

Original Research

Evaluation of Polylocus Spectra of *DNA* Fragments of the *Genus Eguus* Using *ISSR-PCR* Markers

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Abstract

Characterization of the non-coding part of the transcriptome is a supplement to the annotation of the genome of living organisms. According to *ISSR*-fingerprinting data, information is obtained about ancient gene pools, modern gene pools, genetic variability and conservation of breeds and species of farm animals. The aim of the study is to study the molecular genetic component and phylogenetic relationships in the aboriginal *Hutsul horse* breed and fossil remains of ancient horses using *ISSR-PCR* markers. The polymorphism of amplified *DNA* fragments isolated from the blood of *Hutsul horses* and fossil bones of *ancient equids*, flanked by nucleotide repeats ((*ACC*)₆G, (*CTC*)₆C, (*GAG*)₆C, (*GA*)₆CC, (*AG*)₈CG, (*AG*)₈CA, (*GA*)₉C and (*AG*)₉C), was investigated using the *ISSR-PCR* labeling method. Genotyping of 30 *Hutsul horses* revealed 54 loci, 33 of which are polymorphic. The average value of the index, which shows the level of informativeness of the locus, was 0.31. Fragments with a size of 380-400, 500-520 and 600-630 nucleotide pairs are species-specific for the *genus Eguus*, amplicons with a size of 340-350 nucleotide pairs of primer (*GA*)₉C and 470-490 primers (*GA*)₉C and (*GA*)₆CC were found only in fossil remains. The closest genetic distances were between the



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true tarpan and the *ancient domestic horse* - 0.0234; between the *Pleistocene horse* and the *ancient domestic horse* - 0.0385; the most distant were between the *Hutsul horses* and the *true tarpan* - 0.5057. Using *ISSR*-markers, a moderate level of genetic diversity of *Hutsul horses* was established. As a result of the research, unique spectra were found for the *genus Equus* and for *ancient equids*. Genetic distances were calculated to reflect the genetic relationships between the studied horses and fossil remains. The closest were the *tarpan* and the *ancient domestic horse*.

Keywords

Genome; non-coding *DNA* sequences; *ISSR-PCR* markers; fossil bones of the *genus Equus*; polymorphic and conservative amplicons; genetic distances; *Hutsul horse* breed

1. Introduction

For decades, researchers have focused more attention on protein-coding genes. With the accumulation of data on mammalian transcriptomes, the focus of research is now shifting to non-coding *DNA* sequences, *RNA*-coding genes, and their transcripts. Many non-coding transcribed sequences have been shown to have important regulatory roles, but the functions of most remain unknown [1].

The proportion of protein-coding *DNA* in the genome decreases with increasing complexity of the organism. In bacteria, about 90% of the genome encodes proteins. This number decreases to 68% in yeast, 23-24% in nematodes, and 1.5-2% in mammals. Without characterizing the noncoding portion of the transcriptome, no annotation of the genome of a living organism can be complete [2, 3].

Thus, on the example of studying the variability of the genome of horses, along with protein markers in the molecular genetic monitoring of these animals, *DNA* markers are becoming increasingly common. Markers created on the basis of polymerase chain reaction (*PCR*) are combined into two main classes: markers created on the basis of small changes to the nucleotide sequence of *DNA* of unique loci and markers based on changes in the number of repeats of tandem *DNA* sequences. Multilocus *DNA* markers are best suited for establishing the level of polymorphism of the genome as a whole and for differentiating animal breeds. A simpler and more effective method of polylocus genotyping is the use of fragments of microsatellite loci as *PCR* primers, with their help it is possible to assess the polymorphism of dozens of *DNA* fragments flanked by inverted repeats.

Using *ISSR-PCR* markers, it is possible to obtain polylocus spectra of *DNA* fragments, the polymorphism of which reliably distinguishes one breed of horse from another. *ISSR-PCR* markers are now widely used for taxonomic and phylogenetic comparison and as a means of mapping a wide range of organisms. Compared with other methods of multilocus profiling, it is characterized by better reproducibility. Genomic fingerprints of a number of mammals, birds, fish, reptiles and plants have been obtained using *ISSR-PCR* markers [4, 5].

The aim of our work was to use *ISSR* markers to study intraspecific genetic polymorphism of the aboriginal *Hutsul horse* breed and to study the molecular genetic component in the fossil remains

of ancient representatives of the *genus Eguus*, which allows us to determine the corresponding phylogenetic relationships based on the analysis of amplified *DNA* fragments.

The Hutsul breed of horses was formed over 400 years ago in the vast mountainous part of the Ukrainian Carpathians - the Hutsul region. The Hutsul breed is a local mountain breed of riding and pack horses. The population of these horses was formed over the centuries, arriving with people in ancient times (the beginning of the 5th-9th centuries), and therefore researchers of this breed interpret it differently and do not have a common opinion regarding the origin and formation of its features [6]. The formation of the Hutsul breed was influenced by local working horses, and later by horses of the Arabian breed. During the time of the Austro-Hungarian monarchy, whose power extended to the area where the *Hutsul horses* were found, the *Hutsul horse* breed underwent systematic and purposeful selection for use in cavalry. After the First World War and the collapse of the Austro-Hungarian Monarchy, the center of breeding *Hutsul horses* moved to Romania, Czechoslovakia, and Poland.

