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Original Research

Cytogenetic and *ISSR*-Markers Polymorphism in the Population of Local Ukrainian Lebedyn Cows

Lyubov Starodub [*], Nataliia Mokhnachova [*], Ostap Zhukorskyi

Institute of Animal Breeding and Genetics nd. a. M.V. Zubtsya National Academy of Agrarian Sciences, Ukraine; E-Mails: Starodublf@gmail.com; nataliia.mokhnachova82@gmail.com; o_zhukorskiy@ukr.net

* **Correspondences:** Lyubov Starodub and Nataliia Mokhnachova; E-Mails: Starodublf@gmail.com; nataliia.mokhnachova82@gmail.com

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Abstract

The preservation of the fund of local breeds of agricultural animals, which are breeding materials for the creation of new ones and the improvement of existing ones, meets the FAO requirements, which are the protection of biological diversity. The study of the genetic structure of cows of the local Lebedyn breed, which was bred in Ukraine, using cytogenetic and molecular genetic methods, is aimed at establishing information about the structure of the gene pool of these animals and the uniqueness of their genotype. Cytogenetic monitoring showed that the modal number of chromosomes corresponded to the species norm and was 2n = 60, constitutional abnormalities in the form of Robertsonian translocations were not detected, the average frequency of genomic disorders (aneuploidy) was 10.2 ± 2.10%, polyploidy was not manifested, structural disorders (chromosomal breaks) accounted for 3.1 ± 1.88% and asynchronous separation of the centromeric regions of chromosomes – 0.8 ± 0.10%. The average share of cytogenetic parameters of cells was determined: lymphocytes with micronucleus – 3.6 ± 0.61‰, binucleus lymphocytes – 4.3 ± 0.97‰ and mitotic index – 3.7 ± 1.20‰. The genetic structure of Ukraine's local, small-numbered Lebedyn cattle breed was analyzed using 8 *ISSR* systems. All 8 microsatellite primers showed high efficiency. *ISSR* labeling revealed 88 amplified *DNA* fragments and only 18 of them were polymorphic. The

total number of polymorphic loci was 20.45%. 11 species-specific loci for $(ACC)_6G$, 5 loci for $(CTC)_6C$, 10 for $(GAG)_6C$, 7 for $(GA)_6CC$, 10 for $(AG)_8CG$, 9 for $(AG)_8CA$, 13 for $(GA)_9C$ and 14 for $(AG)_9C$ were found in the cows of Lebedyn cow breed. It was established that the experimental animals were characterized by karyotype stability, reduced sensitivity to mutagenic factors of various natures, high genetic consolidation, and reproductive isolation.

Keywords
Genotype of cows of the Lebedyn breed; cytogenetic analysis; *ISSR*-markers; intra-population diversity

1. Introduction

One of the critical problems facing humanity is poverty and hunger, which the development of agriculture can solve. Therefore, the breeding and maintenance of cattle, in particular, is one of the important economic sectors and requires special attention [1, 2]. The Food and Agriculture Organization (FAO), a leading agency of the United Nations (UN), was established to deal with developing rural regions and agricultural production. The organization's motto is: "helping build a world without hunger" [3].

Therefore, nowadays, it's a burning question: how to preserve the gene pool of local breeds of agricultural animals whose level of productivity is inferior to modern breeds, but in their genotype, there are valuable alleles that modern breeds have almost lost. These animals can become breeding material necessary for creating new breeds and improving existing ones [4]. Unfortunately, the genetic potential of animals of local breeds remains beyond the attention of scientists and breeding practitioners. Species specificity and breed specificity of their variability under the influence of factors of different natures (history of creation and breeding, paratypes factors) have not been researched yet [5].

The local domestic breed in Ukraine is the Lebedyn breed of cattle, bred by the reproductive crossing of cows of local breeds (mainly Gray Ukrainian breed) with Swiss bulls and subsequent breeding of the best hybrids among themselves. Currently, according to calculations of population parameters, trends, and risk statuses, the Lebedyn breed is in a state of risk according to FAO recommendations, and according to the European Federation of Animal Science, it is in a state of potential danger [6].

