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Combination of upstream primer-multuplex PCR (UP-mpcr) and capillary electrophoresis for equine genetic analysis

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Abstract

The purity and content of DNA extracted from the sample is important during PCR analysis. In the conditions of our country, there are many cases of working on samples that do not meet the requirements for some reason. In such cases, there is a need to further test and develop other sensitive methods. The upstream-primer multiplex PCR (UP-mPCR technique) is known for its high specificity and fidelity, and has been used for detecting multiple food borne pathogens, meat species testing and detecting different genetically modified organism (GMO) insertions in a plant genome. The purpose of this experiment is to apply the UP-mPCR method on DNA samples that do not meet the quality requirements, and to test it on domestically produced diagnostics.

We combined UP-mPCR with fragment analysis on DNA capillary electrophoresis genetic analyzer by applying fluorescent labelled upstream primers which were tested by amplifying 8 STRs on 23 low-quality equine gDNA samples. These samples had formerly undergone unsuccessful testing by domestic equine genotyping 15-plex kit. Single trial of UP-mPCR on the same samples showed successful amplification and detection of amplicons from 4-6 STRs, and their alleles were genotyped. Combining UP-mPCR and DNA capillary electrophoresis can be helpful in extreme situations such as having limited amounts of sample, or a shortage of multiple fluorescent dye oligonucleotides. There is no former report about the same method as combining UP-mPCR with fragment analysis.

Keywords: Unqualified DNA samples, microsatellites, mPCR, Fragment analysis, typing

Introduction

DNA or RNA-based analysis methods are trending in agriculture, forensics, and biological sciences. Current scientific literature proposes a variety of different biological and molecular methods that can be used to analyze animal populations. For instance, methods have been developed in order to: differentiate various animal breeds and populations [1]; determine parent-offspring relationships; evaluate meat and other raw materials' content; and to conduct veterinary medical examinations regarding animal theft and food adulteration. One such method utilizes microsatellite or short repeated sequence analysis in order to distinguish genetic information segments [2]. A short tandem repeat (STRs) refers to a repeated sequence (loci) in the genome with a variable number of 2-5 sets of nucleotides.

In Mongolia, microsatellite DNA has been identified at 15 positions in the human genome in forensic testing [3], and research has been underway to increase the number of positions to up to 20 [4]. Studies have shown that 0.03% variation in microsatellites or short repeated sequences (STRs) occurs in mammals per generation [5]. That's why STR is widely used in animal genetic analysis because of its high variability.

Animal paternity testing standards have only been approved for horses [6] and no such tests have yet been performed on other animals. We must learn the guidelines and methods of the International Society of Animal Genetics (ISAG), which was established to standardize laboratory analysis methods for animal breeding and population genetic research. More than 700 public and private laboratories worldwide have been registered as members. Every 2 years, they undergo an

equivalency test which allows them to improve their analytical skills [7].

As a part of the ISAG recommendations, a unified set of guidelines for microsatellite sequence nomenclature and phenotyping have been updated. Zanaaspex Ltd. has created analysis kits based on 15 microsatellite sequences for horses. PCR products are amplified by the multiplex PCR (mPCR) method [8]. The kit features 15-20 pairs of primers with 3-4 different fluorescent dyes in the analysis.

Upstream (UP)-mPCR or paired-primer mPCR amplification products are characterized by the fact that one of the unique primer pairs has the same complementary tail at the 5' end, so mPCR can be used to detect the species and origin of various animal meats [10-13], in addition to food-borne pathogenic bacteria [9, 14-16].

In recent years, genetic modification analysis of vegetables has been carried out [17-19] and combined with real-time PCR and pyrosequencing [20-22]. There are currently no studies combining this method with capillary DNA electrophoresis.

Since mPCR has a unique pair of primers for each amplification fragment, increasing the concentration of sample and primer to increase the yield of the reaction on low-quality DNA samples not only improves the results, but also produces more by-products [21-23]. According to ISAG recommendations, 8 STR repeats are added to the 9 equine STRs, and a total of 17 microsatellites are used alphabetically [24-27].

