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DETERMINATION OF CONSENSUS GENOTYPES BY MICROSATELLITES FOR MUSEUM ACCESSIONS OF CATTLE (*Bos taurus*)

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Abstract

DNA analysis of ancient and historical samples, including specimens stored in museum and craniological collections, is an invaluable source of genetic information for reconstructing the origin of local breeds of livestock. Given the high degree of DNA degradation in most of these samples, studies are usually conducted on the mitochondrial genome, since it is present in hundreds or even thousands of copies in a single cell. However, in some cases, the study of mitochondrial DNA (mtDNA) does not allow us to fully trace the demographic history of animal species and breeds, especially when crossbreeding is used in breeding work. An informative tool for analyzing these types of demographic events is the study of microsatellites, or short tandem repeats (STRs). However, in microsatellite genotyping for DNA extracted from museum specimens imposes an increased risk of amplification errors. The aim of our work was to improve the algorithm for determining consensus STR marker genotypes for samples containing highly degraded DNA and to evaluate the effectiveness of the algorithm suggested for cattle craniological museum samples. The material were museum exhibits of cattle skulls dated from the end of the 19th to the first half of the 20th century and stored in the craniological collection of the Liskun Museum of Animal Husbandry (RSAU — Timiryazev Moscow Agricultural Academy). For genotyping, a multiplex panel was used which included 11 microsatellite loci recommended by the International Society of Animal Genetics (ISAG), according to protocols adopted at the Ernst Federal Research Center for Animal Husbandry. The success of amplification for each locus in the sample was assessed by calculating genotyping quality indices (QI). The most frequently occurring genotypes were coded as 1, and the genotypes that differed from those coded as 1 due to allelic drop-out (ADO) or false alleles (FA) were defined as 0. Next, the proportion was calculated of genotypes with the value 1 to the total number of repetitions. The threshold value for QI was set at 0.75. The genotypes that showed a frequency of occurrence above the threshold value for each locus were included in the consensus genotype. The algorithm was tested on 144 museum samples of black-and-white, Turano-Mongolian, pale-and-white and brown cattle. A complete profile (11 microsatellite loci) was obtained for 60.42 % of accessions. The quality of genotyping at most loci (9 out of 11 loci examined) was above 0.950, ranging from 0.951±0.011 at the TGLA122 locus to 0.995±0.003 at the BM2113 locus. An assessment of genotyping efficiency showed that the TGLA53 and BM1818

loci had the lowest genotyping success (74.86 % and 61.45 %, respectively). A positive correlation at the trend level ($r^2 = 0.53$, $p = 0.09$) between the size of alleles at the locus and the proportion of genotyping errors was revealed. Since studying the allele pool of populations is impossible without obtaining correct genotypes, our proposed algorithm, which ensures the probability of correct genotyping $p < 0.001$, can be used when working with museum and other samples containing highly degraded DNA.

Keywords: microsatellites, genotyping errors, consensus genotype, cattle, museum samples

DNA analysis of ancient and historical samples is an invaluable source of genetic information for reconstructing the origins of local breeds [1-3]. Particularly valuable specimens are accessions from museum [4, 5] and craniological collections [6]. Given substantial DNA degradation in most of these samples, the mitochondrial genome is typically studied [7-9], since it is present in hundreds or even thousands of copies per cell. However, in some cases, mitochondrial DNA (mtDNA) analysis fails to fully trace the demographic history of animal species and breeds [10, 11]. For example, creation of some domestic cattle breeds practiced introductory crossings with bulls of foreign breeds [6]. Mitochondrial genome analysis is not helpful in estimating such events and tracing involvement of foreign breeds in the allele pool formation of modern breed populations, since mtDNA has a maternal type of inheritance. An informative tool for analyzing these types of demographic events is the study of microsatellites, or short tandem repeats (STR) [12]. Microsatellite analysis is recognized as the gold standard for pedigree testing in cattle breeding programs [13], and has provided a wealth of information on the genotypes of modern local and transboundary breeds. The availability of large genetic data sets from modern populations is important for the study of historical samples because it can help trace the persistence of historical alleles in modern populations.