In Ukraine, there are no integral scientific and systematic approaches to analyzing the influence of various factors on the formation of the genetic structure of local breeds of domestic animals using cytogenetic and molecular genetic methods. To preserve these breeds of farm animals, it is necessary to obtain knowledge about the structure of their gene pool to obtain inexpensive and effective genetic diversity assessment and differentiation methods.

The cytogenetic analysis allows the assessment of the level of chromosomal instability and the presence of probable mutagenic factors to identify carrier animals of constitutive cytogenetic abnormalities, to single out animals with an increased level of chromosomal instability as a risk group associated with a violation of repair systems and possible hidden inflammatory processes and to predict the reproductive qualities of animals, in particular, the ability to fertilize.

We used inter-microsatellite polymorphism analysis - *ISSR*-analysis (*ISSR*-fingerprinting) to assess the interbreed and interbreed genetic diversity of the Lebedyn breed of cattle. This method belongs to molecular multilocus analysis, which allows simultaneous assessment of polymorphism of dozens of loci and is more informative for assessing the level of genetic diversity of gene pools. *ISSR* markers are poorly studied, especially in agricultural animals, particularly cattle.

The Lebedyn cattle breed is an ancient Ukrainian local breed, a potential source of genes and gene complexes necessary to compensate for the decrease in intraspecific diversity. Before the start of our research, there was practically no characterization of the gene pool of this breed by cytogenetic and *ISSR*-markers. Therefore, such complex studies are being held for the first time.

The work aimed to study the genetic structure of Lebedyn cows using molecular genetic and cytogenetic methods to establish information about the heredity of these animals and the uniqueness of their genotype.

2. Materials and Methods

The material for the assessment of intraspecific genetic variability and stability of the karyotype was blood samples taken from cows of the local Lebedyn breed (n = 33 cows), which are kept in the Boryspil district of the Kyiv region at the "Holosiiyivske" farm, Ukraine.

2.1 Cytogenetic Analysis

Cytogenetic drugs were obtained from lymphocytes of peripheral blood using a standard technique. 10 mL blood was collected aseptically by jugular vein puncture in a sterile vacutainer (Greiner bio-one vacuette containing 1% lithium heparin solution 6 mL of blood).

RPMI-1640 medium, bovine blood serum (preferably embryonic), antibiotic gentamicin, and mitogen - a substance that stimulates the mitotic division of lymphocytes in culture (phytohemagglutinin P) were used for the cultivation of blood cells. The mixture was cultivated in a thermostat at a temperature of +37°C for 48 hours. Two hours before fixation, a colchicine solution heated to 37°C was added to the culture in a final concentration of 0.3-0.5 μg/mL of nutrient medium. For hypotonization, a freshly prepared 0.55% potassium chloride solution was used. After the end of hypotonization, the culture was centrifuged, the supernatant liquid was poured off, and a fixing liquid-cooled to +4°C was added to the sediment, mixing one part of glacial acetic acid with three parts of methyl (or ethyl) alcohol solution. After that, the sediment was resuspended and centrifuged, repeating this operation 2-3 times. Using an automatic dispenser, the cell suspension was applied to clean, cooled glass slides. The glass was dried in the air. After being stained with a ready-made Giemsa dye, the obtained preparations were analyzed for chromosomal variability under the immersion magnification of an Axiostar plus microscope (Carl Zeiss, Germany) 1000 times and photographed [7].

The number of binuclear lymphocytes, mononuclear lymphocytes with micronuclei, and the mitotic index were counted on the same preparations. The frequency of binucleated lymphocytes, lymphocytes with a micronucleus, and the mitotic index were calculated in parts per million (‰), the number per 1000 cells.