The purpose of this study was to perform UP-mPCR using primers attached to fluorescent dyes on low-quality DNA samples extracted from long-degraded blood. We then tested the compatibility of the combined analysis of the products with capillary electrophoresis to investigate and check the quality of domestic products.

Methods and materials

Sampling

In 2019, blood samples were collected from a total of 100 horses of Galshar, Darkhad and Tes pedigree. Samples were transported to the laboratory and preserved until analysis in 2021.

DNA isolation

From 100 coagulated blood samples, the genomic DNA (gDNA) of only 23 horses (Galshar 9, Darkhad 8, Tes 6) was extracted and used in the experiment due to low quality (poor yield and purity of DNA). Around 200–250 µl of coagulated blood samples were used for gDNA extraction by phenol chloroform method.

PCR

We used mono and multiplex PCR with fragment analysis. PCR amplification was performed according to MNS 6427:2013. Allele frequencies associated with a total of 15 STR loci were analyzed according to the manufacturer's instructions (Zanaaspex, Equine STR 15plex Kit). DNA isolated from the horses' upper lip swabs and saliva, which was previously confirmed to be positive by PCR, was used as a positive control (PC).

Table 1 shows the sequence information of the 15 equine STRs, including their position on the genome, their accession numbers, and the length of their PCR products.

Table 1.

List of microsatellite loci used for multiplex PCR

Dyes	Nomenclature of microsatellite	Chromosome	Gene bank registration number	Product size (bp)
	VHL20	30	X75970	~82-102
	HTG4	9	AF169165	~116-140
	AHT4	24	Y07733	~149-175
	HMS7	1	X74636	~187-207
	HMS2	10	X74631	~216-238
	ASB17	2	X93531	~98-114
	HMS1	15	X74630	~114-128
	LEX3	X	AF075607	~137-161
	HTG7	4	AF169291	~179-195
	CA425	28	U67406	~234-258
	HTG6	15	AF169167	~78-108
	AHT5	8	NC_009151	~119-139

	HMS6	4	X74635	~154-170
	ASB23	3	MG601746	~195-225
	ASB2	15	X93516	~249-280

To amplify 8 STRs (Table 2) by fluorescent dye UP-mPCR reaction, primers were selected [22-25] (Shanghai Generay Biotech and Beijing SBS Biotech, China) and dissolved in 10 mM Tris-HCl (pH 8.5). The eight pairs of primers (Table 2) were added to the pre-prepared Taq polymerase felt mixture (Taq polymerase 2X: polymerase 0.25 units/µL, nucleotide 0.4 mM, MgCl2 3.6 mM, KCl 50 mM in 20 mM Tris-HCl, pH 8.5). The reaction was carried out by adding an equal amount of the upstream primers to that of the complementary primers (Table 3). The reaction volume was 25 µl. DNA fragments were amplified as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles consisting of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and an 8 min final extension step at 72°C. PCR products from horse gDNA samples were run on ~1.1% agarose gels in 1X TAE medium.

Table 2.

