

## Genetic diversity study of the Mongolian horse breeds and lines: Results based on Microsatellite marker and Coat color genes polymorphism

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### Abstract

The genetic diversity of Mongolian native horse breeds Mongol, Galshar, Tes, and the lines Darkhad and Undurshil was studied using coat color gene and microsatellite marker polymorphism analyses. A total of 2,000 horses were characterized based on predominant coat color phenotypes and associated genes. Among six coat color genes, the highest average heterozygosity was observed in the Undurshil line, while the Tes breed exhibited the lowest. Microsatellite analysis of 17 markers in 259 horses identified 225 alleles, including 80 private alleles. ASB17 was the most polymorphic marker, whereas HTG4 was the least.

The Galshar breed displayed the highest genetic diversity, with Na (10.4), Ne (0.795), No (0.726), and PIC (0.802) parameters, while the Darkhad line showed the lowest values. Principal coordinate analysis (PCoA) and cluster analysis revealed distinct genetic structures among the populations. The Darkhad line was notably different from other breeds, while the Galshar breed shared genetic similarities with the Undurshil line, and the Mongol breed was genetically similar to the Tes breed. These findings provide critical insights into the genetic diversity and population structure of Mongolian native horse breeds, supporting their conservation and sustainable management.

**Keyword:** Mongolian horse, genetic diversity, DNA microsatellite markers, horse coat color gene

### Introduction

Mongolian horses are perfectly adapted to the local unique and special natural conditions and characterized by their endurance and ancient origin and reflect the traditional way of life and cultural traditions of Mongolian people. The genetic diversity study of Mongolian horse breeds and lines using DNA microsatellite markers and coat color genes will provide valuable genetic data for an assessment of the unique state of the genetic resources of Mongolian horses. For the genetic diversity research of the Mongolian horses is of relevance the investigation of the genetic structure of local breeds and lines in connection with their origin, phylogenetic relationship, and morphological characteristics.

DNA microsatellite markers are widely used for horse genetic diversity study. They are variable, do not carry a functional load, their unique combinations make it possible to establish phylogenetic relationships and calculate

population genetic parameters (number and frequencies of alleles, levels of observed and expected heterozygosity, fixation index, etc.). The investigation of 7 northern European horse breeds and the Mongolian native horse using 26 DNA microsatellite data showed close associations between the Mongolian native horse and northern European horse breeds [1]. Based on 20 DNA microsatellite polymorphisms, the genetic relationship of 13 populations of Japanese, Asian and European horses was studied and revealed the highest heterozygosity in Mongolian horses and Japanese horses were originated from Mongolian horses came through the Korean Peninsula [2]. The study of the genetic structure of 3880 Korean Halla horse population was conducted using 17 microsatellites, and it was found that M and Q of ASB23 marker, G of HMS2 marker, H, L of HTG6 marker, L of HTG7 marker, and E allele of LEX3 marker are unique only in Halla horses [3].

Berber N. et al. studied 14 microsatellites of 201 horses of 4 breeds in Algeria and found 123 alleles, and found the high variability of these breeds and the genetic distance between them is statistically significant [4]. In 2015, Kanshor et al. conducted a study of 15 microsatellite markers in 981 Canadian horses and determined that the Canadian horse is the original horse breed of North America [5]. In 2015, Criscione et al. analyzed 16 microsatellite markers and nucleotide sequences of the D-loop of mtDNA in 61 horses of the ancient breed Salernitano, which currently facing a concrete risk of extinction to set up the basic knowledge for a conservation plan [6]. The coat color of most mammals results from the activity of four basic color genes, A, B, C and E, first discovered in 1954 [7]. It has been studied that the heredity of the coat color of horses is not significantly related to their morphological feature [8]. The melanocortin-1-receptor (MC1R) gene and the agouti-signal-protein (ASIP) gene were amplified and the nucleotide sequence was

determined [9]. The melanocortin-1-receptor (MC1R) gene and the agouti-signal-protein (ASIP) gene determine the primary black, brown and fawn stripes in horses, and these genes have been studied in relation to horse behavior [10]. The leopard spotting gene was detected in early domestic horses dating to 2700-2200 BC, which disappeared during the late Bronze Age, but reappeared during the Iron Age [11].