This little horse is very hardy, resistant to disease, and able to live outdoors on pasture all year round. *Hutsul horses* are short, the average height at the withers for stallions is 139-145 cm, for mares – 137-142 cm. The croup of the horses is well developed, the chest is deep and wide, the head is expressive with large eyes and small mobile ears. They have a very strong back, a muscular neck. The hooves are small and very hard and do not require horseshoes. The color of the majority is bay with a dark belt along the back, but can also be brindle, black, red, gray, mouse-colored. A characteristic feature of the breed is a stripe along the spine and stripes on the legs. In recent years, the use of *Hutsul horses* for recreation and health improvement has become more active in the Carpathian region of Ukraine, aimed at using horses in the medical, biological, processing industries, in cinematography, as well as traditions and rituals [7, 8].

2. Materials and Methods

2.1 Data Retrieval

The research was conducted on the aboriginal Hutsul breed of horses, which are bred in the Carpathians (National Nature Park "Hutsulshchyna" and in private farms) (Figure 1) and on the fossil remains of ancient representatives of the *genus Eguus* (Figure 2).



Hutsul Horse Exhibition, Verkhovynske City

Figure 1 Hutsul horses.



Figure 2 Paleontological findings of fossil remains of horses: **a** – tarsicentral bone (os. *Tarsicentral*) of a *Pleistocene* horse; **b** – *Pleistocene* horse bone found in Novgorod-Siverskyi; **c** – phalanx of a limb of a *true tarpan*; **d** –tooth of a *true tarpan* horse; **e** –tooth of an *ancient domestic horse*; **f** – fragment of a vertebra of an *ancient domestic horse*.

The study was conducted on samples of fossil horse bones from the *Pleistocene* period (about 10 thousand BC), which were found in Ukraine. One bone was found in the village of Buki, Zhytomyr region, during construction work in a quarry in 1960. Another bone was found in Novgorod-Siverskyi, Chernihiv region. Excavations were carried out in 1935. To study the wild *horse tarpan* (7480-7170 thousand BC), a tooth and a phalanx of a limb were used, found in the village of Skybnitsa, Trostyanets district, Vinnytsia region in 1959 by paleontologist V. Danylenko. For the study, the paleontological material was provided by the Kyiv National Museum of Natural Sciences of the National Academy of Sciences of Ukraine, Department of Paleontology.

Studies of the *ancient domestic horse* were carried out on paleontological finds also found in Ukraine: a tooth of a *domestic horse* (Cornea Pottery culture 3-2 millennium BC, Semeniv village, Zdobuniv district, Rivne region). Excavations were carried out by the Lviv Historical Museum, 1958; a fragment of a horse vertebra, Scythian period. VII-III centuries BC, Sukhostav village, Husyatyn district, Ternopil region (Lviv Historical Museum 1952).

2.2 Molecular-Genetic Studies

To analyze the variability of intermicrosatellite *DNA* fragments located between two inverted repeats of the horse genome, the *ISSR-PCR* marker method was used. Genotyping of the studied objects was carried out using 8 *ISSR* primers – $(GA)_9C$, $(AG)_9C$, $(AG)_8CA$, $(AG)_8CG$, $(GA)_6CC$, $(ACC)_6G$, $(GAG)_6C$ and $(CTC)_6C$. To more accurately determine the molecular weight of the detected *DNA* fragments, we used a universal scale. An appropriate molecular weight gradation was applied on the scale. Depending on the zone, a certain step from 10 to 100 nucleotide pairs was used. Thus, 37 zones with a fixed interval were taken into account, which allows for fairly accurate determination of the molecular weight for 37 *PCR*-products. Using a single universal scale allows us to identify patterns in the distribution of fragments and conduct a comparative study of breed and interspecific differences.

To study *DNA* polymorphisms of different groups of horses using *ISSR*-primers, selected amplification temperatures when performing *PCR* (Table 1).

Table 1 Inter-Simple Sequence Repeats used in the work.

No	Primer sequence	Motifs	Annealing temperature
1	5'-ACCACCACCACCACCG-3'	$(ACC)_6G$	64°C
2	5'-CTCCTCCTCCTCCTCC-3'	$(CTC)_6C$	64°C
3	5'-GAGGAGGAGGAGGAGGC-3'	$(GAG)_6C$	64°C
4	5'-GAGAGAGAGAGACC-3'	$(GA)_6CC$	54°C
5	5'-AGAGAGAGAGAGAGCG-3'	$(AG)_8CG$	56°C
6	5'-AGAGAGAGAGAGAGCA-3'	$(AG)_8CA$	54°C
7	5'-GAGAGAGAGAGAGAGA-3'	$(GA)_9C$	56°C
8	5'-AGAGAGAGAGAGAGAGC-3'	$(AG)_9C$	57°C

PCR was performed on an amplifier «Applied Biosystems» (USA), using a set of reagents for *PCR* amplification «Fermentas» (Lithuania) [9].

