ISSN 1817-7204(Print)
ISSN 1817-7239(Online)
УДК 636.293.2.082.12:577.21
https://doi.org/10.29235/1817-7204-2021-59-3-361-365

Поступила в редакцию 15.02.2021
Received 15.02.2021

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GENOTYPING OF "UKRAINIAN" WATER BUFFALOES ACCORDING β-CN (A2-MILK), *CSN3* AND β*LG* GENES

Abstract: Buffalo breeding in Ukraine is an ancient traditional branch of animal husbandry of the Crimean Tatars and Rusyns of Transcarpathia. Basically, "Ukrainian" buffaloes belong to the river buffalo (*Bubalus bubalis*) and are bred for dairy and meat production. Polymorphism of genes associated with dairy productivity will allow breeding buffaloes taking into account the "desired" genotypes in relation to economically useful traits. The paper dwells on studying allelic polymorphisms of beta-casein (β-*CN*), kappa-casein (*CSN3*) and beta-lactoglobulin (β-*LG*) genes in population of water buffaloes bred in Ukraine using PCR followed by restriction hydrolysis of the formed fragments (PCR-RFLP). Results of study of the "Ukrainian" population of water buffaloes are discussed, namely: the frequency of genotypes and alleles at the loci of beta-casein, kappa-casein and beta-lactoglobulin genes. Amplified fragment β-*CN* with the length of 121 bp was digested with *DdeI* restriction enzyme. A feature of the allelic spectrum of the beta-casein gene (β-*CN*) in the studied population was absence of A1 allele. All animals carried the β-CN^{A2A2} genotype of beta-casein gene, respectively, β-CN^{A2} allele frequency was 1.0. For the *CSN3* gene, an amplified fragment in 273 bp was digested with *HinfI* restriction enzyme. A 100% predominance of animals with the most preferred homozygous $CSN3^{BB}$ genotype was revealed. During the β-*LG* gene study process, an amplified fragment with a size of 247 bp was digested with *HaeIII*. It has been determined that the most frequent was allele βLGA and genotype βLGAA of beta-lactoglobulin gene (0.96 and 0.92, respectively). Heterozygous β-LG^{AB} genotype is present in 8 % of buffaloes. The research results are of interest in the field of molecular genetic analysis of the buffalo genome, which are the source of specific properties. The data obtained can be useful for preserving and increasing the genetic diversity of the "Ukrainian" population of water buffaloes, as well as for obtaining valuable products from buffaloes. **Acknowledgments.** This research was performed with supported of the program 31.01.00.03. F "Genetic assessment of animal reference populations for SNP-polymorphism of different DNA loci", SR No. 0121U109254.

Keywords: cattle, water buffalo, A2-milk, genetic markers of productivity, gene, genotype, beta-casein, kappa-casein, beta-lactoglobulin, DNA polymorphism, PCR-RFLP, genotyping, allele, dairy productivity

For citation: Mokhnachova N.B. Genotyping of "Ukrainian" water buffaloes according β-CN (A2-milk), CSN3 and βLG genes. *Vestsi Natsyyanal'nay akademii navuk Belarusi. Seryya agrarnykh navuk = Proceedings of the National Academy of Sciences of Belarus. Agrarian series,* 2021, vol. 59, no 3, pp. 361–365 (in Russian). https://doi.org/10.29235/1817-7204-2021-59-3-361-365

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ГЕНОТИПИРОВАНИЕ «УКРАИНСКИХ» ВОДНЫХ БУЙВОЛОВ ПО ГЕНАМ β-*CN* (А2 – МОЛОКО), *CSN3* И β*LG*