The study on the genetic resources of horse breeds and lines established as a result of natural selection and the creative work of breeders and experts, and the breeding of breeders based on scientific knowledge using molecular genetic parameters will discover the unique genetic structure of the horse breeds which represent the genetic basis for new horse breeds.

The aim of our study was to assess the genetic diversity of Mongolian native horse breeds Mongol, Galshar, Tes and lines Darkhad, Udorshil based on the 17 DNA microsatellite marker and coat color gene analyses.

### Material and methods

In this study were included Horses of Mongol horse breed from Bayantsagaan sum of Tuv aimag, of Galshar breed from Galshar sum of Khentii aimag, of Tes breed from Tes und Zungov sum of Uvs aimag and of Darkhad horse line from Rinchenlkhumbe und Tsagaanuur sum from Khuvsgul aimag and of Undurshil horse line from Undurshil sum of Dundgov aimag.

The coat color determination was carried out on 400 horses of each horse breeds Mongol, Galshar, Tes breeds, and lines Darkhad, Undurshil. The blood samples were collected from 256 horses of these breeds: Mongol (n=50), Galshar (n=50), Tes (n=50) and lines: Darkhad (n=50), Undurshil (n=50).

Main coat color phenotypes and genotypes of horses from breeds and lines included in this investigation were determined as shown in Table 1, frequency of six coat color genes ( $q$ ), homozygosity index ( $H.I$ ), heterozygosity index ( $1 - H.I$ ), and heterozygosity ( $\check{H}$ ) of six coat color genes were calculated using Nozawa's method [12].

Genomic DNAs were extracted from horse blood samples using the ExtraGene Prep kit (IsoGene, Moscow). A set of 17 microsatellite markers

(AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS1, HMS2, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, HTG10, LEX3 and VHL20) approved by ISAG (International Society of Animal Genetics) for equine research were used for the study of horse diversity on STR locus.

17 microsatellite markers were amplified by multiplex PCR: the reagent mix was prepared with 2  $\mu$ l of DNA, 9  $\mu$ l of master mix, and 9  $\mu$ l of primer mix in a total of 20  $\mu$ l, PCR amplification was as follows: initial denaturation for 4 min at 95°C and 4 cycles at 98°C for 30 sec, 59°C for 2 min, 72°C for 1 min 30 sec, 6 cycles of 94°C for 30 sec, 59°C for 2 min, 72°C for 1 min 15 sec, 18 cycles 30 sec at 90°C, 2 min at 59°C, 1 min 15 sec at 72°C, 10 min at 68°C, and storage at 4-12°C. Microsatellite allele lengths were determined using an ABI Prism 3130xl sequencer at Zanaa Specs LLC. Microsatellite genotypes were determined using Gene Mapper™ V6 [13] software. GenAlEx V6.4 [14] and Fstat V2.9.3.2 [15] programs were used to process the parameters of population genetic structure and STRUCTURE V2.3.4 [16] program was used to determine the genetic distance and phylogenetic relationship between horse breeds and lines.

