Genetic Diversity Analysis of Kyrgyz Native Horse and Korean Horses using Microsatellite Markers

Seungchang Kim[1], Bolot Toktosunov[3], Myrzakul Israilov[3], Daehyeok Jin[1], HuiMang Song[1], Jae-Yeong Lee[1], Sangrae Cho[1], Bong-Hwan Choi[1], Sungsil Moon[1], Jae-Young Choi[2], Asankadyr Zhunushov[3*]

[1]Animal Genetic Resources Center, National Institute of Animal Science, RDA
[2]Subtropical Livestock Research Institute, National Institute of Animal Science, RDA
[3]Institute of Biotechnology, National Academy of Sciences

초위성체 마커를 이용한 키르기스 재래마와 한국 말의 유전적 다양성 분석

김승창[1], Bolot Toktosunov[3], Myrzakul Israilov[3], 진대혁[1], 송희망[1], 이재영[1], 조상래[1], 최봉환[1], 문성실[1], 최재영[2], Asankadyr Zhunushov[3*]
[1]농촌진흥청 국립축산과학원 가축유전자원센터, [2]농촌진흥청 국립축산과학원 난지축산연구소, [3]키르기스스탄 국립과학원 생명공학연구소

Abstract The Kyrgyz native horse (KH) is one of the economically and traditionally important livestock in the Kyrgyz Republic, representing the history and values of the Kyrgyz people. The Objectives of this study were to determine the genetic diversity, structure, and characteristics of KH through comparative analysis with Korean horse breeds. The KH and three Korean breeds (Jeju horse, Jeju crossbred, throughbred) were characterized by using 19 microsatellite markers. The mean observed and expected heterozygosities were 0.689 and 0.794, respectively. The mean number of alleles for the KH was 9.474. The polymorphism information content (PIC) showed that six loci (AHT4, TKY297, VHL20, LEX3, ASB17 and HTG10) were highly polymorphic (> 0.8). The Phylogenetic analysis, factorial correspondence analysis (FCA) and admixture analysis showed that four breeds were separated into four different populations. Based on these results, it is considered that steady management planned breeding considering inbreeding is necessary for the continued presevation of KH, and it could be used as a basis for future genetic resource maintenance and conservation planning.

요 약 키르기스 재래마는 키르기스 공화국에서 경제적으로나 전통적으로 중요한 가축 중 하나이며 키르기스스탄 사람들의 역사와 가치를 대표하고 있다. 이 연구의 목적은 국내 말 품종과의 비교 분석을 통해 키르기스 재래마의 유전적 다양성, 구조 및 특성을 파악하기 위한 것이다. 19개의 초위성체 마커를 사용하여 키르기스 재래마와 한국 품종 3종(제주마, 제주 교배종, 서러브레드)의 특성을 분석했다. 관찰된 평균 이형접합도와 기대 이형접합도는 각각 0.689, 0.794로 나타났다. 키르기스 재래마의 평균 대립유전자 수는 9.474개였다. 다형성정보량(PIC)은 6개의 유전자좌(AHT4, TKY297, VHL20, LEX3, ASB17, HTG10)에서 높은 다형성(> 0.8)을 나타냈다. 계통 발생학적 분석, 요인 대응 분석(FCA), 혼합도 분석 결과 네 품종이 네 가지 집단으로 분리된 것으로 나타났다. 이러한 결과를 바탕으로 키르기스 재래마의 지속적인 보존을 위해 근친교배를 고려한 꾸준한 관리 계획 교배가 필요할 것으로 고려되며, 향후 유전자원 유지 및 보전 계획 수립의 기초자료로 활용될 수 있을 것으로 생각된다.

Keywords : Heterozygosity, Horse, Kyrgyzstan, Microsatellite, Polymorphism Information Content (PIC)

1. Introduction

Due to population growth and industrialization, livestock improvement in most countries has been driven by economic considerations, resulting in a decrease in the genetic diversity in breeds [1]. In recent years, the international community has recognized the importance of genetic for the future use, leading to the Convention on Biological Diversity (CBD), which recognizes the biological resources held by countries as inherent national property. As the world recognizes the potential economic value of biological resources, much attention and effort continues to be devoted to their acquisition, conservation, management, characterization and utilization through protection policies for national resources [2-5].