Electrophoretic analysis of the amplification products was performed using a 1.5% agarose gel in 1xTBE buffer with the addition of ethidium bromide to a final concentration of 0.5 µg/ml with the following parameters: current strength 100 mA, voltage 100 V for 1.5 h. An ultraviolet source was used for visualization.

Each spectrum amplicon was considered as one *DNA*-locus. The polymorphism of such a locus (P) was assessed by the presence or absence of an amplicon of the corresponding length in the spectra. The calculation of the Polymorphic Information Content (PIC) index was performed according to the formula for diallelic loci, for which $PIC = 2f(1-f)$, where f is the frequency of one of the two alleles, which is calculated as $f = \sqrt{R}$, where R is the frequency of variants that lack a *DNA* fragment of the corresponding length (homozygote for the recessive allele), since *ISSR* markers are characterized by a dominant type of manifestation. Determination of the polymorphism of loci according to the Polymorphic Information Content (*PIC*) index: loci with a *PIC* value > 0.500 are highly polymorphic; *PIC* within 0.250-0.500 are moderately polymorphic; *PIC* < 0.250 are low polymorphic [10, 11].

To establish the parameters of genetic diversity of *Hutsul* horses, the proportion of polymorphic loci - P , the average per locus genetic diversity - H_s , the Shannon information index - H' , the intrapopulation diversity index - μ , and the proportion of rare loci - h_μ were calculated.

The proportion of polymorphic loci (P) is the number of polymorphic loci divided by the total number of loci (polymorphic and monomorphic).

$$P = n_{pj}/n_{total}$$

where, P = proportion of polymorphic loci; n_{pj} = number of polymorphic loci; n_{total} = total number of loci.

The average genetic diversity per locus was calculated using the formula:

$$H_e = 1 - \sum_{i=1}^n p_i^2$$

where $p_1, p_2, \dots, p_n$ are allele frequencies.

The Shannon-Wiener index for quantifying allelic diversity was calculated using the formula:

$$H' = - \sum_{i=1}^n p_i \times \ln p_i$$

where $p_1, p_2, \dots, p_n$ are allele frequencies.

Calculations of intrapopulation diversity indicators – the average number of morphs/alleles (μ) and the proportion of rare morphs/alleles (h_μ) and their statistical errors were performed using the Microsoft Excel software package according to the following formulas:

$$\mu = (\sqrt{p_1} + \sqrt{p_2} + \sqrt{p_m})^2$$

$$h_\mu = 1 - \mu/m$$

where p is the proportion of polymorphic alleles; m is the number of polymorphic loci.

Statistical analysis was performed using the Statistica 6.0 software package and Excel (Microsoft Office 2007). Statistical data processing was performed in the standard Microsoft Excel package using the integrated StatistixL 2.0 program add-in (<http://www.statistixl.com/>) [12].

2.3 DNA Extraction from Bonetissue

DNA extraction from bone tissue is a lengthy procedure that includes various stages, namely: sample preparation, material decalcification, and DNA purification procedure. The process of DNA extraction from bone tissue was carried out according to the author's method [13].

Construction of dendrograms of genetic distances between horses of different species and breeds using the M. Ney method, carried out using the TreeView X program. Version 0.5.0 <https://treeview-x.en.softonic.com> [14].

2.4 Bioethical Norms

The work was carried out in compliance with the provisions of national and international standards for conducting scientific research using animals: Order of the Ministry of Education and Science, Youth and Sports of Ukraine No. 249 dated March 1, 2012 "On approval of the procedure

for conducting research and experiments on animals by scientific institutions"; WHO Handbook: good laboratory practice (GLP): quality practices for regulated non-clinical research and development (2009); Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

3. Results

3.1 Molecular Genetic Studies of Hutsul Horses

The *ISSR-PCR* labeling method was used to study the polymorphism of *DNA*-fragments flanked by inverted tandem repeats ((*ACC*)₆G, (*CTC*)₆C, (*GAG*)₆C, (*GA*)₆CC, (*AG*)₈CG, (*AG*)₈CA, (*GA*)₉C and (*AG*)₉C) in the genome of *Hutsul horses*. Based on the genotyping of 30 *Hutsul horses*, 54 loci were found, 33 of which are polymorphic. Analysis of the *PIC*-index values, which shows the level of information content of the locus, revealed that the studied *Hutsul* breed individuals according to the amplicon spectrum using the primers (*GA*)₉C, (*AG*)₉C, (*GA*)₆CC, (*ACC*)₆G, (*GAG*)₆C, (*CTC*)₆C, were characterized by moderate polymorphism with a *PIC* value in the range of 0.29-0.41. In the tested animals, the spectrum of amplicons when using the primer (*AG*)₈CA was characterized by a high level of informativeness with a *PIC* value of 0.60. Only when using one primer (*AG*)₈CG, the spectrum of the resulting amplicons was completely conservative (Table 2).

Table 2 Parameters of the used *ISSR*-markers (*Hutsul* breed horses, n = 30).

