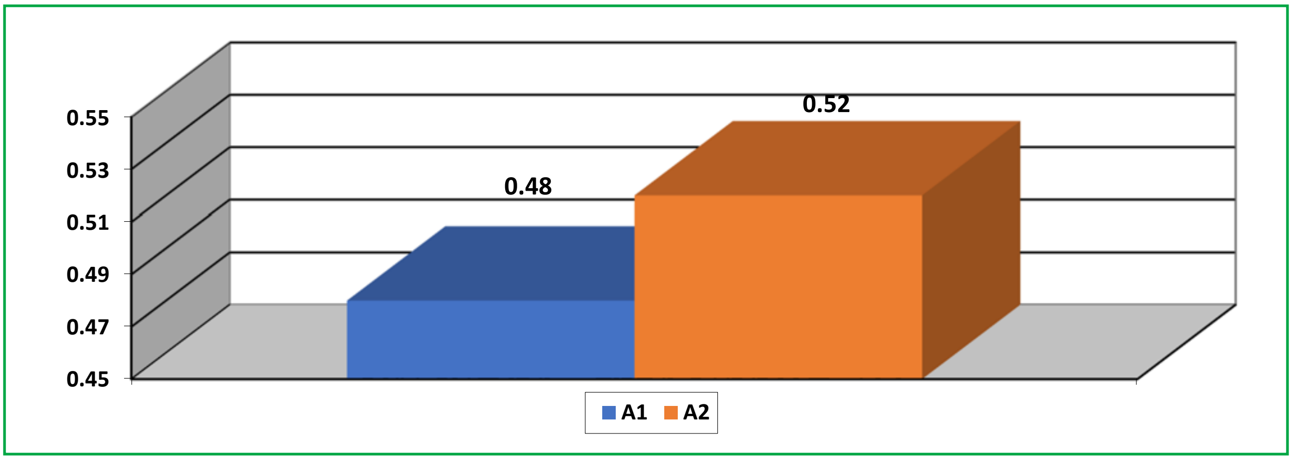


Figure 6 Frequency of beta-casein alleles in sires of the Ukrainian Black-and-White dairy breed (30 sires)

to theoretical heterozygosity, we note a deviation of observed heterozygosity from the expected one with left-sided excess, which also indicates a deficiency of the heterozygote (Table 2), except for Ichnianske LLC.

Most of the Holstein breed sires evaluated by the beta-casein genotype and allowed to reproduce in Ukraine are heterozygous, and one-third have the desired A2A2 genotype (Figure 4).

Among the evaluated sires of the Ukrainian Black-and-White dairy breed, 30% have the desired A2A2 genotype and, therefore, may be used to restore the population of the Ukrainian Black-and-White dairy breed in order to create herds with the A2A2 genotype. The majority – 43% are heterozygous genotypes, which, if used regularly, may also be involved in creating herds with a given genotype. However, their effectiveness will be less than the first ones. Almost a third of sires have the homozygous A1A1 genotype, which may not be used in creating such herds (Figure 5). However, to preserve the Ukrainian Black-and-White dairy breed, they can be widely used on farms.

Almost identical frequencies of homozygous genotypes revealed a slight difference in the frequencies of alleles of the beta-casein gene (Figure 6).

4 Conclusions

The modern population of the Ukrainian Black-and-White dairy breed was formed using mainly sires of the Holstein breed. At the same time, the American and German breeders were predominant. In parallel, the original breed determined the current status of beta-casein gene polymorphism in the studied populations.

The genetic structure of Ukrainian dairy breeders Holstein and Black-and-White allows the formation of homozygous populations for these characteristics in subsequent generations.

The breeding stock of the Ukrainian Black-and-White dairy breed (33–53% of A2A2 homozygous and 27–54% of heterozygous) predicts a significant increase in the frequency of occurrence of individuals with the A2A2 genotype by beta-casein in subsequent generations, especially in case of using homozygous A2A2 sires by beta-casein.

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