**Table 1.**

Horse coat color phenotypes and genotypes

| Main coat color | Phenotypes     | Genotypes |     |     |       |       |     | Horse number |
|-----------------|----------------|-----------|-----|-----|-------|-------|-----|--------------|
|                 | Bay            | E/-       | A/- | d/d | cr/cr | gr/gr | s/s | X1           |
|                 | black          | E/-       | a/a | d/- | cr/cr | gr/gr | s/s | X2           |
|                 | chestnut       | e/e       | A/- | d/d | cr/cr | gr/gr | s/s | X3           |
|                 | Bya-cream      | E/-       | a/a | D/- | cr/cr | gr/gr | s/s | X4           |
|                 | Chestnut-cream | E/-       | A/- | D/- | cr/cr | gr/gr | s/s | X5           |
|                 | Buckskin       | E/-       | A/- | d/d | CR/cr | gr/gr | s/s | X6           |
|                 | Palomino       | e/e       | a/a | d/d | CR/cr | gr/gr | s/s | X7           |
|                 | White          | E/-       | A/- | d/d | cr/cr | GR/-  | s/s | X8           |
|                 | Spotted        | e/e       | a/a | d/d | cr/cr | gr/gr | S/- | X9           |

**Results**

The investigation results of the coat color phenotype and genes of the horses (n=2000) in Mongolian native horse breeds Mongol, Galshar, Tes and lines Darkhad and Undurshil are presented in Tables 2, 3 and Figure 1.

It was observed that horses in breed Mongol have black and chestnut, Galshar breed horses have brown and palomino, Tes breed horses have brown and back skin, Darkhad horses have white and Undurshil (Table 2).

**Table 2.**

Number of main coat color phenotypes of Mongolian horse breeds and lines

| Number of phenotypes | Main coat color | Mongol | Galshar | Tes | Darkhad | Undurshil |
|----------------------|-----------------|--------|---------|-----|---------|-----------|
| X1                   | Bay             | 93     | 56      | 122 | 72      | 32        |
| X2                   | Black           | 52     | 35      | 47  | 33      | 28        |
| X3                   | Chestnut        | 58     | 67      | 34  | 32      | 32        |
| X4                   | Bay-cream       | 54     | 30      | 30  | 9       | 27        |
| X5                   | Chestnut-cream  | 30     | 25      | 39  | 17      | 28        |
| X6                   | Buckskin        | 27     | 46      | 21  | 25      | 57        |
| X7                   | Palomino        | 7      | 19      | 6   | 7       | 9         |
| X8                   | White           | 37     | 41      | 43  | 132     | 110       |
| X9                   | Spotted         | 26     | 6       | 18  | 26      | 29        |

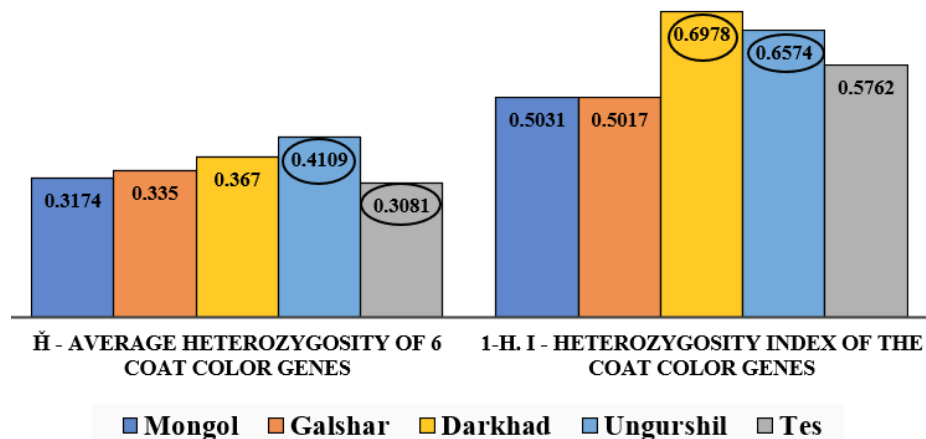
Table 3 shows the of 6 genefrequencies of the dominant coat color in Mongolian, Galshar, Tes breeds, Darkhad and Undurshil lines. In Galshar breed and Undurshil lines Dominant allele frequencies of E and A genes were lower and relative higher frequencies of dominant alleles of CR gene were observed compared to other breeds. The frequency of the dominant allele of the GR gene, which determines the white coat color, was higher in Darkhad and Undurshil lines compared to other breeds and had similar values. Figure 1

shows the average heterozygosity of the 6 coat color genes of horses in the studied breeds and lines, and the heterozygosity index parameters of the coat color genes. It was observed that the average heterozygosity of 6coat color genes of horse is the highest in Undurshil breed and the lowest in Tes breed, and the heterozygosity index of coat genes is the highest in Darkhad and Undurshil breeds and relatively low in the other 3 breeds.