Аннотация: В Украине разведение буйволов является древней традиционной отраслью животноводства крымских татар и русинов Закарпатья. В основном «украинские» буйволы относятся к речному буйволу (*Bubalus bubalis*) и разводятся для молочного и мясного способа производства. Полиморфизм генов, ассоциированных с молочной продуктивностью, позволит вести селекцию буйволов с учетом «желанных» генотипов в отношении хозяйственно полезных признаков. В статье исследованы аллельные полиморфизмы генов бета-казеина (β-*CN*), каппа-казеина (*CSN3*) и бета-лактоглобулина (β-*LG*) в популяции водных буйволов, которые разводятся в Украине, с помощью ПЦР с последующим рестрикционным гидролизом образующихся фрагментов (ПЦР-ПДРФ). Обсуждены результаты исследования «украинской» популяции водных буйволов, а именно: частота генотипов и аллелей в локусах генов бета-казеина, каппа-казеина и бета-лактоглобулина. Амплифицированный фрагмент β-*CN* длиной 121 п.н. разрезали с помощью фермента рестрикции *DdeI*. Характерная особенность аллельного спектра гена бета-казеина (β-*CN*) в изученной популяции выразилась отсутствием алеля А1. Все животные несли генотип β-CN^{A2A2} гена бета-казеина, соответственно аллель β-CN^{A2} встречался с частотой 1,0. Для гена *CSN3* амплифицированный фрагмент размером 273 п.н. обрабатывали рестриктазой *HinfI*. Выявлено 100%-ное преобладание животных с наиболее предпочтительным гомозиготным генотипом $CSN3^{BB}$. В процессе исследования гена β-*LG* амплифицированный фрагмент размером 247 п.н. расщеплялся *HaeIII*. Установлено, что наиболее часто встречались аллель β-LG^{A} и генотип β-LG^{AA} гена бета-лактоглобулина (0,96 и 0,92 соответственно). Гетерозигоный β-LG^{AB}-генотип присутствует у 8 % буйволов. Результаты исследований представляют интерес в области молекулярно-генетического анализа генома буйволов, которые являются источником специфических свойств. Полученные данные могут быть полезными для сохранения и увеличения генетического разнообразия «украинской» популяции водных буйволов, а также с целью получения от буйволов ценной продукции. **Благодарности.** Работа выполнена в рамках программы «Генетика, сохранения и воспроизводства биоресурсов в животноводстве» на 2021–2025 годы, подпрограмма «Генетическая оценка животных референтных популяций за SNP-полиморфизмом разных локусов ДНК», ГР №0121U109254.

Ключевые слова: крупный рогатый скот, водяной буйвол, А2-молоко, генетические маркеры продуктивности, ген, генотип, бета-казеин, каппа-казеин, бета-лактоглобулин, ДНК-полиморфизм, ПЦР-ПДРФ, генотипирование, аллель, молочная продуктивность

Для цитирования: Мохначева Н. Б. Генотипирование «украинских» водных буйволов по генам β-*CN* (А2 – молоко), *CSN3* и βLG / Н. Б. Мохначева // Вес. Нац. акад. навук Беларусі. Сер. аграр. навук. – 2021. – Т. 59, № 3. – С. 361–365. https://doi.org/10.29235/1817-7204-2021-59-3-361-365

Introduction. Buffaloes are a group of closely related genera of ruminant mammals of the Bovids (*Bovidae*) family of the artiodactyls (*Artiodactyla*) type, sometimes combined into one genera Bubalus. Nevertheless, now it is more often common to refer it only to one species - the Asian, or Indian, buffalo (*B. bubalus*), and distinguishe the others in genera Anoa and Syncerus. Domesticated forms of buffaloes belong to the Asian. Recently, more attention has been paid to these animals abroad. This is especially noticeable in countries such as India, Egypt and Italy [1]. In Ukraine, there is an environmental initiative "Preservation of agro-biodiversity of the Carpathian Mountains", aimed at restoring the population of Carpathian buffalo.

In Ukraine, buffalo breeding is an ancient traditional livestock industry of the Crimean Tatars and Ruthenians of Transcarpathia. Basically, "Ukrainian" buffaloes belong to the river buffalo and are bred for dairy and meat production.

Dairy productivity of buffaloes, in comparison with cows, is small - up to 2500 liters. However, it should be noted that the benefits of buffalo milk determined by its composition, which is almost identical to female. Buffalo milk has a more viscous consistency than cow's milk and can be beige or white. This drink has no smell and also has a pleasant taste. The fat content of buffalo milk is 7.6 %, protein 4.3-5.1 %, and carbohydrates about 5 %. This product is very popular in Egypt, Georgia, the Caucasus and Armenia [1, 2].