Specific primers for UP-mPCR of 8 STR

STR short name	Position in chromosome	Registration # STR	Left primer sequence (5`-3`)	Concentration of the left primer	Right primer sequence (5`-3`)	Concentration of the right primer	References	Product size(bp)
UMNe156	15	AF536268	ccttccttccttcccccc**agactagcttcaaattgcccc**	0.02 µM	cctatgcttgaaggagtgtg	0.2 µM	[22]	~120-142
HMS6	4	X74635	ccttccttccttcccccc**gaagctgccagtattcaaccattg**	0.02 µM	ctccatcttgtgaagtgtaactca	0.2 µM	[23]	~169-201
HMS2	10	X74631	ccttccttccttcccccc**cttgcagtcgaatgtgtattaaatg**	0.03 µM	gatctctagctcagtaaatcacagg	0.25 µM	[23]	~240-256
UMNEe222	15	AF536300	ccttccttccttcccccc**accaagctatgagtcaggag**	0.03 µM	agcatcttcatgtcctctgc	0.25 µM	[22]	~267-285
COR008	9	AF083451	ccttccttccttcccccc**aggacactgaagggctgaaag**	0.03 µM	tagatagcgtctggagggttc	0.3 µM	[24]	~315-341
UMNe116	11	AY735236	ccttccttccttcccccc**ctggctaaactcttattcc**	0.03 µM	acatgggagaaaatacacac	0.3 µM	[25]	~382-396
HMS1	15	X74630	ccttccttccttcccccc**acttatcagagaggccctcc**	0.04 µM	gtcatcccactctatcagggg	0.35 µM	[23]	~403-427
UMNe191	12	AF536279	ccttccttccttcccccc**ctgtcctcacttggcatgagtcc**	0.04 µM	ccagatggtgaaacaaggggc	0.35 µM	[22]	~403-460

The upstream primer sequence was 5'-CCTTCCTTCCTTCCCCCC-3' and was ligated to the 5' end with FAM fluorescent dye. The fluorescent UP-MPCR reaction mix was prepared by calculating the reaction volume to 25µl. The productivity of each primer was not tested beforehand.

Table 3.

The fluorescent UP-mPCR master mix

2X Taq Polymerase ready mix	12.5 0µl
A mixture of 8 pairs of primers (Left @ right 1:10)	2,0µl
Upstream primer (with fluorescence dye, 10µM)	2,0µl
gDNA (concentration and quality unknown)	5,0 µl
DDW	3,5 µl
	25,0 µl

One µl of each reaction product was mixed with 9 µl of ionizing solution (Zanaaspex Formamide DNA Solvent) and 0.2 µl of size determinant (Zanaaspex, Orange-800). The mixture was kept at 95°C/5 min and placed on ice for 10 min. Capillary electrophoresis was performed on the ABI 3730xl machine using dedicated buffer and semi-liquid gel (Zanaaspex CE 10X buffer) on Fragment Analysis POP-4 36cm mode and analyzed on Genemapper ID-X program.

Results

Experiment A (conventional PCR results)

By conventional PCR, no product was detected (Figure 1a). Also, the mPCR for 15 STRs did not produce sufficient product in any of the samples, and the results could not be analyzed (Figure 1b).

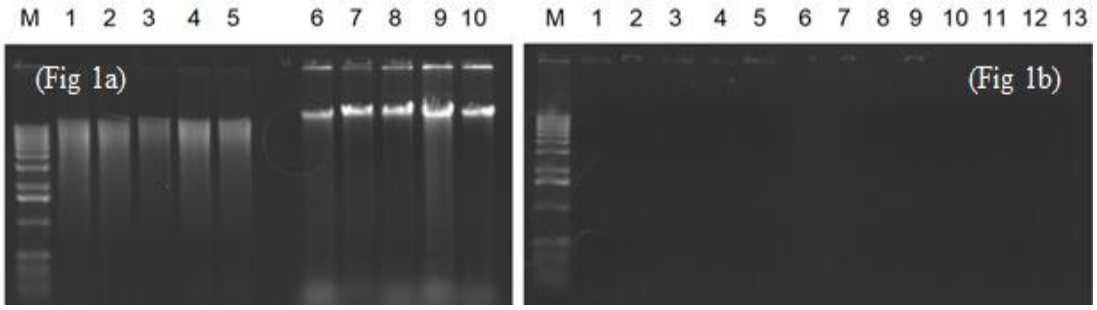

Figure 1a. M- DNA marker; 1-5: DNA of horses; 6-10- Positive control DNA.3 Figure 1b. 1-13: gDNA of Galshar (5), Darkhad (4), Tes (4) horses isolated from coagulated blood.

