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GENETIC ANALYSIS OF SIRES OF LEBEDYN CATTLE AND RELATED POPULATIONS

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Abstract

The population-genetic analysis wascarried outby ISSR-PCR markers of animals of the local Lebedyn breed and breeds that participated in its formation and improvement: Swiss, Ukrainian Grey and original Brown Swiss. ISSR-PCR technology showed a low level of genetic heterogeneity in all animals of investigated breeds having unique species-specific DNA fragments. The low levels of genetic polymorphy and heterozygosity, particularly in the subpopulation of Lebedyn breed animals, give evidence to the presence of genetic erosion and the threat of inbred depression. The results of evaluation of genetic differentiation between breed founders and based on them Lebedyn breed were proved to be used in the breeding programs for its gene pool restoration. Taking into account the results of population-genetic analysis, a fundamentally new scheme of reproduction of the genealogical structure of aboriginal breeds by the reciprocal population reproduction method was developed.

Key words**:** *Lebedyn breed, aboriginal cattle, ISSR-PCR, genetic marker, population-genetic analysis, interbreed differentiation, gene pool conservation*

INTRODUCTION

A great deal of attention worldwide is paid to the biodiversity conservation. The fundamental stage in the preservation of genetic resources involves their phenological and genetic description and its further thorough systematization. The vast majority of cattle breeds is represented by local populations, which differ significantly not only in morphology but also in the corresponding co-adapted genetic complexes that were formed under the influence of natural and artificial selection in the specific breeding conditions. Genesis, further breed improvement and selection of pedigree valuable animals require information concerning their productivity potential, genetic polymorphism level, genotype by the genes of quantitative traits, presence of unique alleles that are typical for this very breed and have adaptive value, and therefore, pay the way for its gene pool conservation [8].

Most scientists believe that genetic diversity conservation requires genetic monitoring in order to study the structure of gene pool objects that need to be preserved and protected by means of modern genetics and biotechnology methods [9].

To confirm this idea [25] states that the main tasks for sustainable conservation of national genetic resources are genetic monitoring, cataloging and certification, creation of gene pool and computer databases of collection farms, genetic banks, genetic selection plans for the conservation and management of natural resources. These studies represent the most complete data on the genetic diversity and the character of differentiation of particular populations, and should form the basis for the programs of scientifically grounded conservation of genetic resources of few in numbers and vanishing farm animals breeds [23, 29].

The problem of genetic resources conservation and breeding acceleration require a special approach to the control of their gene pools. For this purpose, various classes of molecular genetic markers are used [23]. They were created and gradually

changed with the development of scientific methods concerning the determination of polymorphism of certain DNA regions, initially at the level of proteins - their products, and then based on the identification of nucleic acids (DNA and RNA) structural rearrangements. They helped to solve the following important scientific problems: reconstruction of breeds history and genealogy, their distribution, clarification of their gene pool specifics, development of genetically grounded programs for the sustainable use of local breeds and their conservation, mapping of the main genes of quantitative traits in order to use this information in the genomic selection of farm animals [6, 21].

Researchers note that genesis of particular cattle populations and breeds can be traced and even adapted in the process of breed formation by constructing dendrograms of their phylogenetic interactions based on the productivity data as well as the molecular-genetic markers of different classes [8, 9].

Based on the analysis of the polymorphism of the microsatellite sequences for different cattle breeds, it was established that the dendrograms of genetic relatedness created on the basis of the adequately performed cluster analysis reflect the history of breed formation and their identified genealogical relationships. The first cluster includes animals of the old type Ukrainian Simmental and Ukrainian Grey cattle breeds. The author explains it by the fact that blood of Ukrainian Grey breed was added to the Simmental breed in Ukraine. The second cluster combines animals of domestic breeds: Lebedyn, Carpathian Brown and modern type of Ukrainian Simmental. The genetic similarity of these breeds, calculated on the basis of the allelic distribution of microsatellite markers, is explained by the analogy of the methods used for their formation. According to the author, Lebedyn breed was formed on the basis of Ukrainian Grey cattle breed, with Swiss sires used as an upgrading breed. This history of Lebedyn breed origin is proved by [27, 31].

The next cluster is represented by two breeds – European Brown Swiss and Austrian Simmental. The combination of these breeds can be explained by their common geographical origin (Switzerland is the native land for both Swiss and Simmental cattle [8].The similar result is given by [2]. According to his research the animals of Simmental and Alatau cattle breeds are included into the separate cluster in the evolutionary tree of Aulie-Ata cattle breed. Alatau cattle breed was formed by crossbreeding of local cattle with Swiss breed sires [30].