The main problem with microsatellite genotyping for DNA from archaeological and museum samples is amplification errors, in particular, false homozygotes, or allele drop-out (ADO), and false alleles (FA) [14, 15] which are polymerase chain reaction (PCR) artifacts {16]. To improve the genotyping reliability for highly degraded or low-concentration DNA, a multiplex approach based on repeated independent amplifications of each DNA sample has been proposed [15, 17]. Compared to the standard procedure, this technique allows the error to be quantified for each possible genotype [17].

Despite numerous investigations of the cattle allele pool in Russia, until recently there were no protocols for constructing consensus genotypes by microsatellite markers for samples from craniological collections. Therefore, a retrospective investigation of the genetic pool in ancestral populations of local breed was unavailable. Studies of phylogenetic relationships between archaeological samples and modern breeds using STR markers have already been carried out around the world. However, very labor-intensive and expensive methods were used with a large number of repetitions of DNA extraction and amplification of single loci for correct genotyping [2].

This paper is the first to describe and validate a genotyping protocol we have developed for museum specimens that most efficiently provides correct data for microsatellites.

Our goal was to improve the algorithm for determining consensus STR marker genotypes for highly degraded DNA and to evaluate its effectiveness on museum samples of cattle.

Materials and methods. DNA was isolated from museum exhibits of cattle skulls, dated from the end of the 19th to the first half of the 20th century (the craniological collection of the Liskun Museum of Animal Husbandry RSAU—

Timiryazev Moscow Agricultural Academy, Moscow, 1950). The study involved 144 museum specimens of biomaterial from black-and-white, Turano-Mongolian, pale-and-white and brown cattle genotyped in 2019-2021.

Sample preparation and DNA extraction were performed as described [18]. Teeth separated from skulls were washed with detergent and distilled water to remove present-day DNA contamination. Using a Dremel 3000-15 mini-drill (Dremel, USA) with a diamond bur, the tooth was sawed lengthwise at minimum speed, drilling out the powder from the inner part. The powder was dissolved in the lysis solution, impurities that inhibit PCR were washed away, and a purified DNA extract was prepared using commercial kits Prep Filer™ BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific, Inc., USA), COrDIS Decalcine extract (GORDIZ LLC, Russia), M-sorb-bone (Syntol LLC, Russia), QIAamp DNA Investigator Kit (Qiagen, USA) as recommended by the manufacturers. The quality of the resulting DNA was assessed by measuring double-stranded DNA (dsDNA) concentrations (Qubit™ fluorimeter, Invitrogen, Life Technologies, USA) and the $OD_{260/280}$ ratio (NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). Given that increasing the quantity of good quality DNA reduces the risk of erroneous genotyping [19], a dsDNA concentration of at least 1 ng/μl and an $OD_{260/280}$ of 1.6-2.0 were the thresholds chosen for microsatellite analysis. DNA preparations that did not meet these requirements were not involved in the study.

Samples were genotyped using a multiplex panel with 11 microsatellite loci TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA122, INRA23, TGLA126, BM1818, ETH225, BM1824 recommended by the International Society of Animal Genetics ISAG [20] according to the protocols adopted at the Ernst Federal Research Center for Animal Husbandry — VIZH [21].

Multiplex PCRs were run in a final 10 μl reaction mix in PCR buffer with 200 mM dNTP, 1.0 mM $MgCl_2$, 0.5 mM primer mixture (the sequences are given at https://strbase-archive.nist.gov/cattleSTRs.htm), 1 unit Taq polymerase (Dialat LLC, Russia) and 1 μl of genomic DNA (> 1 ng/μl). PCR was performed as follows: initial denaturation at 95 °C for 4 min; 95 °C for 20 s, 63 °C for 30 s (35 cycles); 72 °C for 1 min; the final elongation at 72 °C for 10 min. The size of the resulting fragments was determined (a genetic analyzer ABI3130xl with a GeneScan™ 350 ET ROX™ fragment length standard, GeneMapper™ v. 4 software; Applied Biosystems, USA). Allele sizes were standardized in accordance with ISAG STR typing comparative testing for the species *Bos taurus* 2018-2019.

A modified multiplex approach proposed previously was used as a prototype to determine consensus genotypes [22, 23]. Genotyping quality indices (QI) for each sample/locus were calculated according to S. Miquel et al. [24].