Primer	(GA) ₉ C	(AG) ₉ C	(AG) ₈ CA	(AG) ₈ CG	(GA) ₆ CC	(ACC) ₆ G	(GAG) ₆ C	(CTC) ₆ C	Σ
Number of loci	8	5	9	6	8	5	9	4	54
Length limits	280-930	320-1050	260-1230	300-870	380-1750	380-1050	280-870	680-1750	260-1750
Polymorphic loci	6	4	6	0	4	4	6	3	33
PIC	0.32	0.30	0.60	0	0.41	0.29	0.31	0.29	0.31
P, %	75	80	67	0	50	80	67	75	61.7

The spectra of *DNA* amplification fragments of the *Hutsul horse* breed using 8 *ISSR*-systems based on the following microsatellites: $(GAG)_6C$, $(ACC)_6G$, $(CTC)_6C$, $(GA)_9C$, $(GA)_6CC$, $(AG)_8CA$, $(AG)_8CG$, and $(AG)_9C$ are shown in Figure 3.

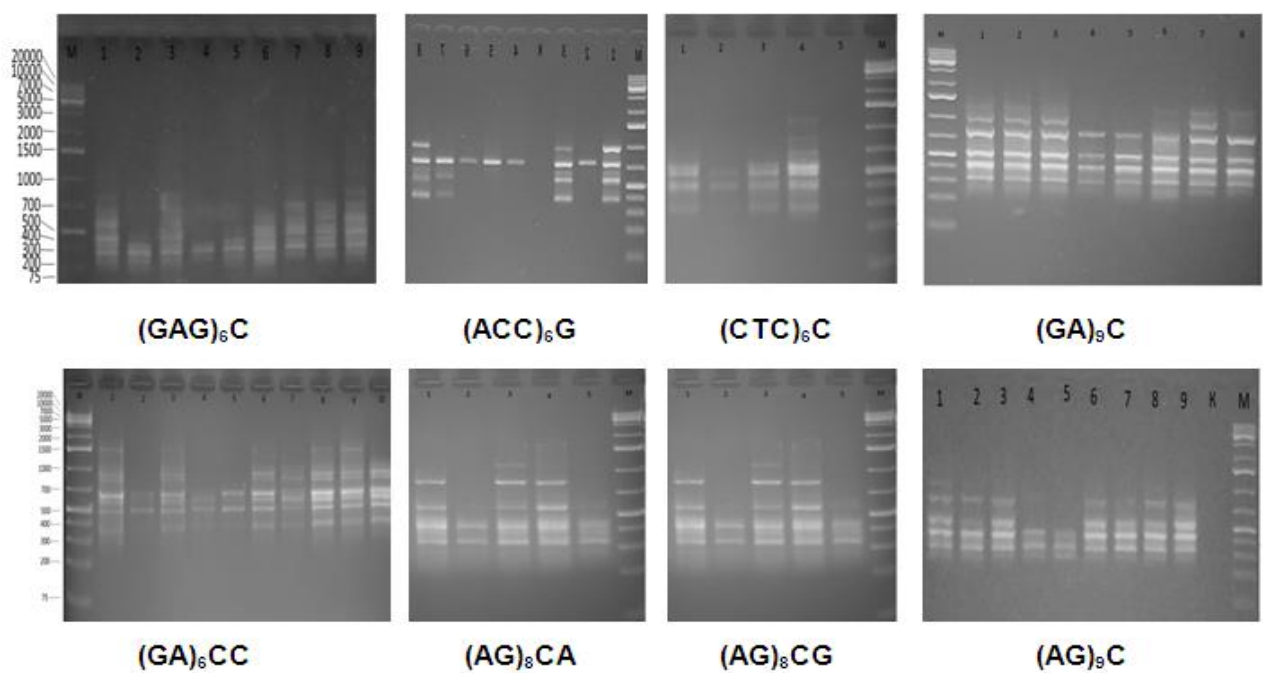


Figure 3 Electrophoregram in 1.5% agarose gel of amplification products of *DNA* samples of *Hutsul horses* by *ISSR-PCR*. M-marker of molecular masses (Gene Ruler™ 1 kb Plus *DNA* Ladder, Fisher Scientific).

Analyzing the parameters of genetic diversity according to *ISSR*- markers of the studied *Hutsul horses*, we conclude that the tested group of animals is relatively prosperous in terms of the detected intrapopulation genetic diversity (Table 3) [15].

Table 3 Main indicators of genetic diversity of *Hutsul horses*.

Breed	P	H _s	H'	μ	h _μ
Hutsul	0.617	0.603	0.311	25.9 ± 3.9	0.20 ± 0.12

Note: P – proportion of polymorphic loci, H_s – average per locus genetic diversity, H' – Shannon information index, μ – index of intrapopulation diversity, h_μ – proportion of rare loci.

3.2 Differentiation of Gene Pools of Different Horse Species Using *ISSR*-Markers

To address the issue of differentiation of gene pools of different horse species using *ISSR*-markers, the spectra of amplification products with 100% expression in the studied animals and fossil remains of bones of ancient horse species were taken into account (Figure 4, Figure 5, Figure 6).