Further research showed [9], that Swiss cattle of European origin and American origin are characterized by certain specificity of the genetic structure. The animals of European and American origin are found in different subclusters, while the animals of Swiss and Simmental breeds of European origin come from one subcluster. By studying the similarity of the breeds of other origin roots, the author established that the animals of Swiss breed and Ukrainian Grey breed are united into one subcluster, in comparison with the cluster of Black-and-White cattle origin.

Other researchers provide the data showing that dairy breeds formed with the participation of Swiss breed (Kostroma) are in the separate cluster from such breeds as Holstein, Kholmogory, Yaroslavl and Red Gorbachovsky [32].

Thus, numerous studies of cattle according to different classes of molecular genetic markers prove the fact that population monitoring with the subsequent cluster analysis is a reliable tool for determining historical and genealogical relationships between breeds. It can be applied as a method for predicting proper combining ability of animals of different populations for the breeding purpose, obtaining the heterosis effect in offspring from these combinations with the desirable productivity traits. Besides, under the conditions of successful selection of informative and highly polymorphic marker systems, this methodological approach can be used as the basis of breeding programs for the restoration of few in numbers and vanishing animal breeds, including Lebedyn cattle breed.

The goal of the research is to conduct the population-genetic analysis of the animals of

the local Lebedyn breed and breeds used for its formation and improvement in order to study their differentiation and genetic relatedness by multilocus ISSR-PCR markers.

MATERIALS AND METHODS

For molecular genetic studies, we used one sperm dosage from five bulls of each of the breeds: Brown Swiss and Lebedyn cattle and one sperm dosage from three bulls of Ukrainian Grey and the original Brown Swiss. To extract the genomic DNA, 50 μl samples from the sperm dosage of each animal of the named above breeds were taken.

The extraction of genomic DNA from sperm was carried out using the commercial standard kit Sorb-B (AmpliSens, RF) with its own modifications. The solution of mucolysin in the quantity of 120 μl per 50 μl of sperm sample was added to the selected sperm sample, the mixture was thoroughly vortexed and incubated in the solid state thermostat at +65°C for 5 min. Further procedure of the extraction of genomic DNA from animal sperm was carried out in accordance with the recommendations of the reagent kits manufacturer. The elution of the extracted DNA was carried out by adding 120 μl of TE buffer to the precipitate with the solution.

DNA amplification with primer ISSR-S1 was performed using the commercial kit Thermo Fisher Scientific (USA). The reaction mixture composition was the following: reaction buffer – 2.5 μl; 100 pM primer - (0,5-1,0) μl; from 2 to 4 activity units of Taq-polymerase – (0.1-0.2) μl; (1-2 ng) DNA sample – (1-3) μl; deionized water (to bring the volume of the mixture to 25μl). Not more than 0.5-1 ng of DNA sample (stock solution of DNA in the ratio of 1:10) was used. The primer synthesis used in the inter microsatellite analysis technique (ISSR) was performed by Fermentas (Vilnius, Lithuania) under order.

The nucleotide structure and temperature condition of the primer was: S1 3'-AGCAGCAGCAGCAGCAGCC-5 ', acting as a forward and reverse initiator of the amplification in PCR.

The amplification was carried out in the thermocycler "Biotherm" (Germany). The amplification program with the primer S1 was: 1 cycle: 94°C - 4 minutes; 2 - 31 cycles: 57°C - 2 minutes; 72°C - 4 min; 94°C - 1 minute; 32 cycles: 57°C - 3 min; 72°C - 7 minutes [23].

Electrophoretic separation of amplified DNA regions in ISSR technique was performed in 2% agarose gel in a single Tris Borate Electrophoresis buffer, according to the methodological recommendations [14]. Gels were coloured with 0.5% solution of bromide ethidium for 10 minutes. The visualization of the electrophoregram was performed on the transilluminator in the ultraviolet spectrum at the wavelength of 340 nm. The gel was photographed with Canon camera using the orange filter. The control of the size of amplification products on the gel electrophoregrams was performed with the use of the molecular weight marker 1 kb - Ledderplus ("Fermentas", Vilnius, Lithuania) and O'Range Rulertm 200 bp DNA Ladder. («Thermo Fisher Scientific", USA). Only those PCR products were analyzed, which were clearly reproduced on the gels in the range of molecular weights relative to the marker: from 200 bp up to 3,000 bp in the course of 3 repetitive amplification reactions. The received profiles were processed in the standard computer program GenAlex6 [20]. The construction of the cladograms was carried out according to the values of genetic distances in the MEGA 4 program [18, 23, 24, 26].