The β-casein composition of milk and dairy products is an important economic feature of dairy animals. The loci of milk protein genes have a direct impact on the quality of milk and the level of milk productivity. β-casein is the second most common cow's milk protein. The cattle β-casein (β-*CN*) gene is mapped on chromosome 6 and has 12 different genetic variants. Genetic variants A1, A2, A3 and B were found in populations of *Bos taurus* and *B. Indicus*. The best known are A1 and A2 - genetic variants. Histidine which is contained in A1-milk has a weak bond, which is easily broken down with the release of the bioactive peptide β-casomorphin 7. Whereas the proline contained in milk A2 has a strong bond and is not destroyed during digestion. According to the literature, A1-milk is associated with type 1 diabetes, coronary heart disease, atherosclerosis and sudden infant death syndrome [3-9].

Kappa-casein (*CSN3*) is a polymorphic gene of cattle that is found in water buffaloes on chromosome 7 and exists in several allelic variants. The most common are alleles A and B, which differ in the replacement of two amino acids at positions 136 (*Thr* → *Ile*) threonine is replaced by isoleucine, and 148 (*Asp* → *Ala*) aspartic acid by alanine. Polymorphism of the kappa-casein gene is associated with the protein content in milk, its coagulation, the amount and yield of protein milk products, in particular the suitability [10-13].

Also the main whey protein - beta-lactoglobulin (β*LG*) is important for the desired composition of milk, its biological value and physicochemical properties. The β*LG* gene is located on chromosome 12 of buffalo and has two of the most common allelic variants, A and B. Allele B of this gene is associated with a high content of protein fraction of casein and fat in milk, and allele A is associated with a high content of whey proteins [14,15].

The value of milk proteins for the food industry has necessitated their comprehensive study. Thus, the aim of this study was to identify polymorphisms of beta-casein, kappa-casein and beta-casein genes in the "Ukrainian" population of water buffalo.

Materials and methods of research. The study was performed on venous blood samples from buffaloes in a total of 66 from LLC "TASBIO" Kozelets district of Chernihiv region in 2020. The company specializes in breeding buffaloes and making food from buffalo milk.

Isolation of genomic DNA was performed from whole blood using a commercial set of "DNA-sorb B" produced by "AmpliSense" (Russia). Gene polymorphism was detected using PCR-RFLP analysis method. Nucleotide sequences of primers for amplification: β-CN (*5*`- CCT TCT TTC CAG GAT GAA CTC CAG – 3` ; 5` - GAG TAC GAG GAG GGA TGT TTT GTG GGA GGC TCT – 3`), *CSN3* (*5*` - GAA ATC CCT ACC ATC AAT ACC – 3`; 5` - CCA TCT ACC TAG TTT AGA TG – 3`), βLG (*5*` - TGT GCT GGA CAC CGA CTA CAA AAA G – 3`; 5` - GCT CCC GGT ATA TGA CCA CCC TCT – 3`). The names of restriction enzymes for restriction of amplification products are shown in Table 1.

T a b l e 1. **Restriction enzymes and genotypes**

Gene	Amplified fragment, bp	Restriction enzyme	Genotypes and restriction fragments, bp		Reference
β-CN	121	*DdeI*	*A1A2*	*121,86,35*	McLachlan, 2006 [16]
			A1A1	*121*	
			A2A2	*86,35*	
CSN3	273	*HifI*	*AB*	*224, 133, 91, 49*	S. Pinger, 1991 [17]
			AA	*133, 91, 49*	
			BB	*224, 49*	
βLG	247	*Hae III*	*AB*	*148, 99, 74*	J. Medrano, 1990 [18]
			AA	*147, 99*	
			BB	*99, 74*	

The PCR mixture consisted of: 2 µl of DNA polymerase buffer, 1 µl of a mixture of three phosphates ("Amplisens", Russia), 0.8 µl of the appropriate primer, 0.2 µl of DNA polymerase ("Fermentas", Lithuania). Genomic DNA was added in an amount of 2.0 µl, and the rest was ddH_2O. The total volume of the DNA mixture was 10 µl.