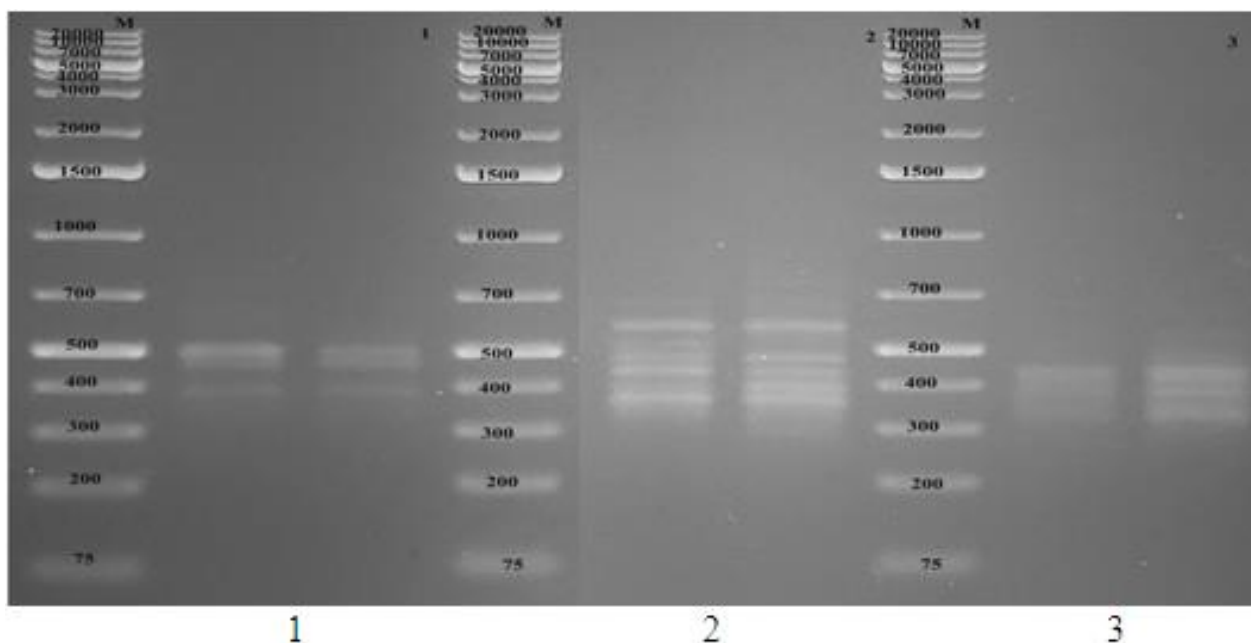


Figure 4 Spectra of DNA amplification fragments of fossil bones of a *Pleistocene* horse with primers: 1 - $(GA)_9C$; 2 - $(GAG)_6C$; 3 - $(AG)_9C$.

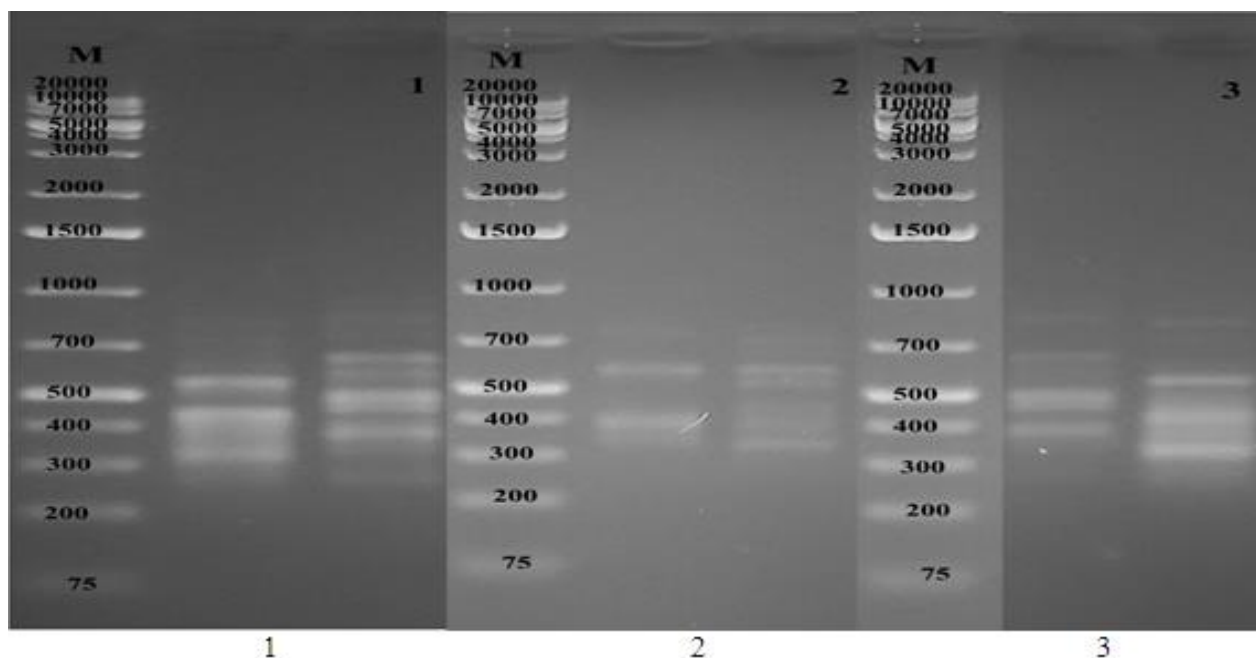


Figure 5 Spectra of DNA amplification fragments of fossil bones of a true tarpan with primers: 1 - $(GA)_9C$; 2 - $(GAG)_6C$; 3 - $(AG)_9C$.