The most frequently occurring genotype at each locus was assigned a code of 1. Genotypes that differed from the most frequent genotype due to allele drop-out (ADO) or false alleles (FA) were considered genotyping errors and designated as 0. The proportion of genotypes with 1 vs. the total number of replicates was calculated. The threshold value for QI was set at 0.75.

Amplification efficiency (+PCR fraction) was calculated as the number of successful PCRs (+PCR) divided by the number of PCR replicates for each locus, expressed as a percentage. To calculate the percentage of unsuccessful amplifications, the percentage of +PCR was subtracted from 100%. The proportion of lost alleles (ADO) and the proportion of false alleles (FA) were calculated using the protocol proposed by T. Broquet and E. Petit [25]. The proportion of ADO for each locus was calculated for heterozygous genotypes (according to the corresponding consensus genotypes) as the number of replicates in which one allele was

lost divided by the total number of +PCRs. FA was calculated for homozygous or heterozygous genotypes as the number of PCRs with spurious alleles divided by the total number of +PCRs.

To analyze genotyping data for false results that affect the estimation of population parameters [26, 27], error rates (ER) were calculated as the number of genotypes differing from the consensus, divided by the total number of +PCRs. The probability of correct genotyping (p) for each locus was calculated as described by G. He et al. [28]. In addition, the means and standard errors of the QI genotyping quality index (M±SEM) values were calculated for each locus. Based on the assessed genotyping quality, Pearson correlation coefficients (r^2) were calculated to assess the relationship between the allele length and the proportion of genotyping errors in loci.

Results. Data on the studied microsatellite loci are summarized in Table 1.

1. Characterization of microsatellite loci for multiplex genotyping of cattle craniological museum samples (the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950)

Locus	BTA	The number of replicates		Allele length, bp	
		min	max	min	max
BM1818	23	13	25	256	280
BM1824	1	10	18	174	190
BM2113	2	12	23	121	143
ETH10	5	14	22	209	225
ETH225	9	19	29	140	160
INRA023	3	10	25	192	222
SPS115	15	17	28	240	262
TGLA122	21	14	38	137	185
TGLA126	20	12	22	105	125
TGLA227	18	9	25	71	103
TGLA53	16	17	40	150	196

N o t e. BTA — *Bos taurus* autosome.

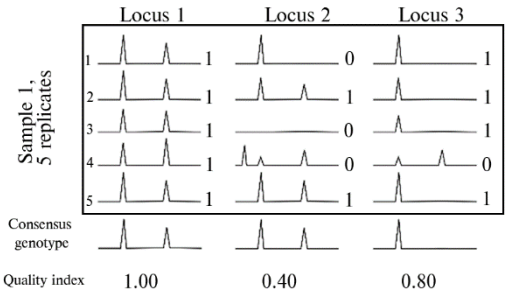

Fig. 1. Scheme for asessing the genotyping quality index (QI) for multiplex genotyping of cattle craniological specimens (the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950).

The scheme for assessment of quality indices for loci is shown in Fig. 1. In locus 1, the profiles of all c clearly show two alleles with different intensities. At locus 2, the most frequent genotype is visualized in replicates 2 and 5 (scored as 1). In replicate 1 for this locus there is an allele dropout (ADO), in replicate 4 a false allele (FA), replicate 3 shows no amplification, so all these replicates are scored as 0. In locus 3 in replicate 4 a false allele is observed (repeat score 0), in all other replicates a clear homozygous profile is visible (score 1). Thus, the QI value for these three loci were 1.00, 0.40 and 0.8, respectively.

Using museum accessions of cattle skulls as an example, we propose a modified algorithm to determine consensus genotypes for complex specimens (Fig. 2). After the initial multiplex amplification of microsatellite loci in duplicate, only those samples in which at least six loci were successfully amplified (+PCR) were selected for further analysis. For such samples, three additional independent PCR replicates were run using the same DNA preparations. Thus, each DNA sample was analyzed in at least five replicates. For samples in which less than six loci were amplified or less than four positive PCR results were obtained at each locus, DNA was re-extracted and PCR were performed as described above.