2.2 Molecular Genetic Studies

In order to confirm the uniqueness and consolidation of the population of cows of the Lebedyn breed, the genetic polymorphism of the studied animals was evaluated using molecular diagnostics (*ISSR*-fingerprinting). *ISSR-PCR* tagging has been used effectively for interspecific genetic variability, taxonomic and phylogenetic comparisons, and mapping a wide range of organisms. In order to confirm the uniqueness and consolidation of the population of cows of the Lebedyn breed, the genetic polymorphism of the studied animals was evaluated using molecular diagnostics (*ISSR*-fingerprinting). *ISSR-PCR* tagging has been used effectively for interspecific genetic variability, taxonomic and phylogenetic comparisons, and mapping a wide range of organisms. To confirm the uniqueness and consolidation of the population of cows of the Lebedyn breed, the genetic polymorphism of the studied cattle was evaluated using molecular diagnostics (*ISSR*-fingerprinting). *ISSR-PCR* labeling is effectively used for interspecies genetic variability, taxonomic and phylogenetic comparisons, and mapping a wide range of organisms.

To study *DNA* polymorphism of cattle using *ISSR*-markers, optimization of the temperature regimes for the polymerase chain reaction was carried out (Table 1).

Table 1 Primer sequences and annealing temperature regimes used in the study.

№	Primer sequence	Motifs	Annealing Temperature
1	5`-ACCACCACCACCACCACCG-3`	$(ACC)_6G$	64°C
2	5`-CTCCTCCTCCTCCTCCTCC-3`	$(CTC)_6C$	64°C
3	5`-GAGGAGGAGGAGGAGGAGC-3`	$(GAG)_6C$	64°C
4	5`-GAGAGAGAGAGACC-3`	$(GA)_6CC$	54°C
5	5`-AGAGAGAGAGAGAGAGCG-3`	$(AG)_8CG$	56°C
6	5`-AGAGAGAGAGAGAGAGCA-3`	$(AG)_8CA$	54°C
7	5`-GAGAGAGAGAGAGAGAGA-3`	$(GA)_9C$	56°C
8	5`-AGAGAGAGAGAGAGAGAGC-3`	$(AG)_9C$	57°C

Polymerase chain reaction (PCR) ran in a Thermal Cycler amplifier (Applied Biosystems, USA). A *PCR* cocktail consists of 2 µL of buffer for DNA polymerase, 1 µL of triphosphate mixture (Amplisens), 0.8 µL of the appropriate primer, 0.2 µL of *DNA* polymerase (Fermentas, Lithuania). Genomic *DNA* was added to 1.2 µL (25 ng). The total volume of the *PCR* mixture was adjusted to 10 µL of ddH_2O [8, 9].

After *PCR* fragments were analyzed by electrophoresis on 1.5% agarose/1X TBE gel stained with ethidium bromide, differentiation of amplicons by size was carried out with the help of a molecular size marker of Gene Ruler 1 kb Plus DNA Ladder (Thermo Scientific™). The bands were visualized under UV light, and the gels were photographed using a Camera.

Each spectrum amplicon was considered as one *DNA* locus. The presence or absence of an amplicon of the corresponding length in the spectra assessed the polymorphism of such a locus (P). Parameters of genetic diversity were determined using the Microsoft Excel computer program.

2.3 Statistical Analysis

All biometric indicators were calculated according to the accepted methods. Statistical analysis was performed using the *Statistica 6.0* software package and *Excel* (*Microsoft Office 2007*). Statistical data processing was carried out in the standard *Microsoft Excel* package using the integrated add-on of the *Statisti XL* 2.0 program (http://www.statistixl.com/) [10].

2.4 Bioethical Norms

Experiments on animals were carried out in compliance with the requirements of the Law of Ukraine "On the Protection of Animals from Cruelty Treatment" (Article 230 of 2006), General Ethical Experiments on Animals", approved by the National Congress on Bioethics and agreed with the provisions of the "European Convention on the Protection of Vertebrate Animals, which are used in experiments and other scientific purposes" (Strasbourg, 1986), permission for the study was obtained from the Commission on the treatment of animals in scientific research Institute of Animal Breeding and Genetics n.d. M.V. Zubtsya NAAS (protocol No. 2 from February 2024).