RESULTS AND DISCUSSIONS

Selective breeding of certain breeds with optimal use of their genetic resource requires synthesis of information from a number of sources, including analysis of the results of molecular genetic studies, that provide objective criteria for assessing the diversity of individuals in the breed and between them. The variety of existing marker systems that are suitable for assessing the genetic variability of biological objects, sets the task for the researchers to choose an optimal type of markers or their combinations to objectively assess the state of the gene pool of breeds. The general concept of applying

marker breeding elements in livestock farming must have a methodological basis - a universal approach to identifying the features of gene pools of any farm animals, high polymorphicity and informative value of the selected genetic markers, their stable reproducibility and low testing cost [13, 15].

This task is currently important concerning the genetic criteria for the estimation of small gene pool populations of cattle and the characteristics of the formed breeds with the aim of their further consolidation, increasing pedigree value and productivity.

The population-genetic analysis of the animals of four breeds (American Swiss, Lebedyn, Ukrainian Grey and original German Brown Swiss) in the technique of inter-microsatellite analysis with the use of the fragment of the anchored microsatellite with a trinucleotide AGC motif gave total 23 amplification products in the size from 250 to 2,000 bp (Table 1).

Table 1. Comparison of frequency and size of DNA fragments in ISSR technology with primer S1 in bulls of four populations*

№ DNA fragment	Size of DNA fragment	Populations of cattle			
		BS	OBV	L	UG
1	2,000	0.000	0.667	0.000	1.000
2	1,500	0.800	0.333	1.000	0.667
3	1,350	0.400	1.000	0.400	1.000
4	1,230	0.600	0.333	0.600	1.000
5	**1,100**	**1.000**	**1.000**	**1.000**	**1.000**
6	1,050	**0.200**	0.000	0.000	0.000
7	1,000	0.400	1.000	0.400	1.000
8	960	0.600	0.000[a]	0.600	1.000*
9	**900**	**1.000**	**1.000**	**1.000**	**1.000**
10	830	0.200	1.000	0.400	1.000
11	800	0.800	1.000	1.000	1.000
12	770	0.600	1.000	0.200	1.000
13	700	1.000	1.000	0.200*	1.000
14	670	1.000	1.000	1.000	**0.000***
15	650	1.000[a]	0.000*	1.000[a]	0.333
16	620	0.400	0.000[a]	1.000*	0.000[a]
17	560	0.000	1.000	0.000*	1.000
18	**530**	**1.000**	**1.000**	**1.000**	**1.000**
19	475	0.000	0.667	0.000	0.667
20	450	0.800	0.667	0.600	0.000
21	350	0.000	**1.000***	0.000	0.000
22	325	0.400	0.667	0.600	1.000
23	250	0.400	1.000	0.600	1.000

Note: the difference is statistically significant upon Fisher's criterion: * - p<0,5; BS – Brown Swiss; OBV – original Brown Swiss; L – Lebedyn breed, UG – Ukrainian Grey breed.
Source: Own results.

The analysis of certain DNA fragments distribution enabled to determine the unique genome features of the representatives of particular breeds.

For example, we found a product of amplification in the polymerase chain reaction (amplicon) in the size of 770 bp. (Fig. 1) with a frequency of 0.60 in animals of Swiss breed [23], 100% of the examined bulls of original Brown Swiss and Ukrainian Grey, and only 0.20 in the bulls of Lebedyn breed.

It is interesting to note that in Lebedyn breed there occurred a selection of animals that were carriers of an alternative allele and its frequency significantly decreased compared with the population of Brown Swiss breed, whose sires participated in the scheme of Lebedynsky breed improvement. Among the representatives of Brown Swiss breed, we found the animals with a rare allele sized 1050 bp with the frequency of 0.2, while the representatives of other subpopulations didn't have it.

DNA fragment sized 960 bp can be considered the marker for the animals of Ukrainian Grey breed, since its frequency was 1.0 being absent in individuals of the original Brown Swiss breed (p <0.5) and having the

frequency of 60% in the selection of animals of Lebedynsky and Swiss breeds.