**Table 3.**

Frequencies of 6 coat color genes in Mongolian horse breeds and lines.

| Breed, line      | 6 coat color gene frequencies |                           |                           |                                   |                                   |                           |
|------------------|-------------------------------|---------------------------|---------------------------|-----------------------------------|-----------------------------------|---------------------------|
|                  | E-e<br>$\{q_E$<br>$\{q_e$     | A-a<br>$\{q_A$<br>$\{q_a$ | D-d<br>$\{q_D$<br>$\{q_d$ | CR-cr<br>$\{q_{CR}$<br>$\{q_{cr}$ | GR-gr<br>$\{q_{GR}$<br>$\{q_{gr}$ | S-s<br>$\{q_S$<br>$\{q_s$ |
| <b>Mongol</b>    | 0.4190                        | 0.4655                    | 0.5717                    | 0.0950                            | 0.0803                            | 0.0585                    |
|                  | 0.5810                        | 0.5345                    | 0.4283                    | 0.9050                            | 0.9197                            | 0.9415                    |
| <b>Galshar</b>   | 0.3799                        | 0.3488                    | 0.5660                    | 0.2083                            | 0.1284                            | 0.0185                    |
|                  | 0.6201                        | 0.6512                    | 0.4340                    | 0.7917                            | 0.8716                            | 0.9815                    |
| <b>Tes</b>       | 0.4726                        | 0.5907                    | 0.6193                    | 0.0689                            | 0.1042                            | 0.0416                    |
|                  | 0.5274                        | 0.4093                    | 0.3807                    | 0.9311                            | 0.8956                            | 0.9584                    |
| <b>Darkhad</b>   | 0.4394                        | 0.5167                    | 0.6850                    | 0.1168                            | 0.2995                            | 0.0832                    |
|                  | 0.5606                        | 0.4833                    | 0.3150                    | 0.8832                            | 0.7005                            | 0.9168                    |
| <b>Undurshil</b> | 0.3169                        | 0.4102                    | 0.5110                    | 0.2619                            | 0.3251                            | 0.1280                    |
|                  | 0.6831                        | 0.5898                    | 0.4890                    | 0.7381                            | 0.6749                            | 0.8720                    |

**Figure 1.** Genetic diversity of Mongolian horse breeds and lines

The results of 17 microsatellite marker polymorphisms representing the genetic structure of Mongolian, Galshar, Tes breeds, Darkhad, and Undurshil horses are shown in Tables 4-7 and Fig. 2- 4.

Table 4 shows the results of 17 microsatellite polymorphisms. A total of 259 horses were genotyped for 17 microsatellites and 225 alleles were detected. Among these 17 microsatellite

markers, polymorphism of ASB17 marker was the highest and of HTG4 marker was the lowest in terms of the expected heterozygosity ( $H_e$ ), observed heterozygosity ( $H_o$ ), polymorphism information content (PIC) and number of alleles. In Mongolian horses, the average number of alleles in these 17 microsatellites was 13.24,  $N_e$  was 0.775,  $H_o$  was 0.723, PIC was 0.783,  $F_{is}$  was 0.065 and  $F_{st}$  was 0.042.

