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COMPARATIVE STUDY OF DIFFERENT METHODS OF DNA EXTRACTION FROM CATTLE BONES SPECIMENS MAINTAINED IN A CRANIOLOGICAL COLLECTION

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Abstract

The development of molecular-genetic methods allows elucidating the origin and demographic history of breeds of farm animals. Samples of bones and teeth maintained in craniological collections can serve as a source of DNA for such studies. The work with historical samples is complicated by the presence of a very low quantity of DNA, the high degree of its degradation and by the contamination of samples by PCR inhibitors. The aim of this work was the comparison of the efficiency of various methods of DNA extraction from historical cattle skulls, suitable for molecular genetic studies. The material was teeth extracted from historical skulls of cattle of the Yaroslavl and Kholmogor breeds stored in the craniological collection of the Liskun Museum of Livestock (Timiryazev Russian State Agrarian University—Moscow Agrarian Academy). At the first stage, we compared various DNA isolation methods implemented in the form of commercial kits, i.e. Prep Filer™ BTA Forensic DNA Extraction Kit («Thermo Fisher Scientific Inc.», USA), COrDIS Extract decalcine («GORDIZ» LLC, Russia), M-sorb-bone («Syntol» LLC, Russia), QIAamp DNA Investigator Kit («Qiagen», USA), with the modification of the amount of bone material and conditions of lylis. Based on preliminary research results, we selected for more detailed studies two kits, the QIAamp DNA Investigator Kit («Qiagen», USA) which implements the technology of column with silica gel membrane, and Prep Filer™ BTA Forensic DNA Extraction Kit («Thermo Fisher Scientific Inc.», USA) which is based on using magnetic particles. The quantitative and qualitative characteristics of the obtained DNA were evaluated by measuring the concentration of double-stranded DNA using a Qubit™ fluorimeter («Invitrogen, Life Technologies», USA) and determining the ratio of the absorption at 260 nm and 280 nm ($OD_{260/280}$) on a NanoDrop 8000 instrument («Thermo Fisher Scientific, Inc.», USA). The suitability of the obtained DNA extracts for molecular genetic studies was assessed based on the multiplex analysis of 11 microsatellite loci (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA122, INRA23, TGLA126, BM1818, ETH225, BM1824) as well as genome-wide genotyping on high-density DNA chips containing 777 thousand SNPs (Bovine HD BeadChip, «Illumina, Inc.», USA). Concentrations of double-stranded DNA (dsDNA) obtained using QIAamp DNA Investigator Kit and Prep Filer™ BTA Forensic DNA Extraction Kit ranged from 0.146 ng/μl

to 2.060 ng/μl and from 0.110 ng/μl to 13,600 ng/μl, respectively, and averaged 0.83±0.23 ng/μl and 2.75±1.33 ng/μl. The correlation coefficient (*r*) between the concentrations of dsDNA in isolations DNA obtained by two different methods was 0.84. Analysis of microsatellites showed that each of the samples has its own unique genotype which differs from other historical and modern samples of individuals. Efficiency of SNP genotyping (Call Rate) of the historical samples was 0.533-0.878 and 0.958-0.977 for DNA preparations produced using QIAamp DNA Investigator Kit и Prep Filer™ BTA Forensic DNA Extraction Kit, respectively. The results of microsatellite analysis and SNP genotyping, on the one hand, indicate the suitability of the obtained DNA for polymorphism research, on the other hand, confirm the compliance of the laboratory in which this analysis was performed with the authenticity criteria for working with ancient DNA. Conducting large-scale studies of historical samples using different types of DNA markers will clarify the origin and demographic history of domestic cattle breeds and develop effective programs for their conservation.

Keywords: historical DNA, craniological collection, DNA extraction, microsatellite analysis, SNP genotyping, cattle, local breeds

Involving historical and fossil specimens in researches is a way of studying evolution and demographic history of farm animal breeds [1]. Although after the death of an organism, DNA is destroyed in the process of enzymatic reactions, the molecules can persist for hundreds and thousands of years under favorable conditions [2, 3].