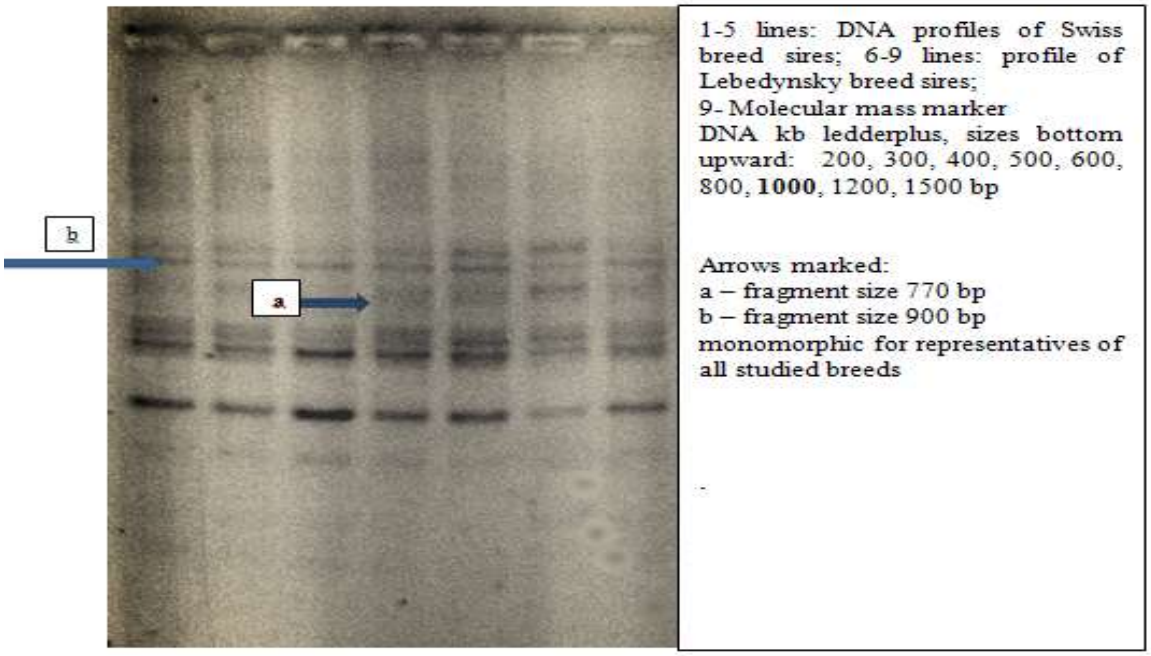

Fig. 1. Electrophoregram of DNA amplification products of animals of Swiss and Ukrainian Grey breeds obtained with ISSR-S1 primer in 2% agarose gel
Source: own results.

DNA fragment sized 960 bp can be considered the marker for the animals of Ukrainian Grey breed, since its frequency was 1.0 being absent in individuals of the original Brown Swiss breed ($p < 0.5$) and having the frequency of 60% in the selection of animals of Lebedynsky and Swiss breeds. The amplicon sized 700 bp occurred in only 20% of Lebedyn breed animals, the frequency of this allele in the individuals of other studied subpopulations was 1.00 ($p < 0.5$). A specific breed feature for the sires of Ukrainian Grey breed was the absence of the DNA fragment with a size of 670 bp, while it was found in the representatives of the other three breeds. In all sires of Lebedyn breed the allele with a size of 620 bp was identified being absent in the individuals of the original Brown Swiss and Ukrainian Grey breeds ($p < 0.5$), while in the subpopulation of Brown Swiss breed this DNA fragment was detected in 40% of individuals. A highly informative genetic marker for animals of the original Brown Swiss breed can be considered a presence of an allele of 350 bp, which occurred in all representatives of the studied selection and was absent in the bulls of other breeds ($p < 0.5$).

By the results of the conducted population-genetic analysis of the animals of different breeds by the arrangement of ISSR-PCR markers, essential statistically significant differences between them on a number of parameters were revealed (Table 2). The maximum number of amplification products obtained was characteristic of bulls of the original Brown Swiss breed – 16.667, while this indicator for animals of Brown Swiss and Lebedyn breeds was the same and significantly lower – 12.600 ($p < 0.01$). The level of calculated intra-group similarity, which in a certain way could serve as a mathematical model for assessing the degree of population's genetic consolidation, was the maximum in the animals of Ukrainian Grey breed (0.959) with the minimum value of this indicator for individuals of Brown Swiss breed (0.730), however this difference was not statistically probable. Noteworthy is the fact that the value of the theoretically calculated heterozygosity level in bulls of Ukrainian Grey breed was only 3.7%, while this

indicator in animals of Brown Swiss breed was 28.3% ($p < 0.01$).