3. Results

3.1 Cytogenetic Studies

Monitoring of the karyotype of the maternal herd of Lebedyn breed cows showed that in all the experimental animals, the modal number of chromosomes corresponded to the species norm and was equal to 60 (2n = 60) (Table 2). Carriers of constitutional anomalies in the form of Robertsonian translocations were not detected in any examined animal.

Table 2 Variability of the karyotype of Lebedyn breed cows,%.

Cytogenetic indexes	Aneuploidy	Chromosomal breaks	Asynchrony of separation of the centromeric regions of chromosomes
M ± m	10.2 ± 2.10	3.1 ± 1.88	0.8 ± 0.10

The analysis of genomic disorders of chromosomes revealed the presence of metaphase plates with aneuploidy, the average value of which was M ± m = 10.2 ± 2.10%. A wide range of individual variability in aneuploidy was established for the experimental cows of the Lebedyn breed - from 0 to 15.5%, most of whose cells were hypoploid, with a set of 55 to 58 chromosomes. Multiple doublings of the haploid number of chromosomes and polyploidy were not detected.

The karyotypic variability of structural violations of chromosomes manifested in chromosome breaks ranged from 0 to 12.5% (M ± m = 3.1%). The average frequency of asynchrony of separation of the centromeric regions of chromosomes, considered a prerequisite for chromosome loss, in cows of the Lebedyn breed was low and amounted to 0.8% (Figure 1).

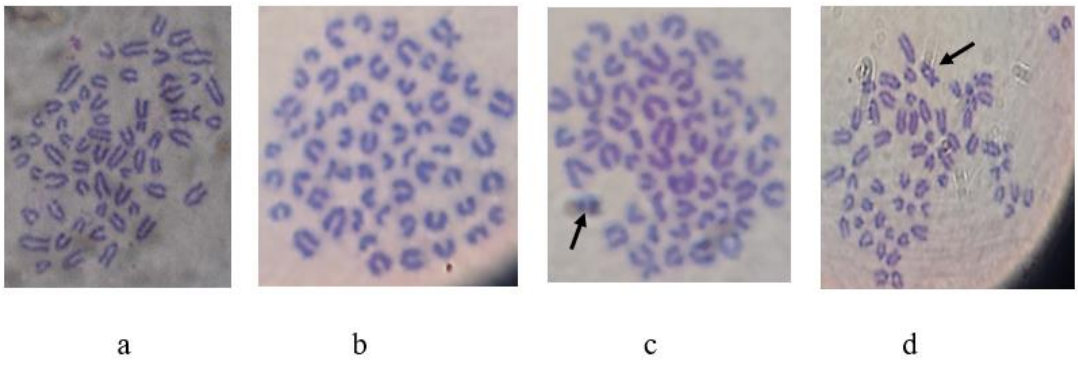

Figure 1 Karyotype variability of cows of the Lebedyn breed: a) karyotype is normal, 2n = 60; b) aneuploidy, 2n = 54; c) asynchrony of separation of the centromeric regions of chromosomes; d) chromosomal break; magnification ×1000 times.

The correlation between the frequency of cells with asynchrony of separation of the centromeric regions of chromosomes and aneuploidy in the experimental animals did not reach statistical significance. This confirms the opinion about the existence of several different mechanisms of the formation of aneuploid cells in lymphocyte culture.

For a more complete assessment of somatic mutagenesis of the experimental Lebedyn cows, a micronucleus test was conducted, according to which it was established that the cytogenetic parameters of the cells varied in the following range: the proportion of binucleated lymphocytes from 2.0‰ to 5.5‰, lymphocytes with a micronucleus - from 1.2‰ to 4.6‰ and the mitotic index - from 1.7‰ to 5.2‰. (Table 3).