Amplification of total DNA with primers was performed on a programmed four-channel thermocycle "Tertsyk" ("DNA technology", Russia) according to the following program: 95 °C, 5 minutes - 30 cycles: 95 °C - 10 seconds, 58 °C - 30 seconds, 72 °C - 30 seconds. The last step is 72 °C - 5 minutes.

PCR products were treated with specific restriction endonucleases (Table 1) according to the scheme: H2O - 3.5 µl, 10x enzyme buffer - 1.0 µl, restriction enzyme - 0.5 µl and 5.0 µl of amplification per 10.0 µl of the working mixture. Visualization of the results was performed using method of electrophoretic distribution of DNA fragments in 3 % agarose gel in 1xTBE buffer, followed by detection using a transilluminator TUV-1 in ultraviolet light 312 nm.

The dimensions obtained in PCR, or as a result of restriction products were detected using molecular weight markers: GeneRuler TM 50 bp DNA Ladder, GeneRuler TM, 100 bp DNA Ladder, "Fermentas". Detection of the results was performed by photographing the gels with a digital camera. Statistical analysis was performed using the software package Statistica 6.0 and Exel (Microsoft Office 2007).

Results of the research. With the help of PCR-PDFR the diagnosis was made and the results of the study of the "Ukrainian" population of water buffaloes were obtained, namely: the frequency of genotypes and alleles at three loci (beta-casein, kappa-casein and beta-lactoglobulin) (Table 2).

Analysis of the results revealed that according to the beta-casein (β-CN) gene, all buffaloes were carriers of the β-CN^{A2A2} genotype, which is considered "desirable" (Fig. 1). It is the local breeds of cattle and

T a b l e 2. **Frequency of alleles and genotypes according to loci of beta-casein, kappa-casein and beta-lactoglobulin genes in water buffaloes**

Gene	Genotype	Frequency of genotypes	Frequency of alleles		H_0	H_e	χ^2	F_{is}
β-*CN*	A1A1	-	A1	-	0	0	-	-
	A1A2	-						
	A2A2	1	A2	1				
CSN3	AA	-	A	-	0	0	-	-
	AB	-	B	1				
	BB	1						
β***LG***	AA	0.92	A	0.96	0.08	0.077	0.04	-0.042
	AB	0.08						
	BB	-	B	0.04				

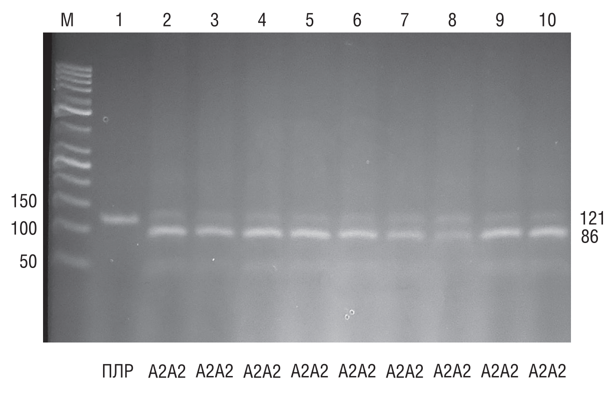

Fig. 1. Electrophoretic analysis of restriction products in the determination of genotypes according to β-CN: M - marker of molecular weights; the genotypes of the samples are shown in the figure

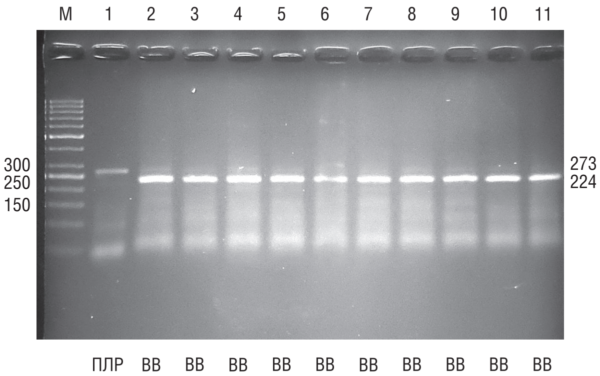

Fig. 2. Electrophoretic analysis of restriction products in the determination of genotypes according to *CSN3*: M - marker of molecular weights; the genotypes of the samples are shown in the figure