The main problem in the study of fossil DNA is the contamination of extracts or reagents with modern DNA molecules. Almost from the very beginning of work with fossil samples, the question arose as to whether the resulting DNA belonged to historical samples. As a result of numerous studies, the following so-called authenticity criteria have been formulated: conducting a reaction in each batch of samples with a "pure extract", i.e. without using fossil DNA; examining each sample in duplicates with subsequent comparison of the results; controlling the length of amplified fragments; the presence of polymerase chain reaction (PCR) products longer than 500-700 bps may cause suspicion [4-6]. Compliance with the above criteria is a prerequisite when working with fossil samples. The minimum requirements for working with ancient DNA are considered to be the presence of a physically isolated work area in which all studies are carried out before the amplification stage [7]; the use of negative controls during amplification and avoiding (if possible) positive control, since it carries a risk of contamination [8, 9]; the reproducibility of results for different DNA extracts of the same sample [10].

Since soft tissues are very poorly to preserve, bones of historical and fossil specimens are mainly used to extract DNA [11, 12]. The inner part of the temporal bone and the cement layer in the roots of the teeth are the best to extract DNA while no systematic difference in the DNA content between the two substrates was found [13]. However, bone tissue is extremely difficult to grind and dissolve. In addition, ancient bones and teeth often contain a large number of PCR inhibitors that are co-extracted with DNA [12, 14, 15]. Ancient DNA is quite severely damaged [16-18], so overly aggressive sample treatments, such as high temperatures or strong detergents, should be avoided [19]. There are several ways to extract ancient DNA, including sedimentation in water-ethanol or water-isopropanol solutions [12, 15], DNA concentration in membranes and separation by DNA molecules binding to silicon dioxide [20-22].

Regardless of the DNA extraction method, sample preparation involves several steps. First, the bones (teeth) are washed with detergent and distilled water, and then 2-3 mm of tissue is ground off to remove surface contamination and modern DNA. Purified bone fragments are treated with UV light (λ = 254 nm) for 30 minutes and mechanically ground into fine flour. It is followed by the steps of dissolving the bone powder in a lysing solution, washing away impurities

that inhibit PCR, and obtaining a purified DNA extract. Lysing solutions may differ in composition, but usually contain proteinase K, ethylenediaminetetraacetic acid (EDTA), and the sodium salt of N-lauroyl sarcosinate. A distinctive feature of the bone powder lysis process is the duration of incubation. For effective cell dissolution, lysis is recommended at a given temperature and continuous stirring for at least 24 hours. Increasing the lysis time to 48 hours or more is only appropriate for large sample weight portions and solution volumes. Otherwise, it does not lead to a significant improvement in the quality and does not increase the yield of the final product. It is followed by purification of DNA from lysates, for which various methods are used.

The simplest method is to sediment DNA with ethanol or isopropanol, wash the sediment to remove impurities from the solution, and dissolve the DNA in bidistilled water or buffer. The main advantages of this method are its rapidity and low cost. Disadvantages include high labor intensity, increased probability of sample loss or cross-contamination if several samples are processed simultaneously [23, 24].

To obtain pure DNA preparations, the use of columns with a silica gel membrane is effective. Nucleic acids are selectively bound to the membrane, and impurities are removed by the successive addition of washing buffers and centrifugation. At the final stage, an eluting buffer is used, which washes out the nucleic acids from the membrane. The advantages of the method include low labor intensity and low probability of errors of the researcher, good quality of the resulting DNA. It is also not necessary to use reagents that are dangerous to humans and the environment, such as phenol and chloroform. The method has been implemented in several commercial kits: Pure Link® Genomic DNA Mini Kit (Thermo Fisher Scientific, Inc., USA); Nucleo Spin® Tissue (Macherey-Nagel GmbH & Co. KG, Germany); QIAamp DNA Investigator Kit (Qiagen, USA) [25], which allows standardizing the DNA extraction process. The disadvantage of using commercial kits is their relatively high cost.