**Table 4.**

Results of 17 microsatellite polymorphisms identified in 259 horses of 5 horse breeds and lines

| Locus   | Allele number | He    | Ho    | PIC   | Fis    | Fst   |
|---------|---------------|-------|-------|-------|--------|-------|
| VHL20   | 12            | 0.824 | 0.839 | 0.832 | -0.017 | 0.024 |
| HTG4    | 8             | 0.587 | 0.547 | 0.593 | 0.067  | 0.019 |
| AHT4    | 14            | 0.812 | 0.744 | 0.820 | 0.083  | 0.070 |
| HMS7    | 16            | 0.715 | 0.714 | 0.721 | 0.001  | 0.163 |
| HTG6    | 14            | 0.696 | 0.666 | 0.703 | 0.042  | 0.028 |
| AHT5    | 11            | 0.736 | 0.701 | 0.744 | 0.048  | 0.092 |
| HMS6    | 8             | 0.796 | 0.779 | 0.804 | 0.022  | 0.019 |
| ASB23   | 16            | 0.817 | 0.779 | 0.825 | 0.047  | 0.021 |
| ASB2    | 12            | 0.808 | 0.714 | 0.817 | 0.117  | 0.026 |
| HTG10   | 15            | 0.836 | 0.766 | 0.845 | 0.084  | 0.055 |
| HTG7    | 13            | 0.743 | 0.743 | 0.751 | 0.000  | 0.018 |
| HMS3    | 14            | 0.814 | 0.677 | 0.823 | 0.169  | 0.030 |
| HMS2    | 13            | 0.765 | 0.816 | 0.772 | -0.067 | 0.028 |
| ASB17   | 20            | 0.886 | 0.898 | 0.895 | -0.013 | 0.020 |
| LEX3    | 13            | 0.841 | 0.451 | 0.853 | 0.464  | 0.022 |
| HMS1    | 12            | 0.720 | 0.703 | 0.726 | 0.022  | 0.023 |
| CA425   | 14            | 0.778 | 0.749 | 0.786 | 0.037  | 0.059 |
| Average | 13.24         | 0.775 | 0.723 | 0.783 | 0.065  | 0.042 |

**He** -expected heterozygosity, **Ho** -observedheterozygosity, **PIC** -polymorphism information content, **Fis** -inbreeding index, **Fst** - Fixation Index

The comparison of Mongolian horse breeds and lines according to 17 microsatellite markers were shown at Table 5.

According to the total microsatellite marker's allele number, the Galshar breed had the highest number of alleles and the Darkhad line had the lowest number. The Galshar breed and Undurshil

line had the highest average number of alleles (Na) and private alleles, while Darkhad line had the lowest. The expected and observedheterozygosity (He, Ho) and PIC -polymorphism information contenting the Galshar breed were higher compared to other breeds and lines, while in the Darkhad line was the lowest.

**Table 5.**

Comparison of Mongolian horse breeds and lines based on 17 microsatellite markers

| Breed, line | NA  | Na     | Ne    | He    | Ho    | PIC   |
|-------------|-----|--------|-------|-------|-------|-------|
| Mongol      | 155 | 9.176  | 5.147 | 0.785 | 0.745 | 0.793 |
| Galshar     | 176 | 10.471 | 5.564 | 0.795 | 0.726 | 0.802 |
| Tes         | 152 | 9.000  | 4.973 | 0.770 | 0.687 | 0.778 |
| Darkhad     | 130 | 7.765  | 4.364 | 0.745 | 0.725 | 0.752 |
| Undurshil   | 163 | 9.588  | 5.315 | 0.780 | 0.731 | 0.788 |
| Average     |     | 9.200  | 5.072 | 0.723 | 0.775 | 0.783 |

**NA**-total allele number, **Na** - average allele number, **Ne** -private allele, **He** - expectedheterozygosity, **Ho** - observedheterozygosity, **PIC** -polymorphism information content

The private alleles of microsatellite markers found in horse breeds and lines were represented in Table 6. A total of 80 private allelic genes were detected, and the most private alleles were detected in the Galshar breed. It was observed that all the studied horse breeds and lines have private alleles of markers such as VHL20, HTG6, ASB23, ASB17 and HMS1.