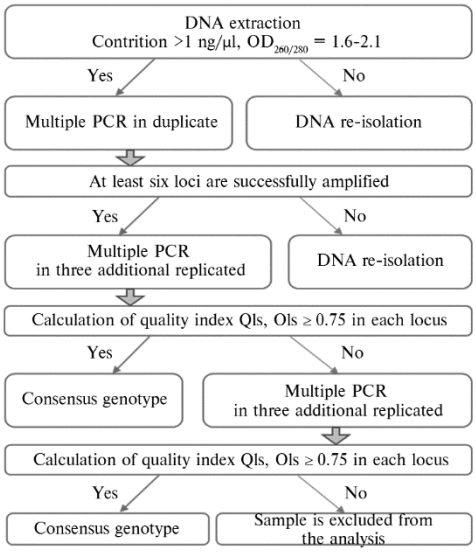

Fig. 2. Scheme for determination of consensus genotypes by microsatellites for cattle craniological specimens using multiplex genotyping (the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950).

As Figure 2 shows, samples having a QI = 0.75 or higher at each locus were considered correctly genotyped and were used in further analysis. For samples with a QI value less than the established threshold (QI < 0.75) at any of the loci, three additional multiplex PCRs were performed using the same DNA preparations, after which the quality indices were recalculated. In case the quality indices were low again (QI < 0.75), DNA was re-isolated and the multiplex PCR was performed as described above if a particular sample is extremely valuable, for example when the breed under study is represented by only 1-2 individuals, or were excluded from further analysis.

2. Efficiency of genotyping by microsatellites of cattle craniological specimens (n = 144, the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950)

Number of successfully genotyped loci	Number of specimens, n	Specimen proportion, %
11	87	60.42
10	21	14.58
9	9	6.25
8	3	2.08
7	3	2.08
6	3	2.08
< 6	27	18.75

The dsDNA concentration in the total sample varied from 1.01 to 63.40 ng/μl, the $OD_{260/280}$ from 1.64 to 2.00. As shown in Table 2, for 60.42% of the 144 genotyped specimens, a complete profile for 11 microsatellite loci was obtained, 14.58 and 6.25% of specimens were successfully genotyped for 10 and 9 microsatellite loci, respectively. For 18.75% of specimens, 5 or fewer loci were successfully genotyped, and as a result, these samples were not further investigated.

3. Quality of genotyping by microsatellite loci (QI index) of cattle craniological specimens (n = 144, the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950)

Locus	QI (M±SEM)	QI = 1		0,75 ≤ QI < 1,00		QI < 0,75		p
		1	2	1	2	1	2	
TGLA227	0.966±0.009	110	87.30	11	8.73	5	3.97	p < 0.001
BM2113	0.995±0.003	122	96.83	3	2.38	0	0.00	p < 0.001
TGLA53	0.759±0.030	68	53.97	20	15.87	38	30.16	p < 0.001
ETH10	0.972±0.008	112	88.89	10	7.94	4	3.17	p < 0.001
SPS115	0.953±0.011	104	82.54	18	14.29	4	3.17	p < 0.001
TGLA122	0.951±0.011	107	84.92	10	7.94	9	7.14	p < 0.001
INRA23	0.952±0.013	107	84.92	14	11.11	5	3.97	p < 0.001
TGLA126	0.960±0.009	106	84.13	15	11.90	5	3.97	p < 0.001
BM1818	0.700±0.040	82	65.08	5	3.97	39	30.95	p < 0.001
ETH225	0.953±0.017	115	91.27	5	3.97	6	4.76	p < 0.001
BM1824	0.961±0.013	113	89.68	7	5.56	6	4.76	p < 0.001

N o t e. 1 — number of samples, n, 2 — proportion of samples, %; p — probability of correct genotyping according to G. He et al. [27].

As Table 3 shows, the quality index of genotyping, assessed by the average values of the quality index QI, in most loci, 9 out of 11 studied, was above 0.950, ranging from 0.951±0.011 for the TGLA122 locus to 0.995±0.003 for the BM2113 locus. In two loci, TGLA53 and BM1818, the genotyping quality index was significantly lower, QI = 0.759±0.030 and QI = 0.700±0.040, respectively. The proportion of specimens with genotyping quality below the threshold value of 0.75 for these loci was maximum and amounted to 30.16% for TGLA53 and 30.95% for BM1818. The probability of correct genotyping for all studied loci was $p < 0.001$.