A promising method of nucleic acid purification is the use of magnetic particles as sorbents made from various synthetic polymers, biopolymers, porous glass or based on inorganic magnetic materials such as iron oxide [26]. DNA reversibly binds to the surface of magnetic particles and after a series of washings and removal of impurities is easily removed from the sorbent using an eluting buffer. This method is convenient, technological, and suitable for preparing samples for PCR amplification. However, product losses are possible due to irreversible sorption on the carrier, as well as during numerous washings, which is critical when working with small amounts of DNA in the sample [27]. The method has been successfully used in commercial Prep Filer™ BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific, Inc., USA); Quick-DNA/RNA MagBead (Zymo Research, USA).

When studying fossil samples of human bones, the advantage of the DNA extraction method using silica gel columns is shown concerning both the amount of DNA obtained [28, 29] and the size of fragments [29]. Thus, when using silica gel columns, a higher concentration of DNA was obtained in 68.4% of samples, while using QIAquick PCR Purification Kit® (Qiagen, USA) – in 21.05% of samples [28].

Historical skulls and their parts preserved in craniological collections are of interest as material for research on the demographic history of domestic animal breeds. The craniological method proposed in 1865 by Rütimeyer was the main method in the study of domestic animals origin in the late 19th to first half of the 20th century [30]. It led to the creation of well-documented craniological

collections, but only a few of them have survived to the present day. One of these collections, collected by Prof. Adametz, which contains about 1,300 skulls of old breeds of large domestic animals in Central Europe, is kept in the Natural History Museum Vienna (Naturhistorisches Museum Wien); https://www.nhm-wien.ac.at/en/research/1_zoology_vertebra-tes/archaeo-zoological_collection).

A unique collection of skulls of domestic and imported to old Russia foreign breeds of domestic animals (cattle, pigs, horses) collected by Prof. Liskun at Russian State Agrarian University—Moscow Timiryazev Agricultural Academy. The collection contains more than 700 animal skulls, including 350 cattle of 41 breeds and breed groups [31].

However, the complications in using material of craniological collections for DNA extraction is due to the fact that the technique of a skull accession preparation includes simmering for a few hours, in some cases bleaching in ammonia solution with hydrogen peroxide, and treating with phosphoric acid or chloramine to give a natural shade. Such treatment leads to significant degradation of nucleic acids, so when using historical specimens as a source of DNA, it is necessary to optimize the technique that allows obtaining DNA preparations that are suitable for quantitative and qualitative characterization of various types of polymorphisms. It is especially critical to obtain a sufficient amount of nuclear DNA, represented by only two copies per cell, in contrast to mitochondrial DNA the copy number of which is from 100 to 10,000 per cell [32].

In this paper, we obtained for the first time DNA preparations suitable for molecular genetic research using cattle skull accessions dated to the first half of the 20th century and subjected to thermal and chemical treatment prior to depositing in collections.

The work objective was to compare the effectiveness of various methods of DNA extraction from historical cattle skulls.

Techniques. The material was teeth from historical skulls of cattle of the Yaroslavl and Kholmogor breeds stored in the craniological collection of the State Museum of Stock-Breeding of Russian State Agrarian University—Timiryazev Moscow Agricultural Academy. All the standards set by the criteria for the authenticity of ancient DNA were met during the research [33].