Table 3 Results of the micronucleus test in cows of the Lebedyn breed.

Indexes	Binuclear lymphocytes, ‰	Lymphocyte with a micronucleus, ‰	Mitotic index, ‰
M ± m	4.3 ± 0.97	3.6 ± 0.61	3.7 ± 1.20

Note: M ± m is the average

The micronucleus test results showed the presence of micronucleus of different sizes (from 1/3 to 1/8 of the central nucleus) (Figure 2).

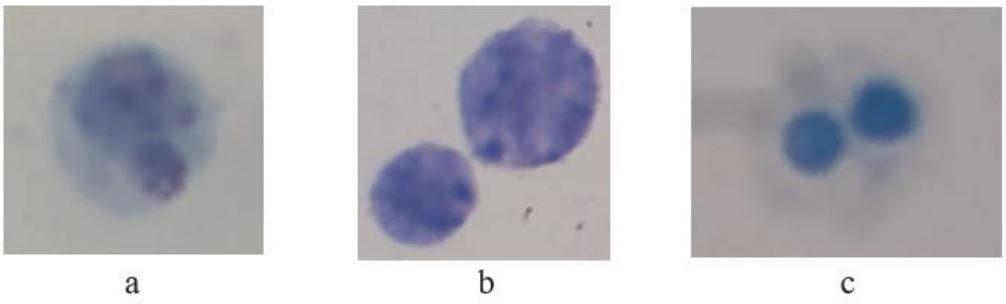

Figure 2 a) a lymphocyte with a micronucleus formed due to clastogenic action; b) a lymphocyte with a micronucleus formed as a result of aneugenic action; c) binuclear lymphocyte.

The proportion of lymphocytes with micronuclei of different sizes in the experimental animals was approximately the same. It did not exceed the spontaneous level of cytogenetic parameters of cells characteristic of cattle [11]. The difference in average values for this feature was statistically insignificant. In our opinion, the genetic potential of Lebedyn breed cows is characterized by a low level of cytogenetic variability, which may be due to the peculiarities of their repair systems the presence and activity of antioxidants in tissues.

The average proportion of binucleated lymphocytes (4.3‰) exceeded the mitotic index (3.7‰). However, the difference in average values between the proportion of binucleated lymphocytes and the level of cell division - the mitotic index - is insignificant, which indicates the absence of an increased radiation background [12]. The indicators of the micronucleus test correspond to the spontaneous level characteristic of cattle [13].

3.2 Molecular Genetic Studies

As a result of the research, an analysis of the genetic structure of the local, small-numbered Lebedyn cattle breed of Ukraine was carried out using 8 *ISSR*-systems based on the following microsatellites: $(ACC)_6G$, $(CTC)_6C$, $(GAG)_6C$, $(GA)_6CC$, $(AG)_8CG$, $(AG)_8CA$, $(GA)_9C$ and $(AG)_9C$ and the following results were obtained. The spectra of the amplification products used as primers of different microsatellite loci as primer firing sites and amplification of the sites located between their inverted repetition were significantly different. The difference was in the number of amplicons obtained, their length (in nucleotide pairs), and their polymorphism (Figure 3).