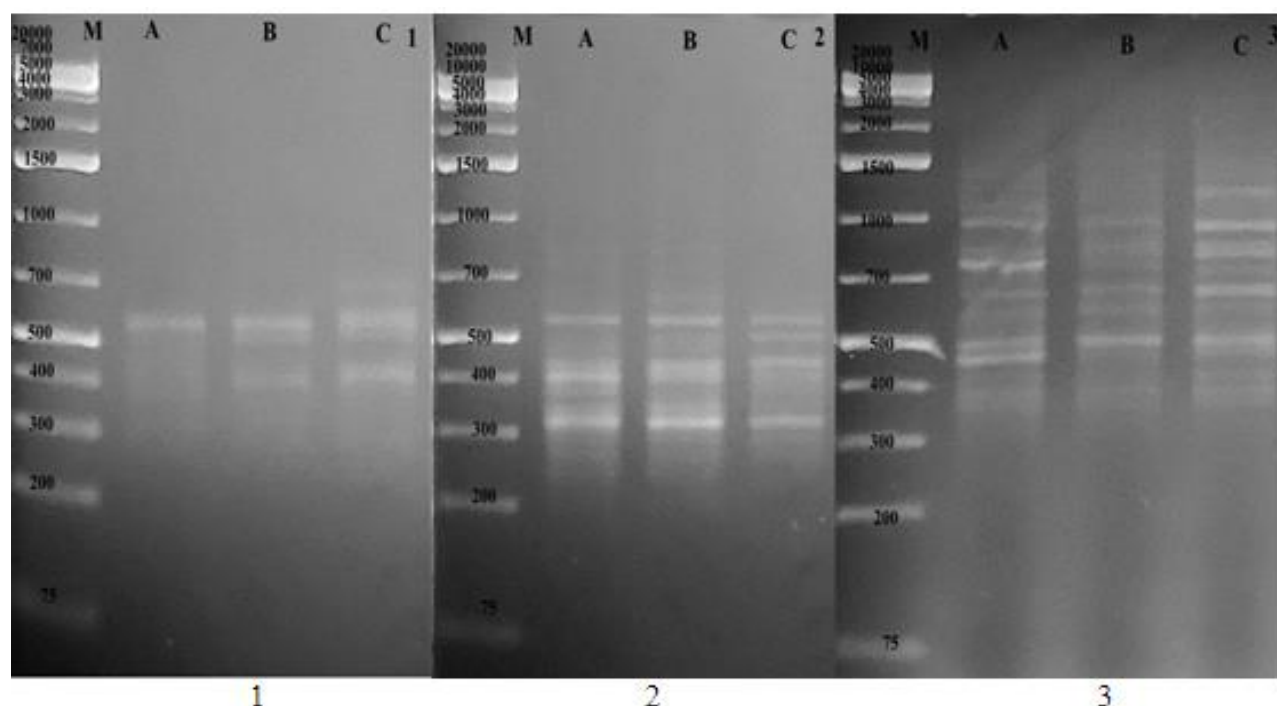


Figure 6 Spectra of DNA amplification fragments of fossil bones of *Pleistocene horse* and *true tarpan* with primers: 1 - $(AG)_8CA$; 2 - $(AG)_8CG$; 3 - $(GA)_6CC$. A, B – *Pleistocene horse*; C – *true tarpan*.

The obtained spectra of DNA amplification products of the *Hutsul horse* breed and the spectra of DNA amplification products from the fossil remains of the *Pleistocene horse*, the *true tarpan* and the *ancient domestic horse* belonged to the “light” (A-25 – A-35) and “medium” (A-13 – A-25) groups on the scale of the dimension of ISSR-fragments in nucleotide pairs, which can be attributed to the specific characteristics of the *genus Equus* (Figure 7). The smallest number of 27 fragments (DNA loci) obtained using 8 primers $(GA)_9C$, $(AG)_9C$, $(AG)_8CA$, $(AG)_8CG$, $(GA)_6CC$, $(ACC)_6G$, $(GAG)_6C$ and $(CTC)_6C$, the frequency of which in the studied animals was 1000, (i.e. conservative loci) was in *Hutsul horses*. The spectra of the amplification products of the fossil remains of the *true tarpan* and the *ancient domestic horse* were 46-47 fragments, and in the *Pleistocene horse* - 36 fragments. These data confirm the moderate genetic diversity inherent in the *Hutsul* breed of horses, compared to other studied horses.