Table 2. Main genetic-population characteristics of the sires of four subpopulations by the results of ISSR-S1 multilocus analysis

Breeds (N)	Genetic-population records				
	Average number of amplicons	Level of intragroup similarity	Heterozygosity	Number of genetic loci	Share of polymorphic loci
BS (5)[1]	12.600±0.400[a]	0.730	0.283[a*]	9.818	0.389
OBV (3)[2]	16.667±0.667[a**]	0.860	0.111[a]	14.998	0.133
L (5)[1]	12.600±0.872[a]	0.780	0.194	10.557	0.242
UG (3)[2]	16.334±0.334[a*]	0.959	0.037[**]	15.749	0.048

Note: the difference is statistically significant upon Fisher's criterion: [*] - $p<0,5$; BS – Brown Swiss; OBV – original Brown Swiss; L – Lebedyn breed, UG – Ukrainian Grey breed
Source: own results based on [23].

By the number of the identified genetic loci with S1 primer in the technique of inter-microsatellite analysis the animals of Ukrainian Grey and Swiss breeds also showed the biggest difference with the researched indices of 15.749 and 9.818, respectively. The genetic parameter "Share of polymorphic loci" confirmed significant differences between subpopulations of animals of Brown Swiss and Ukrainian Grey breeds with data values of 0.389 and 0.048, respectively. Thus, according to the results of the analysis, the genetic uniqueness of an aboriginal ancient Ukrainian Grey breed and a catastrophic bottleneck of genetic variability, associated with the use of a limited number of sires and a critically small effective population size as a whole, were shown.

The informative molecular markers and developed mathematical models for assessing the population situation, as shown by a number of scientists on different types of farm animals, can

evaluate not only the actual contribution of genetic information, inherited by the animals of formed breed from the representatives of upgrading breeds, but also the degree of their divergence in further selection and natural adaptation [10]. One of the key research tasks was to evaluate the genetic differentiation between the breed founders and based on them Lebedyn cattle breed. The results of such an assessment should become a scientific basis for the breeding programs of rational use and restoration of the gene pool of the disappearing Lebedyn cattle breed.

The cladograms were created based on the genetic similarity indices and calculated on their basis genetic distances after the conducted cluster analysis. The evaluation findings of genetic relationships of the micro-populations of different cattle breeds are given in Table 3.

Table 3. Similarity levels and genetic distances between the representatives of different cattle populations according to the results of inter-microsatellite analysis with ISSR-S1 primer*

I / DN	BS	OBV	L	UG
BS	1.000 / 0.000	0.641	0.743	0.617
OBV	0.445	1.000 / 0.000	0.601	**0.809**
L	0.297	0.509	1.000 / 0.000	**0.595**
UG	0.482	**0.212**	**0.520**	1.000 / 0.000

Note: I – genetic similarity index, upper diagonal; DN – genetic distance, lower diagonal.
BS – Brown Swiss; OBV – original Brown Swiss; L – Lebedyn breed, UG – Ukrainian Grey breed
Source: own results.

The highest genetic similarity index according to the results of DNA typing of animals in the ISSR-PCR technique with trinucleotide anchored primer $(AGC)_6C$ was fixed between the subpopulations of animals of original Brown Swiss and Ukrainian Grey breeds (0.809), the calculated genetic distance being 0.212. The lowest level of genetic similarity by the chosen genetic marker was fixed between animal samples of Lebedyn and Ukrainian Grey breeds with the value of 0.595, the corresponding genetic distance being 0.520.

As the rate of evolution events in the populations of farm animal breeds is uneven and depends on the degree of both artificial and natural selection pressure, we chose Neighbor-joining (NJ) method for conducting cluster analysis and minimization of mistakes in the topology of constructed dendrograms [22].

The structure of the constructed cluster (Figure 2) consists of two subclusters. The structure of the first subcluster includes animals of Lebedyn and Brown Swiss breeds, the configuration of another subcluster is formed by Ukrainian Grey and original Brown Swiss breeds.