Figure 1. Conventional PCR result of unqualified DNA samples

The reaction products were read on an ABI 3730xl device and the recorded fragment lengths were analyzed with the Genemapper ID-X software (Figure 2).

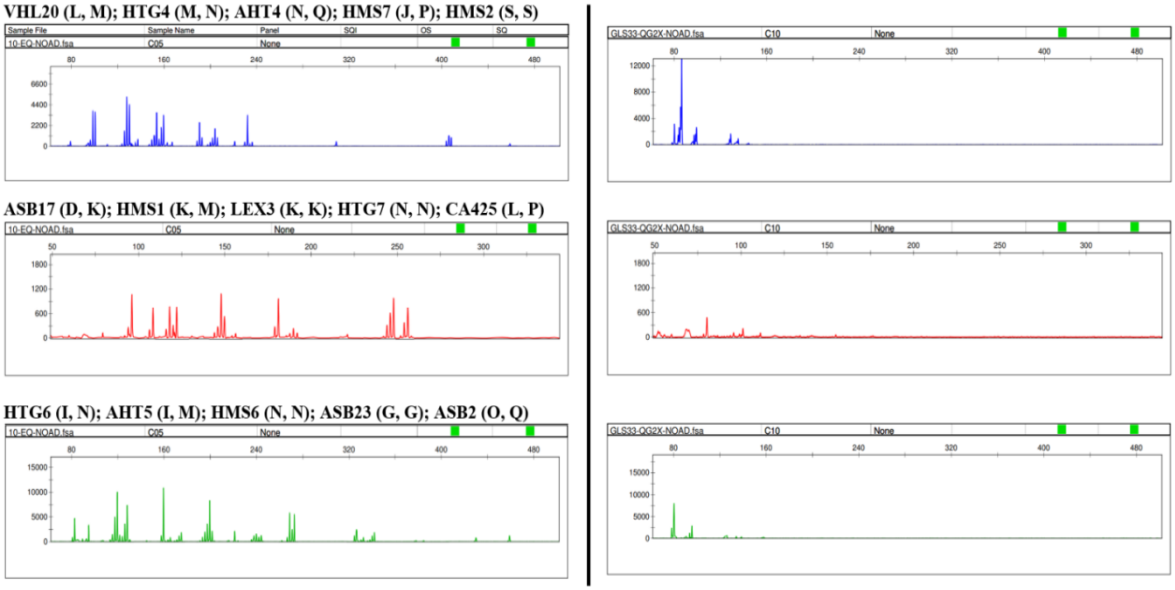

Figure 2. Results of 15 equine STR typing products analyzed by the ABI3730xl DNA genetic analyzer

Figure 2 (left side) showed the amplification of 3 different fluorescent dye products from gDNA isolated from horses, and each of the 15 STRs patterns were clear. In Figure 2 (right side), the results of the reaction on gDNA isolated from clotted blood are insufficient, due to the poor yield and purity of DNA isolated from blood.

Result of experiment B (Up-mPCR results)

Using fragment analysis, 8 horse STR products were successfully amplified from 23 low-quality DNA samples, as well as 4-6 products of Galshir and Tes horse samples. All fragments with a length of 0-665 nucleotides were registered and checked with FAM dye (Figure 3). Reaction products were read on an ABI 3730xl instrument, and recorded FAM-stained fragment lengths were calculated using Genemapper ID-X software.