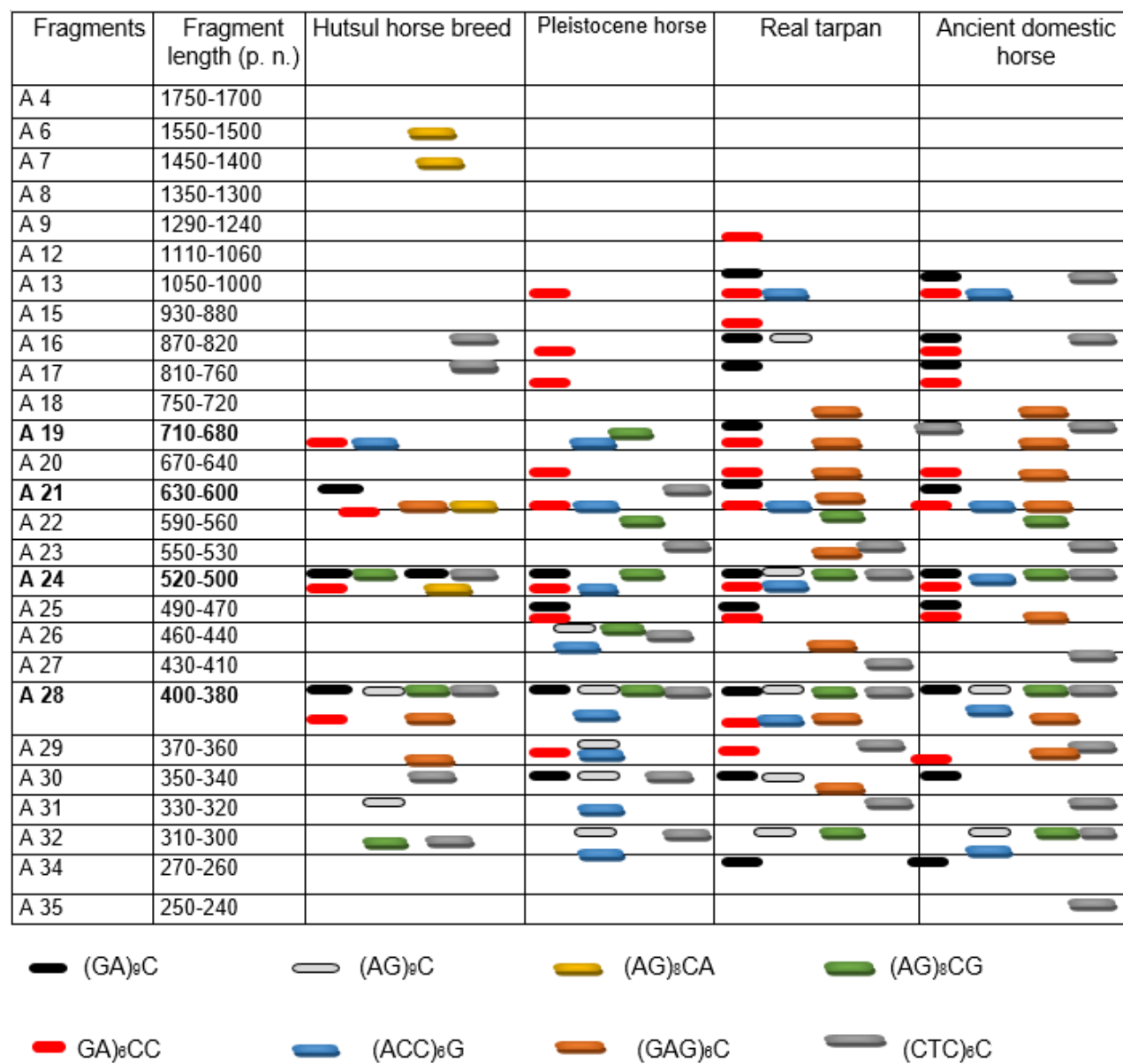


Figure 7 Interspecific differences between *Hutsul horses* and fossil remains of *Pleistocene horses*, true tarpans, and *ancient domestic horses* in the spectra of *PCR* products (frequency = 1.0) obtained by the *ISSR-PCR* method.

Fragments of 380-400 and 500-520 nucleotide pairs were present in the spectra of amplification products of all studied representatives of the *genus Eguus*, at least for 4 primers. Therefore, these fragments are species-specific spectra of *PCR*-products of the studied breed and fossil remains, obtained by the *ISSR-PCR* method.

Loci of 600-630 nucleotide pairs appeared in all studied samples of amplification products at least for 3 primers, which can be considered species-specific for the *genus Eguus*. Amplicons with a size of 340-350 nucleotide pairs of primer (GA)₉C and 470-490 nucleotide pairs of primers (GA)₉C and (GA)₆CC were identified only in the fossil remains of the *Pleistocene horse*, the *true tarpan*, and the *ancient domestic horse*.

As a result of the work, it was determined that DNA molecule amplicons with a size of 340-350 bp of the primer (GA)₉C and 470-490 bp of the primers (GA)₉C and (GA)₆CC were found only in the fossil remains of the *Pleistocene horse*, the *true tarpan*, and the *ancient domestic horse*.

3.3 Genetic Relationships between Studied Horses and Fossil Remains of Ancient Equids

To reflect the genetic relationships between the studied horses and fossil remains of *ancient equids*, pairwise genetic distances were calculated based on the presence of DNA-amplicons of the appropriate length (nucleotide pairs) for (GA)₉C, (AG)₉C, (AG)₈CA, (AG)₈CG, (GA)₆CC and (ACC)₆G, obtained by the *ISSR-PCR* method [16]. Only DNA spectra of intermicrosatellite repeats, the frequency of which was 1.0, were analyzed (Table 4).