The Nei's genetic distances between the studied horse breeds and lines are shown at Table 7. It was observed that the Galshar breed and the Undurshil line are the most distant from the Darkhad horse line. The genetic distance between the Mongolian and Tes breeds and between the Galshar breed and the Undurshil breed was the closest.

**Table 6.**

Private allelic genes in Mongolian horse breeds and lines

| №            | Locus | Mongol    | Darkhad   | Tes       | Galshar   | Undurshil |
|--------------|-------|-----------|-----------|-----------|-----------|-----------|
| 1            | VHL20 | K         | R         | L         | S         | H         |
| 2            | AHT4  |           | N, G      | M, Q      | D         | O, E      |
| 3            | HMS7  |           | Q, J, K   |           | I         |           |
| 4            | HTG6  | K         | H, P      | R         | F, U, K   | L         |
| 5            | HMS6  |           |           |           | Q         | R         |
| 6            | ASB23 | Q         | L, I, O   | R         | F, N, G   | M         |
| 7            | ASB2  | G         | C         |           |           |           |
| 8            | HTG10 | T         | J, M      |           | H         | E         |
| 9            | HTG7  | T, Q      | P         |           | I         | F, J      |
| 10           | HMS3  | K         | S         |           | L, H      | N         |
| 11           | HMS2  |           |           | O, N      | M, J      | G, P      |
| 12           | ASB17 | J         | V         | W, U      | T         | F, G      |
| 13           | LEX3  | I         | J         |           | G         | Q, R      |
| 14           | HMS1  | L         | N         | I         | H         | R         |
| 15           | CA425 |           |           | H         | Q, D      | F         |
| <b>Total</b> |       | <b>11</b> | <b>19</b> | <b>11</b> | <b>21</b> | <b>18</b> |

**Table 7.**

Nei's genetic distances between breeds and lines

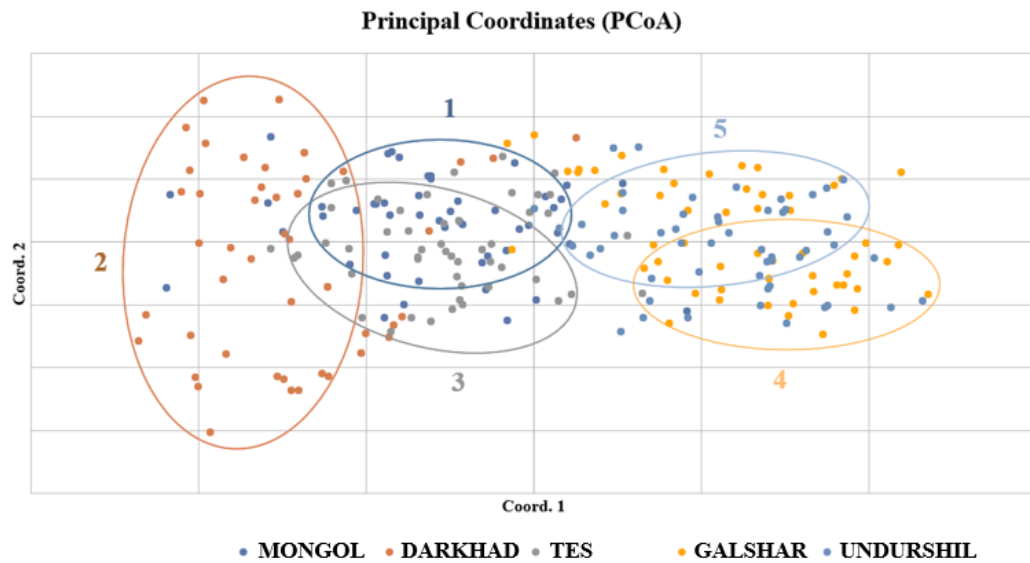
| Breed, line      | Mongol | Darkhad | Tes   | Galshar | Undurshil |
|------------------|--------|---------|-------|---------|-----------|
| <b>Mongol</b>    | ***    |         |       |         |           |
| <b>Darkhad</b>   | 0.155  | ***     |       |         |           |
| <b>Tes</b>       | 0.078  | 0.137   | ***   |         |           |
| <b>Galshar</b>   | 0.272  | 0.399   | 0.237 | ***     |           |
| <b>Undurshil</b> | 0.251  | 0.370   | 0.211 | 0.077   | ***       |