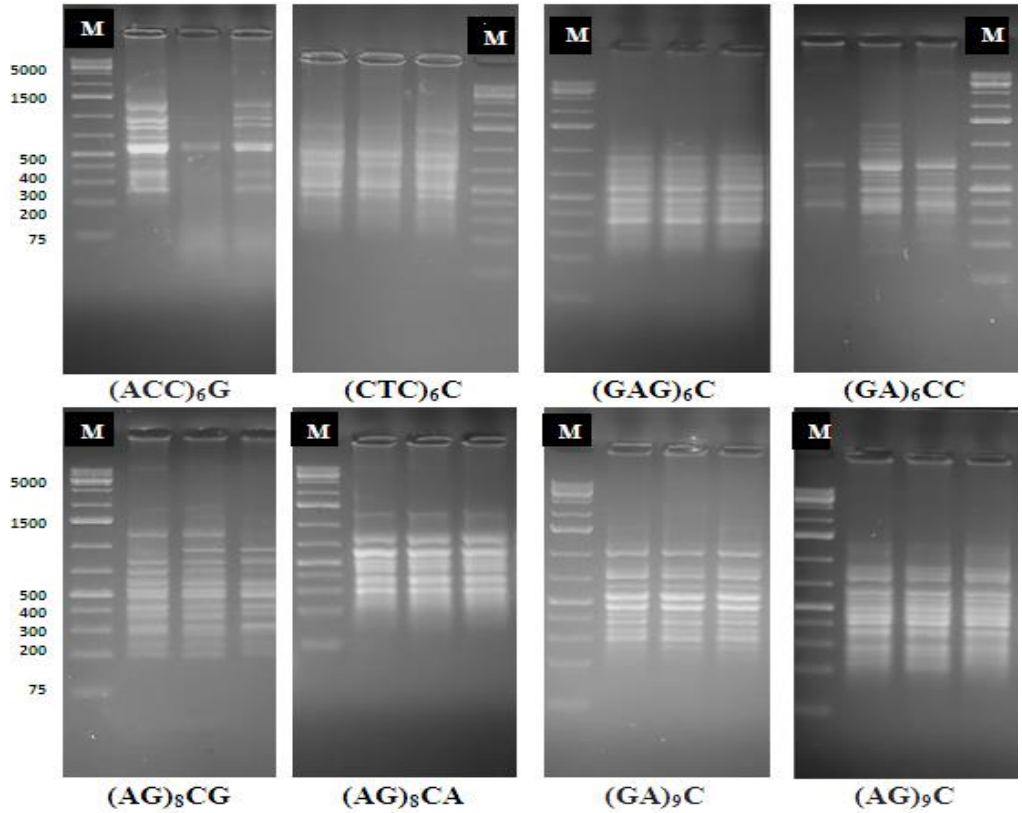

Figure 3 Electrophorogram in a 1.5% agarose gel of amplification products of *DNA* samples of Lebedyn breed animals by the *ISSR-PCR* method. M-marker of molecular weights (*Gene RulerTM 1 kb Plus DNA Ladder, Fisher Scientific*).

The tested 8 *ISSR*-primers contained sequences of di- and trinucleotide microsatellite motifs with the addition of an anchor nucleotide at the 3-d end. All 8 tested *ISSR*-primers showed high efficiency in the study of the Lebedyn breed of cattle in Ukraine. The spectra obtained using di- and trinucleotide microsatellite motifs as primers were equally saturated with amplicons.

ISSR-marking of Ukraine's small local Lebedyn cattle breed revealed 88 amplified *DNA*-fragments, of which only 18 were polymorphic. The total number of polymorphic loci shared was 20.45% (Table 4).

Table 4 Parameters of the used *ISSR*-markers.

Primer	$(CTC)_6C$	$(ACC)_6G$	$(GAG)_6C$	$(GA)_6CC$	$(AG)_8CG$	$(AG)_8CA$	$(GA)_9C$	$(AG)_9C$	Σ
Number loci	6	14	10	10	12	9	13	14	88
Borders length	410-1450	200-1350	240-1050	300-1450	200-1290	280-1450	280-1550	220-1350	200-1550
Polymorp-hic loci	2	7	0	5	3	0	1	0	18

The number of amplified *DNA* fragments varied depending on the primer from 6 to 14, and their size - from 200 to 1550 bp. Primers revealed the most significant number of loci, 14 each $(ACC)_6G$ and $(AG)_9C$. If in $(ACC)_6G$ – 7 loci turned out to be polymorphic, then in $(AG)_9C$ all amplicons are conservative. The fewest loci were detected using microsatellite $(CTC)_6C$ – 6 fragments, of which only 2 are polymorphic.