Table 4 Genetic distances between *Hutsul* horses and fossil bones of the *Pleistocene horse*, *true tarpan*, and *ancient domestic horse* using the average distance method based on *ISSR* analysis data.

Species/breed	Hutsul	Pleistocene horse	Real tarpan	Ancient domestic horse
Hutsul	****			
Pleistocene horse	0.2623 ± 0.2160	****		
Real tarpan	0.5057 ± 0.2180	0.0720 ± 0.0350	****	
Ancient domestic horse	0.2991 ± 0.2180	0.0385 ± 0.0140	0.0234 ± 0.0080	****

According to the results of the calculations, it was found that when comparing the pairwise genetic distances of the tested horses and the fossil remains of *ancient equids*, the fossil remains of the *true tarpan* and the *ancient domestic horse* $D_N = 0.0234$, as well as the *Pleistocene horse* and the *ancient domestic horse* $D_N = 0.0385$, were the closest to each other, and the genetic distances of the *Hutsul* breed horses and the fossil remains of the *true tarpan* $D_N = 0.5057$ were the most distant from each other.

4. Discussion

Such indicators as the percentage of polymorphic loci (less than 30%), the average per locus genetic diversity according to Nei (less than 0.044), the Shannon information index (less than 0.063) may indirectly indicate inbreeding depression in the *Hutsul* population or its strict isolation [17]. Analyzing the parameters of genetic diversity according to *ISSR* markers of the studied breed of *Hutsul* horses, it can be concluded that the tested group of animals is relatively prosperous in terms of the detected intrapopulation genetic diversity. High μ values may indicate a significant uneven distribution of allele frequencies [18]. According to the results of assessing the state of the gene pool of the studied horses, it was established that moderate genetic diversity is inherent in the horses of the *Hutsul* breed.

The moderate genetic diversity inherent in the *Hutsul* breed of horses is also explained by the number of DNA loci that appeared with a frequency of 100%, i.e. conservative loci obtained using 8 primers. The total number of loci was 54, of which 33 loci are polymorphic.

The moderate genetic diversity inherent in *Hutsul horses* is also explained by the number of *DNA* loci that appeared with a frequency of 100%, i.e. conservative loci obtained using 8 primers. The total number of loci was 54, of which 33 were polymorphic loci.

The obtained data indicate that in the gene pool of the currently existing aboriginal breed of *Hutsul horses* there is a tendency to eliminate some short interlocus fragments from their genome compared to ancient forms under the influence of evolutionary events. Similar results were obtained by other scientists [19], who studied the genetic polymorphism of horse populations using *ISSR* markers.

The significant genetic differences between the *Hutsul horse* breed and ancient equidae are explained by the fact that the gene pool of the aboriginal *Hutsul horse* breed was formed through complex crossing of local mountain horses with breeds introduced to the region. This included horses from Bukovina, Galicia, Hungary, and horses of eastern origin.

By accumulating *ISSR* fingerprinting data of gene pools of agricultural species, it is possible to obtain reliable mathematical criteria for assessing the consolidation of breeds by many dozens of loci. The study of a large number of species and breeds will contribute to the creation of a data bank of their gene pool characteristics. This will make it possible to form an idea of the ancient gene pools, modern gene pools, identify the extinction and genetic variability of breeds, determine tactics and strategies for the management and conservation of breeds and species of agricultural animals. The *ISSR* - analysis method is noteworthy for characterizing *DNA* polymorphisms, although it does not allow for the determination of heterozygosity. To determine heterozygosity, codominant markers are required: STR, SNP, some AFLP.

5. Conclusion

Comparative studies of 101 polylocus spectra of *DNA* fragments flanked by microsatellite loci (*ISSR-PCR*) of the *Hutsul horse* population and *ancient equidae* were performed. Conservative loci, variable regions, and breed-specific characteristics (spectra) at the genomic and gene pool levels were identified, which indicate significant genetic differentiation of the studied horses. The established patterns can serve as material for monitoring and analysis in studies of breed phylogenesis.

As a result of the study, the obtained values of the proportion of polymorphic loci of *Hutsul horses* (0.617) were able to confirm the diversity of fragments for most of the analyzed loci. At the same time, some of them were found in fossil even-toed ungulates, (amplicons of size 340-350 bp of primer $(GA)_9C$ and 470-490 bp of primers $(GA)_9C$ and $(GA)_6CC$, in particular tarpans, *ancient domestic horses* and *Pleistocene horses*).

There is a loss of fragments in the genome of the *Hutsul horse* compared to ancient horses. This may be due to several evolutionary processes, such as natural selection, mutations, genetic drift, migrations and domestication.

Author Contributions

Lyubov Starodub participated in the assessment of genetic polymorphism of different horse populations using *ISSR* markers, writing the article; Nataliia Mokhnachova participated in the extraction of *DNA* from horse blood, extraction of *DNA* from fossil bones of ancient horses, genotyping; Ostap Zhukorskyi evaluated the final results of the study.

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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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