The results of principal coordinate analysis (PCoA) and cluster analysis of the distribution of different microsatellite genotypes in Mongolian horse breeds and lines are presented in Figures 2 and 3.

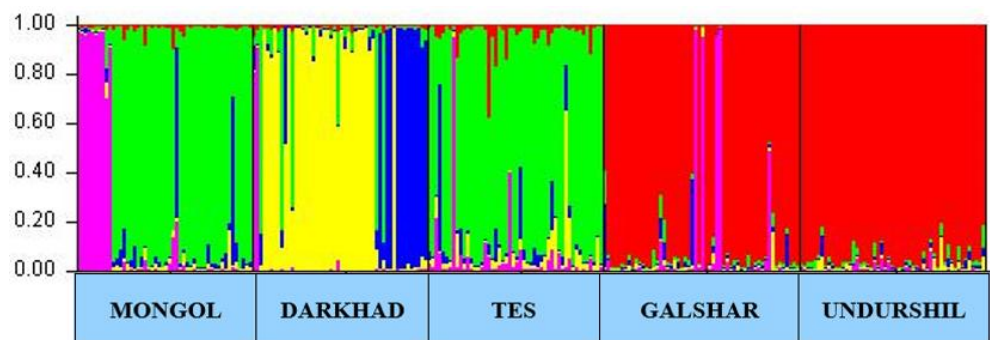
It was observed that the genetic structure of Darkhad horse line is significantly different from other breeds and the genetic structure of the Galshar horse breed is similar to the Undurshil

line. The Mongol breed is close to the Tes breed in terms of its genetic structure.

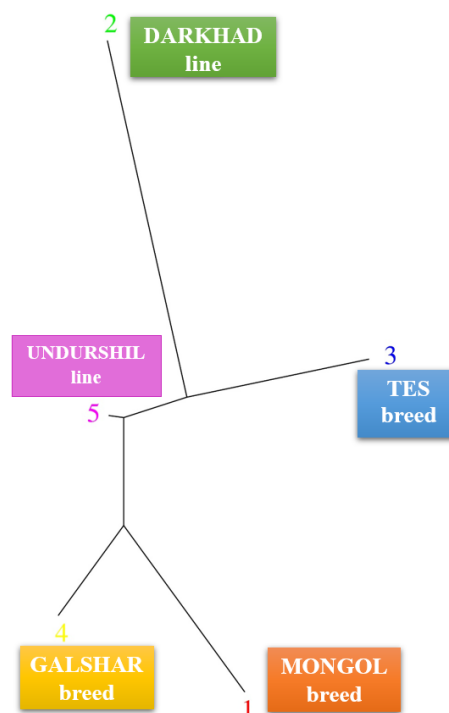
According to the phylogenetic tree created by the Structure program the genetic structure of the Darkhad line and Tes breed was most distant compared to the horse of Mongol breed. The Galshar breed and the Undurshil line were phylogenetically relatively close to the horse of Mongol breed (Figure 4).



**Figure 2.** Results of coordinate analysis of Mongolian horse breeds and lines



**Figure 3.** Results of cluster analysis of Mongolian horse breeds and lines



**Figure 4.** Phylogenetic tree of five Mongolian horse breeds and lines.

## Discussion

The study on the distribution of coat color phenotypes in native Mongolian horse breeds and lines in accordance with their genotypes revealed that the widest distributed coat color is in the Tes horse breed bay and palomino, in Darkhad horse lines white color, in Galshar breed spotted color and in Undurshil line bay and black color.