To assess the genetic diversity of the population of Lebedyn cows, an analysis of polymorphism of *DNA* fragments by *ISSR*-markers was carried out: $(ACC)_6G$, $(CTC)_6C$, $(GAG)_6C$, $(GA)_6CC$, $(AG)_8CG$, $(AG)_8CA$, $(GA)_9C$ and $(AG)_9C$ (Table 5).

Table 5 *DNA* polymorphism of the Lebedyn breed of cows by *ISSR*-markers.

№	*ISSR*-markers	*P*	H_S	*I*	μ	h_μ	*PIC*	*K*
1	$(ACC)_6G$	0.28	0.314	0.137	26.832	0.92	0.205	7
2	$(CTC)_6C$	0.22	0.177	0.084	2.62	0.56	0.150	4
3	$(GAG)_6C$	0	0	0	0	0	0	10
4	$(GA)_6CC$	0.28	0.313	0.134	13.47	0.347	0.204	5
5	$(AG)_8CG$	0.18	0.114	0.055	6.40	0.47	0.115	9
6	$(AG)_8CA$	0	0	0	0	0	0	9
7	$(GA)_9C$	0.04	0.058	0.027	0.5	0.96	0.032	12
8	$(AG)_9C$	0	0	0	0	0	0	14
Average		0.125	0,125	0.166	9.964	0.651	0.141	

Note: *P* – share of polymorphic loci, H_S – average gene diversity per locus, μ – the average number of alleles per locus, *I* – the Shannon heterogeneity index, *PIC* – locus polymorphism index, h_μ – the proportion of rare loci, *K*- the number of conservative loci

According to the population genetic analysis results, the $(AG)_8CG$ marker (PIC = 0.115) turned out to be the least polymorphic. According to this primer, 9 conservative loci were detected. In general, conservative loci were found in all *ISSR*-markers, but only 3 microsatellite primers ($(GAG)_6C$, $(AG)_8CA$, $(AG)_9C$) amplified exclusively conservative fragments, that is, they were monomorphic.

Along with common loci, species-specific fragments were also identified in our study. To determine the species-specific pattern in domesticated species, it was proposed to use only fragments that occur with a frequency of 0.4 and above (Figure 4) [14].