Teeth were cleaned of mechanical impurities, washed with detergent and distilled water to remove surface contamination and modern DNA. Then, they were irradiated with ultraviolet light (λ = 254 nm) for 30 minutes. Using a table-top MBS240/E electric saw (Proxxon, Germany) equipped with a bimetallic blade, the teeth were cut longitudinally into two parts. After that, fine powder was drilled from the inside of the teeth using a Dremel 3000-15 mini drill (Dremel, USA) with diamond boron. Drilling was performed at minimum speeds (~ 8,000 rpm) with breaks to prevent excessive heating of the dentin and destruction of DNA under the influence of high temperatures. The resulting bone powder was poured into pre-irradiated Eppendorf-type sterile tubes with a Safe-Lock system (1.5 ml).

At the first stage, various methods of DNA isolation implemented in the form of commercial kits were compared with modifications of the amount of bone material used and lysis conditions: Prep Filer™ BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific, Inc., USA), COrDIS Extract decalcin (LLC (OOO) GORDIZ, Russia), M-Sorb-kost (LLC (OOO) Syntol, Russia), QIAamp DNA Investigator Kit (Qiagen, USA).

For more detailed research, QIAamp DNA Investigator Kit, which implements the technology of selective DNA binding on a silicon membrane, and Prep Filer™ BTA Forensic DNA Extraction Kit, based on the use of magnetic

particles, were selected. To obtain comparable results, the extraction protocols recommended by the manufacturers for each set were modified. Sample weight portions resulted in a single mass (100±3 mg), the lysis time was increased to 24 h at a temperature of 56 °C and the rotation speed was 1100 rpm. A further increase in lysis time to 48 hours did not have a significant positive effect on the quality and quantity of the resulting DNA. The elution time for QIAamp DNA Investigator Kit extraction was increased to 30 minutes.

The quality of the obtained DNA preparations was evaluated as follows: the total DNA concentration and the drug absorption ratio were measured at $\lambda = 260$ nm and $\lambda = 280$ nm using a NanoDrop 8000 device (Thermo Fisher Scientific, Inc., USA); the concentration of double-stranded DNA was determined on the Qubit™ (1.0) fluorimeter (Invitrogen, Life Technologies, USA).

To assess the suitability of the obtained DNA preparations for molecular genetic research, a multiplex analysis was performed on 11 microsatellites (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA122, INRA23, TGLA126, BM1818, ETH225, BM1824) using a panel developed at the Federal Science Center for Animal Husbandry named after Academy Member L.K. Ernst, as well as full-genomic genotyping for about 777 thousand SNPs (single nucleotide polymorphisms) on high-density DNA chips (Bovine HD BeadChip, Illumina, Inc., USA). PCR amplification was performed on a SimpliAmp Thermal Cycler (Life Technologies, USA). Polymorphism of microsatellite markers was studied on the capillary genetic analyzer ABI3500 (Applied Biosystems, USA) using the software Gene Mapper v. 5 (Applied Biosystems, USA). Microsatellite profiles of the representatives of the modern breeds were taken from the database of the Ernst Federal Science Center for Animal Husbandry.

SNP was examined using the iScan® system scanner (Illumina, Inc., USA) and the software supplied with the device.

A phylogenetic tree for modern and historical bovine samples was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method based on genetic distances of Nei (Ds) [34] calculated from microsatellites in the Populations program, 1.2.32 [35], and visualized in the SplitsTree program, 4.13.1 [36].

Results. Kits based on magnetic particles (Prep Filer™ BTA Forensic DNA Extraction Kit, COrDIS Extract decalcin and M-Sorb-kost), in general, allowed obtaining DNA preparations with a higher concentration (2.94-9.80 ng/μl of double-stranded DNA, the value of $OD_{260/280}$ = 1.00-1.64). However, the purity of extracts obtained using spin columns with a silicon membrane (QIAamp DNA Investigator Kit) was higher at a lower concentration of the extracted DNA (0.56-6.42 ng/μl) ($OD_{260/280}$ = 1.47-1.90). QIAamp DNA Investigator Kit and Prep Filer™ BTA Forensic DNA Extraction Kit were selected for a more detailed study.