The genetic structure of Mongolian horse breeds and lines was estimated due to polymorphism of 17 microsatellite markers and it revealed the parameters of the genetic diversity in Mongolian horses were high (Na-9.2, Ne-5.07, He-0.78, Ho-0.72, PIC-0.78). There are 225 alleles of 17 microsatellite markers in Mongolian horses, and D allele [17] of AHT4 microsatellite marker, which was first detected in Tes horses, is also found in Galshar horses. These our study results compared with the results on 14 microsatellite markers (Na-8.73, He-0.773, Ho-0.757, PIC-0.737) in a joint study with Korean scientists [18] were higher despite of observed heterozygosity. According to the expected heterozygosity (He) results of Mongolian horse breeds and lines,

## Conclusion

The average heterozygosity of 6 coat color genes in Mongol, Galshar, Tes breeds and Darkhad and Undurshil lines was the highest in Undurshil and the lowest in Tes breeds. Heterozygosity index of the coat color gene was the highest in the Darkhad breed and the lowest in the Galshar breed.

Genotyping of 17 microsatellite markers in 5 Mongolian horse breeds and lines detect in total 225 alleles and 80 private alleles. The variability of ASB17 marker was the highest and of HTG4 marker was the lowest in terms of the expected heterozygosity (He), observed heterozygosity (No), polymorphism information content (PIC) and number of alleles.

The distribution of various genotypes of microsatellite makers in Mongolian horse breeds and lines showed that the Darkhad line genetic structure is different from other horse breeds and lines and Galshar breed is genetically similar to Undurshil breed and Mongol horse breed is similar to Tes breed.

## Conflict of interests

The authors declare no conflict of interests.

## Authors' Contribution

A.Ts. Performed laboratory and field experiments, data analysis, and prepared the original draft, B.O. head of the horse genetics

comparing with 6 breeds and lines of foreign countries, Mongol horse breed (0.785) are close to Iranian Kurdish (0.783) and Korean Jeju (0.782) horse breeds and Darhad line (0.745), Tes breed (0.77), Galshar breed (0.795) and Undurshil breed (0.78) horses have higher values than Russian Rusver (0.709), Korean local horses (0.734), Japanese Hokkaido (0.636) and English thoroughbred horses (0.674) [19].

The polymorphism information content (PIC) of the Mongolian horse breeds and lines studied was compared with six foreign breeds and lines. The results showed that Darkhad horses (0.752) had PIC values similar to the Iranian Kurdish (0.75) and Korean Jeju (0.749) horse breeds. In contrast, the Mongol breed (0.793), Tes breed (0.778), Galshar breed (0.802), and Undurshil horses (0.788) demonstrated higher PIC values than the Russian Rusver (0.669), Korean local horses (0.7), Japanese Hokkaido (0.518), and English Thoroughbreds (0.625) [19].

When comparing the phylogenetic relationships of these 5 breeds and lines, the Galshar breed and the Undurshil line were relatively close to the Mongol breed, the Tes breed was relatively distant, and the Darkhad line was the most distant from the Mongol breed.

All of the Mongolian breeds developed from Mongol breed by selection based on specific phenotypic features for the agricultural and the farming purpose. Main reason of this process is the landscape and the climate of Mongolia. The southern and the eastern parts of Mongolia are mainly Gobi Desert, but the northern and western parts consist of high mountains. Some special horse breeding processes were started in XX century and still going on. This may have an explanation of high similarity of some breeds and lines to each other. Results of this study could be useful for the equine breeders of the Mongolia in the future.

research team; assisted with laboratory experiments and data analysis, Ts.Ts. conducted laboratory analysis,



S.T. led the field research and coordination with local authorities; collected blood samples, conducted biometric analysis, and contributed to

writing, review, and editing. All authors have read and approved the final manuscript.

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