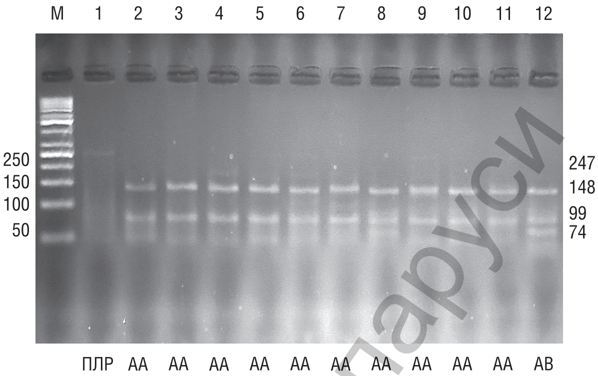

Fig. 3. Electrophoretic analysis of restriction products in the determination of genotypes according to βLG: M - marker of molecular weights; the genotypes of the samples are shown in the figure

representatives of wild species of the genus Real bulls (*Bos*): *Bos taurus L.* (domestic bull), *Bos indicus L.* (zebu), *Bos bonasus L.* (European bison), *Bos bison L*. (American bison), *Bos bubalis L*. (house water buffalo) being the carriers of rarest alleles [19, 20].

The data obtained from the study of the kappa-casein gene (*CSN3)* indicate that all animal studied had the *CSN3*^{BB} genotype (Fig. 2). According to the literature, milk that contains the *B*-allelic variant of kappa-casein has a higher protein content and higher cheese yield, as well as better coagulation properties. Hard cheeses of high quality can be cooked only from milk of cows with the *BB* genotype on kappa-casein. Due to the lack of an alternative allele in one of the herds, the fixation index and the criterion of conformity for the breed were not determined at all.

The results of the analysis of the polymorphism of the beta-lactoglobulin gene of buffalo are shown in table 2. The restriction endonuclease *HaeIII* revealed the presence of two alleles *A* and *B* and two genotypes *AA* and *AB* (Fig. 3). It was found that the vast majority of animals (0.92) are homozygous for allele *A* and only 8 % are carriers of heterozygous genotype *AB*. The βLG^{BB} genotype was not detected in the studied herd. Allele *A* is associated with bigger milk yield and protein yield. This allows to conclude that in herd occurs intensive selection on increasing milk productivity occurs in herd. Actual (H_0 = 0.08) and expected (H_E = 0.077) heterozygosity did not differ. The predominance of homozygous genotypes (AA = 0.92) over heterozygous ones impacted the deviation of genetic equilibrium (F_{IS} = 0.042).

Conclusions

1. Analysis of the results of study of the “Ukrainian” population of water buffaloes according to the beta-casein gene (β-*CN*) showed that 100 % of the animals were carriers of the β-*CN*$^{A2/A2}$ genotype. This makes it possible to recommend buffaloes for creation of herds producing A2 milk.

2. In the studied sample of buffaloes, a 100 % predominance of animals with the *CSN3*^{BB} genotype was revealed, which is associated with a higher protein content in milk and a higher yield of cottage cheese and cheese.

3. For the beta-lactoglobulin (β-*LG*) gene - 92% of the buffaloes studied had the β-*LG*^{AA} genotype, the rest of the animals (8%) are carriers of the β-*LG*^{AB} heterozygous genotype. The frequency of occurrence of β-*LG*^{A} and β-*LG*^{B} alleles was 0.96 and 0.04, respectively.

The research results are of interest in the field of molecular genetic analysis of the buffalo genome and study of biodiversity of cattle. The data obtained can be used as an additional criterion in breeding programs in order to preserve and increase the genetic diversity of the “Ukrainian” population of water buffaloes, as well as to obtain valuable products from buffaloes.

Acknowledgments. This research was performed with supported of the program 31.01.00.03. F "Genetic assessment of animal reference populations for SNP-polymorphism of different DNA loci", SR No. 0121U109 254.

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