Significant differences in DNA concentration were found between samples and between preparations obtained from the same sample using different methods (Table 1). Concentrations of double-stranded DNA isolated using QIAamp DNA Investigator Kit and Prep Filer™ BTA Forensic DNA Extraction Kit ranged from 0.146 to 2.060 ng/μl and 0.110 to 13.600 ng/μl, respectively, averaging 0.83±0.23 and 2.75±1.33 ng/μl. In 8 of the 10 samples, the concentration of double-stranded DNA was 1.43-6.60 times higher when using the Prep Filer™ BTA Forensic DNA Extraction Kit compared to the QIAamp DNA Investigator Kit, while in the two remaining samples it was 1.76-2.80 times lower. The correlation coefficient (*r*) between the concentrations of double-stranded DNA in preparations obtained by two different methods was 0.84.

1. Concentration and purity of DNA preparations obtained from historical samples of Yaroslavl and Kholmogor cattle teeth using various methods

Sample No.	QIAamp DNA Investigator Kit			Prep Filer™ BTA Forensic DNA Extraction Kit		
	Qubit, ng/µl	NanoDrop, ng/µl	$OD_{260/280}$	Qubit, ng/µl	NanoDrop, ng/µl	$OD_{260/280}$
1	0.434	141.50	0.82	0.246	5.60	1.70
2	2.060	23.02	1.71	13.600	79.40	1.87
3	0.308	9.12	1.46	0.110	70.68	1.96
4	0.316	33.80	1.83	1.260	26.51	1.67
5	0.318	14.70	1.58	0.454	17.38	0.67
6	0.334	16.44	1.54	0.540	18.75	1.59
7	1.590	19.13	1.73	2.400	40.09	1.74
8	0.884	15.19	1.72	2.600	42.48	1.64
9	0.146	12.40	1.53	0.378	19.65	1.54
10	1.910	16.57	1.90	5.880	64.23	1.88

N o t e. Double-stranded DNA concentration (a Qubit fluorometer, Invitrogen, Life Technologies, USA) and total DNA concentration (a NanoDrop 8,000 device, Thermo Fisher Scientific, Inc., USA) were measured. $OD_{260/280}$ (absorption ratio at λ = 260 nm and λ = 280 nm, measured on the NanoDrop 8000 device) indicates purity of DNA preparation.

The obtained data show that the main factor affecting the success of DNA isolation is the safety of the sample, which is consistent with the results of other studies [28, 37, 38]. However, unlike some other papers [29], the authors obtained higher concentrations using the magnetic particle method. This is likely due to the severe degradation of DNA in the process of preparing skulls for deposition in collections. As a result, the extracted DNA consists of very short fragments that are better held by magnetic particles than by a silica gel membrane during the washing process.

Microsatellite profiles of historical animal samples of the Yaroslavl and Kholmogor breeds were obtained to assess the suitability of the selected DNA for molecular genetic research and to confirm that the laboratory meets the authenticity criteria. Genotypes of all 11 microsatellite loci studied were determined (Fig. 1). At the same time, it should be noted that the peak height decreases with increasing the allele length of microsatellites, which indicates a strong degradation of the resulting DNA, which is represented mainly by low-molecular fragments.