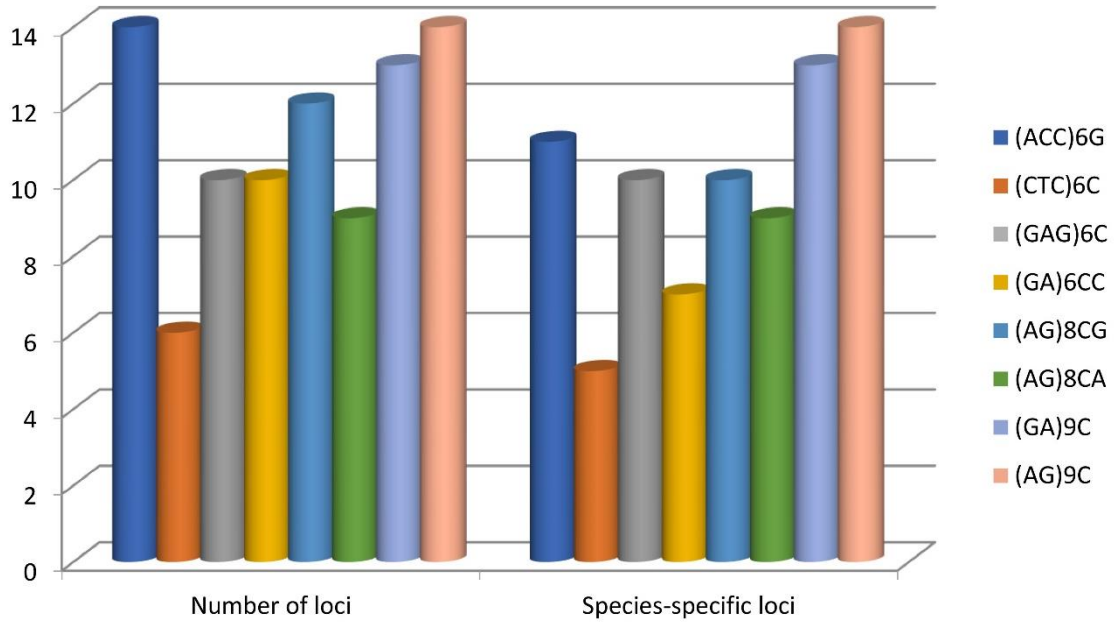

Figure 4 Distribution of *ISSR*-fragments according to the studied primers.

4. Discussion

The use of indicators of cytogenetic variability makes it possible to find out the presence of factors of mutagenic influence on the body and the body's adaptation to specific environmental conditions. The criterion for assessing genotoxic effects is an increase in the frequency of metaphase plates with chromosomal aberrations, genomic disorders, and changes in cell cytogenetic parameters. The study of non-constitutive karyotypic variability of chromosomes in somatic cells allows for predictive analysis of the genetic quality of the generation obtained from the experimental individuals. There is also an associative relationship between chromosomal instability and reproductive function [15], also observed in animals. Such studies have theoretical and practical significance for preserving local breeds of agricultural animals. Gene pools of local domestic cattle breeds are the primary object of systematic, complex scientific research.

A wide range of variability characterized the percentage of cells with aneuploidy in the experimental cows and was somewhat higher in comparison with cattle of milk-meat productivity [16]. One of the reasons for the increased frequency of metaphases with aneuploidy may be the intensive use of cows and the individual genetic polymorphism of this variability. The spectrum of structural chromosomal disorders did not differ from spontaneously formed ones, corresponding to the norm characteristic of the species as a whole.

The size of the micronucleus, which is formed in peripheral blood lymphocytes, serves as an indicator of aneugenic or clastogenic mutation. The size of micronucleosis (MN) resulting from aneugenic mutations of a more extensive MN that appears due to clastogenic action. The absence of a statistically significant difference between micronuclei of different sizes leads to a decrease in the sensitivity of Lebedyn breed cows to genotoxic effects and their adaptation to living conditions.

Based on the results of a molecular genetic study of the population of the Lebedyn breed of cattle, a system for assessing the level of gene polymorphism by markers was developed *$(ACC)_6G$, $(CTC)_6C$, $(GAG)_6C$, $(GA)_6CC$, $(AG)_8CG$, $(AG)_8CA$, $(GA)_9C$* and *$(AG)_9$*C to confirm the uniqueness and consolidation of populations as a basis for their inclusion in national programs for the conservation of biological resources. Analysis of the assessment of the state of the Lebedyn breed gene pool using *ISSR*-markers established a low level of genetic diversity: the proportion of polymorphic loci (P = 0.125), the average genetic diversity per locus (H_S = 0.195), while the Shannon index was 0.166. These results indicate a high degree of gene consolidation and possible reproductive isolation of the population of the studied animals.

5. Conclusions

The studied animals were in ecologically clean conditions relative to the level of radionuclide pollution. They were characterized by karyotype stability, reduced sensitivity to mutagenic factors of various natures, high genetic consolidation, and reproductive isolation.

Author Contributions

Lyubov Starodub took part in setting up the culture and obtaining metaphase plates of chromosomes, analysis of cytogenetic preparations, writing the article; Nataliia Mokhnachova participated in the isolation of *DNA* from the blood of cows, evaluation of genetic polymorphism of the cow population with the help of *ISSR*-markers, writing of the article; Ostap Zhukorskyi assessed the final results of the study.

Competing Interests

The authors have declared that no competing interests exist.

Data Availability Statement

Please note that all data of the study are held in Institute of Animal Breeding and Genetics nd. a. M.V. Zubtsya National Academy of Agrarian Sciences in accordance of the provisions of the applicable legislation and may be accessible by you on a codified basis upon request.

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