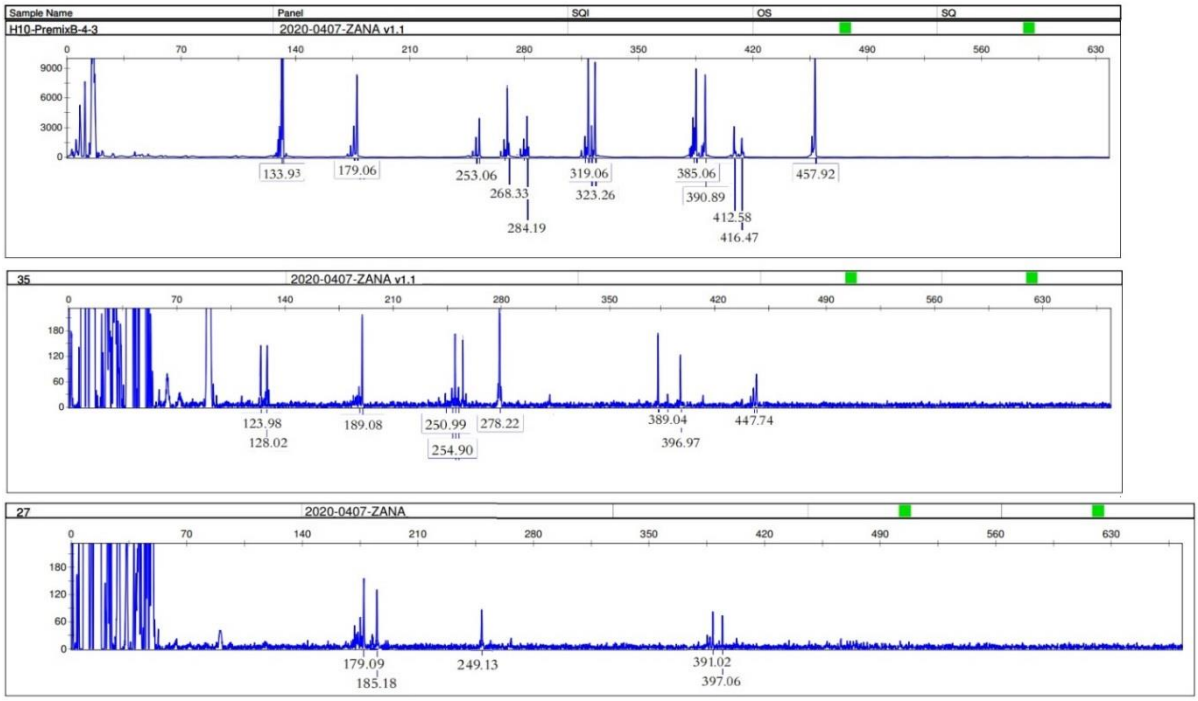

Figure 3a - Control sample; Figure 3b - gDNA product (6 STRs) of Galshir horse (#35); Figure 3c - gDNA product (3 STRs) of a Tes horse (#27).

Figure 3. Results of fluorescent UP-mPCR products analyzed by the ABI3730xl DNA genetic analyzer

HMS6, HMS2, and HMS1 STR loci were determined by purifying the residue of the reaction product from horse #10 (control), cloning, and DNA sequencing to determine the (CA)n double nucleotide repeats of UMNe156, UMNe222, COR008, UMNe116, and UMNe191 STRs. Thus, it was possible to calculate the number of repeats and the product length of the 5 STRs compared to other horses (Table 4). Among all 23 analyzed individuals, the incidence of each of the 3 STRs, HMS6, HMS2 and UMNe116, was completely determined for each type. Among all horses, M type on HMS6, M type on HMS2, and (CA)19 type on UMNe191 were the highest, respectively. The occurrence of the above 3 STRs in all horses, as well as in Galshar, Darkhad and Tes pedigree, could not be statistically confirmed.

Table 4.