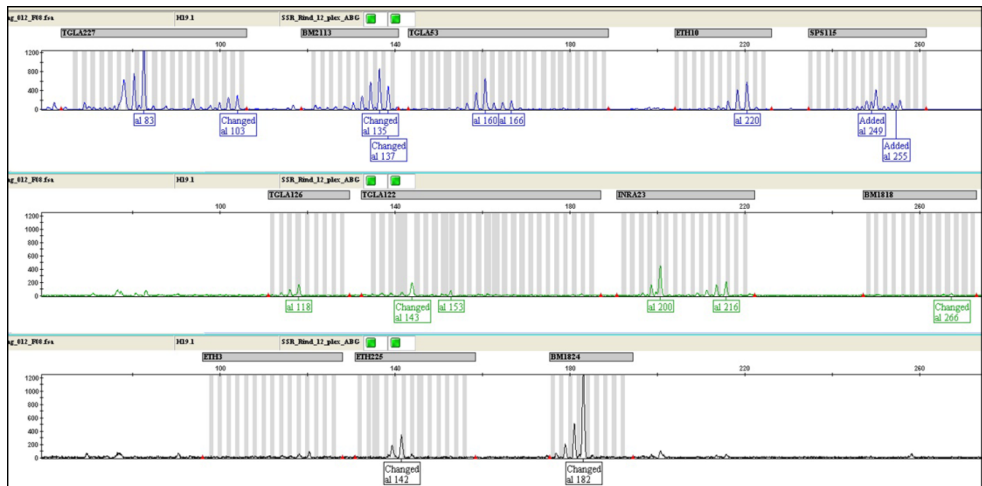

Fig. 1. Capillary electrophoresis-based multiplex analysis of microsatellite profile for 11 microsatellite loci of a Kholmogor animal historical craniological sample (H19.1**):** A — TGLA227 (alleles 83, 103), BM2113 (135, 137), TGLA53 (160, 166), ETH10 (220, 220), SPS115 (249, 255); Б — TGLA126 (118, 118), TGLA122 (143, 153), INRA23 (200, 216), BM1818 (266, 266); В — ETH225 (142, 142), BM1824 (182, 182). X axis corresponds to loci and fragments (peaks) detected; the size of the fragments (al 83, Changed al 103, Changed al 135, etc.) increases along the axis to the right). Y axis corresponds to the peak height. The color indicates different tags for the analyzer. See the full Figure 1 on http://www.agrobiology.ru.

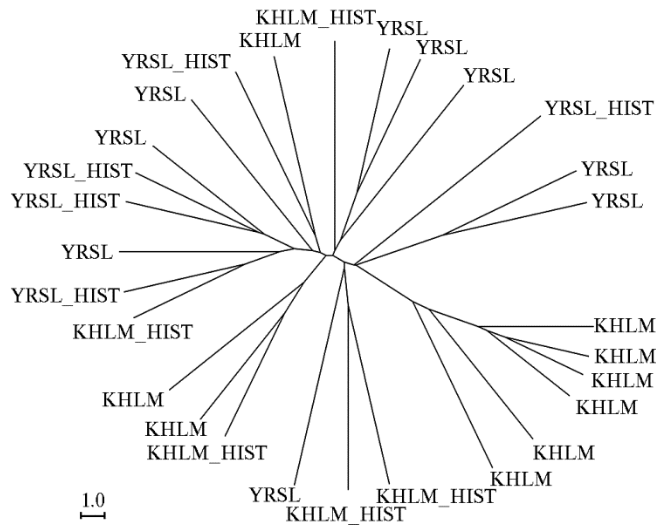

Fig. 2. Phylogenetic tree constructed from microsatellite markers based Nei [34] genetic distances (Ds) for modern and historical cattle samples: KHLM — modern Kholmogor breed, YRSL — modern Yaroslavl breed, KHLM_HIST — historical sample of Kholmogor breed, YRSL_HIST — historical sample of the Yaroslavl breed. UPGMA method, Populations program 1.2.32 [35], visualization with SplitsTree program 4.13.1 [36].

Analysis of microsatellite profiles of historical samples showed that each of the samples had its own unique genotype, which differed from the genotypes of other historical and modern samples. On a phylogenetic tree constructed on the basis of genetic distances of Nei [34] calculated from microsatellites, it was manifested in the formation of its independent branch by each sample (Fig. 2). In all five historical samples of the Yaroslavl breed and three of the five historical samples of the Kholmogor breed, private alleles were identified that were not present in other studied populations. The results of microsatellite analysis confirm that the laboratory where these studies were conducted meets the criteria for working with ancient DNA.