4. Efficiency and distribution of errors in genotyping of cattle craniological specimens by microsatellite loci ($n = 144$, the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950)

Lous	Number of specimens, *n*	No amplification, %	ADO, %	FA, %	ER, %
TGLA227	125	4.33	4.20	0.73	4,23
BM2113	125	3.49	0.53	0.14	0,58
TGLA53	110	25.14	15.53	2.43	14,37
ETH10	126	3.63	3.93	0.43	2,17
SPS115	122	7.96	5.48	1.21	4,40
TGLA122	123	4.05	5.78	1.75	6,26
INRA23	121	8.38	3.89	0.76	3,66
TGLA126	122	7.40	4.60	0.90	4,22
BM1818	94	38.55	4.78	0.68	2,05
ETH225	120	9.36	2.51	0.31	2,00
BM1824	123	6.15	2.32	1.19	2,68
For 11 loci (*M*±SEM)		10.77±0.35	4.68±0.30	0.94±0.12	4.11±0.24

N o t e. Number of samples, *n* — number of animal skulls for which a genotype for the corresponding locus was idetified; ADO is the proportion of dropped alleles, FA frequency is the proportion of false alleles; ER is — total error rate.

An assessment of the genotyping performance for 11 microsatellite loci (Table 4) showed that the largest proportion of specimens with no amplification were detected for the BM1818 and TGLA53 loci, 38.55% and 25.14%, respectively. The BM1818 locus was successfully genotyped in a total of 94 specimens. At the other least successfully genotyped locus, TGLA53, consensus genotypes were obtained for 110 specimens, but the overall genotyping error rate (ER) was 14.37%, which was more than 3 times the average for the 11 loci examined. The number of repeated PCRs to successfully obtain a consensus genotype or to make a decision to exclude a DNA preparation varied from 5 to 8, which is comparable to the results reported for samples with DNA low concentrations or severe degradation [29-31].

We hypothesized that the high proportion of genotyping errors detected in the TGLA53 and BM1818 loci may be associated with the length of the amplified fragments, which serves as a limiting factor when working with degraded DNA. Calculations of r^2 showed a positive correlation between the length of alleles and the proportion of amplification errors, significant at the trend level ($r^2 = 0.53$, $p = 0.09$). Other researchers, when analyzing DNA from museum feather samples [32] or skin fragments from stuffed animals [33], also revealed a relationship between the size of the amplified fragment and the success of amplification.

Worldwide, the ancient DNA polymorphisms are currently studied primarily by sequencing mtDNA fragments [34, 35] or the entire genome [36], however, STR markers continue to be used to analyse complex samples [37]. This report does not present our data on mtDNA and whole-genome sequencing of museum specimens, since we aimed to describe the protocol for obtaining consensus genotypes using microsatellites as a type of DNA marker that remains the most used and accessible to a large number of laboratories.

Investigation of the population allele pool is impossible without obtaining

correct genotypes. The algorithm we described provides a probability of correct genotyping $p < 0.001$, and, therefore, can be used when working with accessions and other samples of highly degraded DNA. We also note that when studying complex samples, e.g., non-invasive samples from wild animals, museum samples, pilot studies are important to preliminaryly calculate the likelihood of genotyping errors and their probable impact on the population parameters [26, 27].

Thus, we propose a modified protocol for genotyping museum craniological specimens in which the DNA is highly degraded due to long-term storage. A multiplex panel of 11 microsatellite markers is used for analysis in one tube with calculation of the quality of genotyping and obtaining the consensus genotype for each locus separately, regardless of the other loci genotyping success in the same amplification repeat. Additionally, the success of genotyping each sample was assessed based on the average quality index in the first two amplification repeats, and a decision was made on the DNA sample prospects. The described modifications reduce the cost and labor intensity of the analysis, since, by quantifying the success of genotyping each locus and calculating the average quality index for each individuum, low-quality DNA samples were removed from the analysis at the initial stages, minimizing the number of PCR repetitions to obtain accurate genotyping data.

So, a modified multiplex PCR analysis method we suggest allows us to obtain, based on STR markers, correct consensus genotypes for cattle craniological speimens dating from the late 19th to the first half of the 20th century. We expect that the approach outlined in the article will help reduce the labor intensity and cost of historical DNA analysis and promote the involvement of museum specimens in studies of the genetic relationships between breeds of farm animals.

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