Results of fragment analysis and comparative control on gDNA samples from Galshar, Darkhad and Tes pedigree horses (n=23)

Samples*	The length of the product and (the number or pattern of repeats of CA pairs of nucleotides)															
	UMNe156		HMS6		HMS2		UMNe222		COR008		UMNe116		HMS1		UMNe191	
Galshir #1	-	-	183.21 (P)	183.21 (P)	239.09 (I)	247.06 (P)	264.91 (7)	278.19 (14)	-	-	385 (19)	388.99 (21)	-	-	-	-
Galshir #7	-	-	179.15 (N)	181.1 (O)	240.85 (M)	253.01 (S)	268.19 (9)	269.92 (10)	313.02 (19)	313.02 (19)	385.11 (19)	385.11 (19)	416.59 (M)	416.59 (M)	453.88 (9)	456 (10)
Galshir #23	-	-	187.04 (R)	189.15 (S)	238.95 (L)	251.18 (R)	264.05 (7)	270.33 (10)	-	-	383.1 (18)	390.99 (22)	-	-	447.82 (6)	457.95 (11)
Galshir #29	-	-	173 (K)	181.02 (O)	231.22 (H)	240.96 (M)	266.21 (8)	278.2 (14)	-	-	380.94 (17)	382.89 (18)	-	-	447.87 (6)	449.99 (7)
Galshir #33	-	-	173.1 (K)	177.12 (M)	233.04 (I)	253.06 (J)	270.22 (10)	278.07 (14)	-	-	385.08 (19)	391.12 (22)	-	-	457.79 (11)	463.97 (14)
Galshir #35	123.98(9)	123.98(9)	189.08 (S)	189.08 (S)	250.99 (R)	254.9 (T)	278.22 (14)	278.22 (14)	-	-	389.04 (21)	396.97 (25)	-	-	447.74 (6)	447.74 (6)
Galshir #39	-	-	179.16 (N)	181 (O)	238.89 (L)	253.06 (S)	278.19 (14)	278.19 (14)	-	-	397.02 (25)	397.02 (25)	410.6 (J)	416.53 (M)	-	-
Galshir #46	133.98(14)	135.92(15)	177.01 (M)	177.01 (M)	238.98 (L)	240.89 (M)	268.16 (9)	276.25 (13)	-	-	383.02 (18)	385.07 (19)	-	-	-	-
Galshir #49	-	-	184.94 (Q)	184.94 (Q)	235 (J)	249.79 (T)	-	-	-	-	387.08 (20)	389.1 (21)	-	-	-	-
Darkhad #2	-	-	175.11 (L)	185.01 (Q)	233.3 (I)	255.07 (T)	270.04 (10)	270.04 (10)	-	-	383.05 (18)	389.04 (21)	-	-	-	-
Darkhad #3	-	-	177.18 (M)	179.24 (N)	237.01 (K)	241.04 (M)	278.1 (14)	282.18 (16)	-	-	385.21 (19)	389.11 (21)	-	-	455.92 (10)	464.02 (14)
Darkhad #5	-	-	175.03 (L)	177.02 (M)	241.1. (M)	251.04 (R)	266.5 (8)	278.3 (14)	317.1 (21)	317.1 (21)	385.08 (19)	390.85 (22)	-	-	-	-
Darkhad #16	-	-	173.17 (K)	180.99 (O)	241.02 (M)	249.1 (Q)	274.41 (12)	282.28 (16)	-	-	385.24 (19)	385.24 (19)	408.49 (I)	410.39 (J)	456.01 (10)	458.11 (11)
Darkhad #22	-	-	174.97 (L)	184.94 (Q)	234.89 (J)	234.89 (J)	278.12 (14)	278.12 (14)	-	-	389.08 (21)	389.08 (21)	-	-	456.08 (10)	456.08 (10)
Darkhad #24	131.86(13)	131.86(13)	173.09 (K)	177.03 (M)	241.13 (M)	251.14 (R)	-	-	-	-	382.96 (18)	390.96 (22)	-	-	-	-
Darkhad #26	-	-	179.1 (N)	180.97 (O)	241.16 (M)	253.12 (S)	282.22 (16)	282.22 (16)	-	-	394 .98 (24)	397.02 (25)	-	-	447.9 (6)	452.1 (8)
Darkhad #48	-	-	175.01 (L)	175.01 (L)	241.08 (M)	239.03 (L)	270.19 (10)	282.31 (16)	-	-	379.41 (16)	391.04 (22)	-	-	-	-
Tes #14	-	-	177.09 (M)	179.15 (N)	241.1 (M)	241.1 (M)	278.09 (14)	282.3 (16)	-	-	385.14 (19)	385.14 (19)	-	-	-	-
Tes #16	-	-	177.22 (M)	177.22 (M)	231.11 (H)	231.11 (H)	268.2 (9)	276.1 (13)	-	-	391.03 (22)	397.22 (25)	-	-	451.96 (8)	463.91 (14)
Tes #20	123.97(9)	133.82(14)	180.98 (O)	184.97 (Q)	240.94 (M)	247 (P)	-	-	-	-	383 (18)	385.05 (19)	416.63 (M)	416.63 (M)	451.89 (8)	460.04 (12)
Tes #25	-	-	175.21 (L)	177.15 (M)	237.08 (K)	237.08 (K)	278.29 (14)	282.15 (16)	-	-	396.98 (25)	396.98 (25)	-	-	458 (11)	458 (11)
Tes #27	-	-	179.09 (N)	185.18 (Q)	249.13 (Q)	249.13 (Q)	-	-	-	-	391.02 (22)	397.06 (25)	-	-	-	-
Tes #28	-	-	173.14 (K)	177.02 (M)	237.11 (M)	241.02 (M)	278.2 (14)	278.2 (14)	-	-	387.15 (20)	397.08 (25)	-	-	-	-
Positive sample	133.93(14)	133.93(14)	179.06 (N)	179.06 (N)	253.06 (S)	253.06 (S)	268.33 (9)	284.19 (17)	319.06 (22)	323.26 (24)	385.06 (19)	390.89 (22)	412.58 (K)	416.47 (M)	457.92 (11)	457.92 (11)
Samples*-Unqualified DNA samples																