The efficiency of full-genomic genotyping of SNP when using DNA isolated using magnetic particles was significantly higher than when using DNA isolated using silicon spin-columns (Table 2). It may be because, for SNP analysis, the size of DNA fragments (as opposed to concentration) is not a critical factor. When analyzing microsatellite loci, the success of amplification is determined by the degree of DNA degradation [18].

2. Effectiveness of whole-genome genotyping DNA from Kholmogor cattle skull historical material with high-density Bovine HD BeadChip DNA chips (Illumina, Inc., USA)

Sample No.	Qubit, ng/μl	NanoDro, ng/μl	$OD_{260/280}$	Call Rate	DNA extraction
1	5.32	35.68	1.87	0.958	Prep Filer™ BTA Forensic DNA Extraction Kit
2	4.52	26.19	1.96	0.970	Prep Filer™ BTA Forensic DNA Extraction Kit
3	6.84	33.10	1.83	0.970	Prep Filer™ BTA Forensic DNA Extraction Kit
4	4.94	65.71	1.90	0.977	Prep Filer™ BTA Forensic DNA Extraction Kit
5	2.94	21.64	1.96	0.641	QIAamp DNA Investigator Kit
6	1.18	21.15	1.86	0.533	QIAamp DNA Investigator Kit
7	6.84	35.15	1.85	0.775	QIAamp DNA Investigator Kit
8	4.48	24.75	2. 00	0.878	QIAamp DNA Investigator Kit

Note. Double-stranded DNA concentration (a Qubit fluorometer, Invitrogen, Life Technologies, USA) and total DNA concentration (a NanoDrop 8,000 device, Thermo Fisher Scientific, Inc., USA) were measured. $OD_{260/280}$ (absorption ratio at λ = 260 nm and λ = 280 nm, measured on the NanoDrop 8000 device) indicates purity of DNA preparation. Call Rate means the proportion of genotyped SNPs of the total number of SNPs on the DNA chip.

The main problems in molecular genetic studies of ancient and historical

samples are low DNA concentrations and high degradation in the extracts [13, 14, 18, 22]. The small length of the remaining target fragments may be a significant limitation for analyzing microsatellites with a longer fragment length (200 bps or more) [41, 42]. For unambiguous interpretation of the obtained profiles in some works [42, 43], it is recommended to amplify each sample at least three times. SNP markers are less demanding on the degree of DNA degradation. Studies have shown [29, 44] that high-performance sequencing and full-genomic genotyping of SNP on DNA chips will significantly expand the range of data processing procedures used and make more complete use of the information contained in historical DNA.

Thus, our findings show the possibility of successfully isolating DNA suitable for molecular genetic research from historical bovine skulls from craniological collections. Comparison of two different methods of DNA extraction based on the use of columns with a silica-gel membrane (QIAamp DNA Investigator Kit) and magnetic particles (Prep Filer™ BTA Forensic DNA Extraction Kit) revealed the advantage of the second method. In 8 of the 10 studied samples, the concentration of double-stranded DNA was higher when using the magnetic particle method, and in two samples — when using silica gel columns, while the average values of the DNA concentration were 2.75±1.33 and 0.83±0.23 ng/μl, respectively. Analysis of the genotypes of historical samples from 11 microsatellite loci shows that each of the 10 studied samples of the Kholmogor and Yaroslavl breeds has its own unique genotype that differs from other samples. The study of the effectiveness of full-genomic SNP genotyping shows the advantage of a set based on the use of magnetic particles (Prep Filer™ BTA Forensic DNA Extraction Kit). Assuming that the length of fragments in this type of analysis is not a critical factor, this method is preferred due to obtaining higher concentrations of DNA. In general, large-scale studies of historical samples using different types of DNA markers will be a significant addition to the results of studying modern representatives of breeds. It will help to clarify the origin and demographic history of domestic cattle breeds to develop effective programs for their preservation.

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