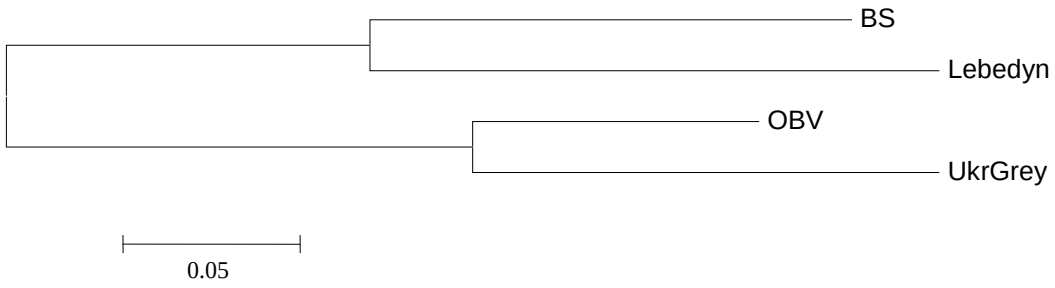

Fig.2. Dendrogram made on the basis of data from the ISSR-typing of four breeds by Neighbor-joining method in MEGA 4 program
Source: own result.

Whereas presence of animals of Lebedyn breed and Brown Swiss breed in the same subcluster can be explained by the fact that starting from 1970s the sires of the last one were used to improve Lebedyn breed, presence of animals of Ukrainian Grey and original Brown Swiss breeds in this cluster requires further research. Genetic similarity of animals without genealogical relationships can only be explained by marking of certain gene fragments that are associated with adaptation to the local housing conditions, since it is known that DNA fragments that are synthesized in ISSR technology may contain sites of structural parts of genes, located between microsatellite sequences [7]. It is possible that the detected low levels of genetic variability typical for aboriginal limited in size breeds had a significant influence on the value of the genetic distance between the unrelated breeds of Ukrainian Grey and original Brown Swiss. Besides, the results of our population-genetic analysis confirm the fact that the animals of Swiss breed of European and American selection differ significantly.

In the pedigree of some sires of Lebedyn breed (Rogiz 5002 and Final 1008) there are sires of both American and Austrian selections. In the pedigrees of sires of modern Swiss breed there are bulls of Laddi, Suprima, Vigata, Hilla, Kontsentranta, Distinkshna, Lailasana, Eleganta lines.

It was no coincidence that the original Brown Swiss breed was included in the list of studied breeds, as the sires of Swiss breed from Germany and Switzerland were brought to Ukraine in the early twentieth century for the improvement of local cattle, and such low influence of these animals on the genetic structure of Lebedyn breed was somewhat unexpected. To answer these questions and to make firm conclusions, it is necessary not only to expand the sample of animals of the above-mentioned breeds, but also to use more primers for ISSR technology and marker

locus-specific systems of other classes for further research.

Thus, on the whole the four studied subpopulations of four cattle breeds showed a low level of genetic diversity having certain differences between them. Reduced levels of genetic polymorphism and heterozygosity, especially in the selection of Lebedyn breed animals, potentially pose the risk of genetic diversity decrease, loss of unique alleles and increase of inbreeding in the next generations. Consequently, our studies emphasized the importance of developing special breeding programs for the breeds with a small number of animals by means of genetic information needed to plan mating and therefore to prevent the loss of a unique genetic diversity of local animal breeds.

Numerous studies have shown that ISSR markers serve as a reliable methodological approach to the evaluation of phylogenetic relationships between different types of farm animals and adequately reflect the difference between them, both in terms of genealogy and productivity characteristics.

Despite the fact that the ISSR technology of fingerprinting is considered to be less suitable for conducting population studies, compared to SSR (STR), due to the dominant type of alleles and because it creates some difficulties in determining the size of amplified fragments and their separation in agarose gel, making it impossible to accurately estimate the heterozygous state of the identified loci, it at the same time permits to search for new genes of quantitative traits and create more accurate SNP markers on the basis of detected amplicons, carries a huge informative load when conducting population studies of farm animals at minimum costs and is irreplaceable especially for poorly-studied biological objects [3, 5, 16, 17, 19].

Equally important task for further research must be the choice of highly informative markers for assessing the genetic specificity of breeds using standard microsatellite panels approved by the international organizations ISAG and ICAR to conduct monitoring studies of all existing populations of each cattle breed [1, 4, 23, 28].