The HMS6, HMS2 and HMS1 STR lengths previously identified in the control samples were compared to other horse STRs, and their patterns were determined and lettered according to ISAG procedures. For other STRs, the sequence of 5 (CA)n pairs of nucleotides in the control sample was determined by comparing the sequence of the other samples. For example, in Galshar #1 horse, the STR named UMNe222 had 7 and 14 repeated (CA)n pairs of nucleotide patterns (inherited from father and mother), and UP-mPCR reaction shows 264.91 and 278.19 pairs. It can be seen from the table (4,5) that fragments with the length of nucleotides were amplified simultaneously.

Table 5.

STR patterns of 23 horses analyzed

HMS6

Types	(CA) repetition #	Occurrences
K	13	0.1087
L	14	0.1304
M	15	0.2391
N	16	0.1304
O	17	0.1304
P	18	0.0435
Q	19	0.1304
R	20	0.0217
S	21	0.0652

UMNe116

Types	(CA) repetition #	Occurrences
379	16	0.02173913
381	17	0.02173913
383	18	0.130434783
385	19	0.260869565
387	20	0.043478261
389	21	0.152173913
391	22	0.152173913
395	24	0.02173913
397	25	0.195652174

NMS2

Types	(CA)repetition #	Occurrences
H	15	0.0652
I	16	0.0652
J	17	0.0870
K	18	0.0870
L	19	0.0870
M	20	0.2826
P	21	0.0435
Q	22	0.0652
R	23	0.0870
S	24	0.0652
T	25	0.0652

Considering a total of 23 horses as a group, there were 9 types of HMS6, 10 types of HMS2, and 9 types of UMNe116. M on HMS6, M on HMS2, and (CA)19 on UMNe116 had the highest occurrences, respectively.

Discussion

Genetic experiments are rare in agricultural studies conducted in Mongolia. At the very least, there is a great need for genetic research and DNA analysis data on horse origins, breeds and pedigrees. Studies show that methods for confirming and recording predictions of unspecified breeds, pedigrees, and breeding areas will continue to be STR-based in the future [6,8,9,10,11]. Agricultural specialists and researchers need to study the occurrence and distribution of STR patterns in horse and other animal genealogy data across the country. Furthermore, DNA-based testing will be essential in forensics, food safety, and product traceability systems.

Because UP-mPCR or mPCR amplification products with paired primers are characterized by the fact that one of the unique primer pairs has the same additional tail at the 5' end, mPCR can be used to determine the species and origin of animal meat [10-13], food-borne pathogenic bacteria widely used in detection [14-16].

In recent years, the genetic modification of vegetables has been analyzed [17-19] and combined with real-time PCR and pyrosequencing [20-22]. When using mPCR with a unique pair of primers for each amplification fragment, increasing the sample and primer concentration to increase the reaction efficiency on low-concentration, low-quality DNA samples has been shown to improve results, as well as to produce more by-products [21-23]. In the present study, due to the impossibility of immediate analysis, there was a need to work on low quality and over-coagulated samples that had been transported long-distance and preserved long term.

According to the guidelines of the International Society of Animal Genetics (ISAG), this study revealed 9 patterns of HMS6, 10 patterns of HMS2, and 9 patterns of UMNe116 from STRs in Galshir, Tes, and Darkhad horses. It was determined that M on HMS6, M on HMS2, and (CA)19 on UMNe116 had the highest incidence, respectively. In the future, it is possible to make the results of the analysis unquestionable if the allelic ladder is prepared and used in parallel to carry out extensive research.

The smaller the STR repeat unit, the more likely it is to change in length during reaction amplification, which can make testing and analysis difficult. Therefore, double-nucleotide repeats can be converted to 4- or 5-nucleotide repeat variants, similar to human assays. For this to be done, in the near future it is necessary to identify STRs with 4-5 nucleotide units from animal genealogy data using bioinformatics methods.

During the experiment, the primers were reacted with 5 times the normal amount of DNA because DNA yield and purification not enough compared to the DNA sample with a hypothetically large amount. A control DNA sample was calculated to be 100 ng. By estimating the amount of signal recorded, we were able to find that the amount of unused dye-binding primers is ∼5 times greater than the total number of successfully amplified products. This indicates that the use of upstream primers is very low and further improvement of the reaction is required.

The purpose of this study was to perform UP-mPCR with primers attached to fluorescent dyes on low-quality DNA samples extracted from long-degraded blood. This methodology allowed us to test the suitability of combining the products by capillary electrophoresis and to check the quality of domestic diagnostic products. The aim of this study, which worked on horse DNA, was achieved by using a combination of reagents produced by Zanaaspex. These innovative products, such as PCR and DNA isolation buffers and dye-based primers, are of practical importance.

Fragments UMNe146, COR008 and HMS1 from poor quality DNA samples were not successfully amplified. However, by adjusting the content of each pair of primers and changing the reaction recipe and the optimization conditions, it is possible to amplify 8 fragments equally. Depending on the length of the amplification product that is designed, a small number (4-7) of fragments of 100-600 nucleotides in length can be attached to a single-dyed primer to stabilize the reaction.

It is important to standardize the foreign nomenclature related to the methodology of this article in Mongolian, but due to the lack of common understanding between experts in the field and the difficulty in exchanging information, it is reflected in common terms and foreign nomenclature.

Conclusion

The results of this experiment on equine DNA showed that a small number of STR marker tests can be tested and put into practice, rather than using the time-consuming, expensive, multi-dye multiplex reaction..

This experiment is believed to be the first innovative piece of work in which the UP-mPCR reaction is coupled with a capillary electrophoresis analysis by dyeing primers. In the future, it is possible to further develop this technique by adjusting the reaction conditions and the concentration of primers, and to conduct the same experiments and studies on other animal species.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

BM, NN, EZ, SL designed and performed the study; EZ supervised the experiment, BM, NN, SL analyzed data; all authors discussed the results and contributed to the final manuscript.

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