DNA-typing of aboriginal animal breeds to identify unique genetic adaptation complexes and alleles associated with the economically important characteristics is particularly relevant [11, 12], and in general will be defined as the main task of further research and development of effective strategies for the conservation of the gene pool of Lebedyn cattle breed.

A small number of Lebedyn breed sires, whose sperm is stored at the breeding centres, makes it impossible to avoid inbreeding, which can lead to unwanted breeding consequences. Based on the results of genetic studies, we proposed a fundamentally new scheme for the reproduction of the genealogical structure of the herds of local (aboriginal) breeds, namely, by the population reciprocal reproduction method (Fig. 3).

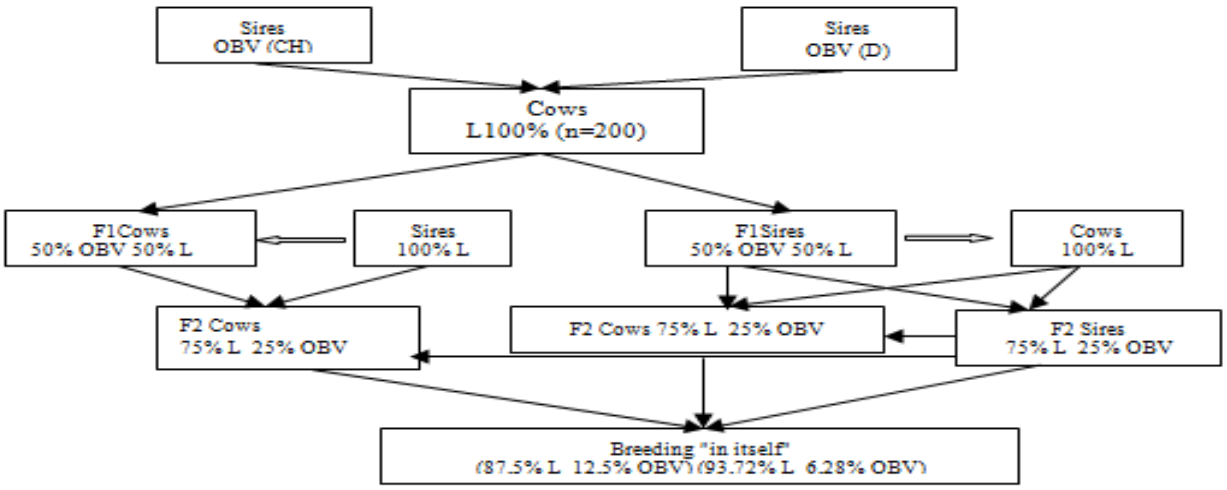

Fig.3. The flowchart of the population reciprocal reproduction method
Source: own results.

As it was mentioned, Ukrainian Grey breed and various Swiss breeds were the parental breeds in forming Lebedyn breed. Based on the results of the conducted cluster analysis with the genetic distances calculation, the use of the sires of original Brown Swiss breed will not only expand the genealogical structure of the small aboriginal breed without significantly affecting its genetic specificity and adaptive properties, but will also help to avoid inbreeding depression when using the sperm of purebred sires from the National Bank of Genetic Resources.

CONCLUSIONS

The conducted population-genetic analysis of the four subpopulations of four cattle breeds (Brown Swiss, the original Brown Swiss, Lebedyn and Ukrainian Grey) has overall demonstrated a low level of genetic heterogeneity. We note the presence of specific DNA fragments, which are detected in the technology of inter-microsatellite analysis, which completely coincides with previously obtained results [23]. We note, that reduced levels of genetic polymorphism and heterozygosity potentially create the threat of genetic diversity decrease, loss of unique alleles and inbreeding depression in the next generations of Lebedyn breed population. The importance of using the information obtained in the development of regional programs for the restoration of the gene pool of the endangered Lebedyn breed with the involvement of animals of improving breeds of foreign breeding has been proved.

The conducted genetic studies showed that Lebedyn breed and original Brown Swiss breed are combined in the same cluster, but in different subclusters, which can be a result of a single-vector selection of these breeds aimed at increasing adaptability to local breeding conditions. This fact allows the use of sperm of original Brown Swiss breed sires on the breeding stock of Lebedyn breed, making it possible to expand its genealogical structure and prevent unwanted inbreeding with the further use of the generative materials of Lebedyn breed sires stored in the National Bank of Genetic Resources.

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