Molecular biological characterization is being actively conducted by many researchers to identify the genetic characteristics of the resources, such as origin, breed formation, genetic diversity, and flexible relationship with other breeds, using various genetic markers based on DNA polymorphism [6]. Various genetic markers are being used to study the population structure of breeds and genetic variation among breeds. Markers related to polymorphisms such as Restriction Fragment Length Polymorphism (RFLP), Variable Number of Tandem Repeats (VNTR), Single-Strand Conformational Polymorphism (SSCP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Microsatellite marker (MS marker), Single nucleotide Polymorphism (SNP), have been discovered [7]. MS markers are tandem repeats consisting of short units of 1 to 6 base pairs in length. During DNA replication, the length of microsatellite DNA sequences is prone to change at a faster rate than the average point mutation rate, and tends to expand as the number of copies increases [8-10]. Because microsatellites are highly polymorphic, homologously inherited to the next generation, and highly reproducible, markers are important tools for determining genetic variation, individual identification, and population structure [11,12]. Also, informative microsatellites have historically been used as markers for paternity testing of various horses [13,14]. MS markers can be good indicators for genetic resource diversity assessment due to their convincing methods and sufficient information. Therefore, MS markers are one of the reference tools for conservation and utilization of animal genetic resources.

Kyrgyzstan in Central Asia maintains a breed of Kyrgyz domestic livestock. In particular, the Kyrgyz native horse (KH) is one of the economically and traditionally important livestock in the Kyrgyz Republic, representing the history and values of the Kyrgyz people. KH is a breed adapted to the harsh environment of Kyrgyzstan with great endurance and adaptability to the mountainous region, and is essential for maintaining the country's natural ecosystem, helping to cultivate the land and maintaining ecological balance. However, the breed is disappearing under the influence of various foreign introduced breeds. One of the efforts to conserve these breeds is to obtain information about their genetic characteristics. Objective breed classification based on genetic uniqueness is required as a first step to formulate breeding policies in an effective and meaningful way and prioritize breeds for conservation [15]. However, the genetic background of these breeds and their inter- and intra-breed genetic relationships have not been clearly elucidated. The aim of this study is to collect genetic information of KH populations using MS marker analysis and to evaluate their genetic diversity through comparative analysis with Korean horse breeds (Jeju horse; JH, Jeju crossbred; JC and Thoroughbred; TB).

2. Material and Methods

2.1 Animal and DNA extraction

Blood of KH was collected randomly around Lake Issyk-Kul in Kyrgyzstan, avoiding parent-offspring or siblings. Animals received care in accordance with the standard guideline for the Care and Use of Laboratory Animals provided by the National Institute of Animal Science Animal Care Committee, and the experiment was conducted with approval from the Animal ethics committee and Operation rule of animal experiment ethics in the National Institute of Animal Science (Approval number: NIAS-2022054). Genomic DNA was extracted from blood samples using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's procedure in national academy of science of Kyrgyz Republic. DNA samples of Korean horse breeds were obtained from the Subtropical Livestock Research Institute in Jeju island.

2.2 Analysis

2.2.1 Genotyping of Microsatellite

The MS markers used in this study were a selection of 19 markers (AHT4, AHT5, ASB2, ASB17, ASB23, ASB25, CA425, HMS1, HMS2, HMS3, HMS5, HMS6, HMS7, HTG4, HTG7, HTG10, LEX3, TKY297 and VHL20) from panel recommended by the International Society for Animal Genetics (ISAG). Allele sizes were adjusted according to ISAG nomenclature. Detailed data for each marker regarding amplified region and chromosome length can be found in FAO [16]. To obtain information from the 19 marker set, multiplex PCR was performed using 10 ng/ml of template DNA in 1 ㎕, a primer mixture containing various MS markers set in 8.25 ㎕, 10 × buffer 3 ㎕, and 10 mM of dNTPs in 2.5 ㎕, HS prime Taq premix (Genetbio, Korea) in 8 ㎕ of distilled water, and a final volume of 20 ㎕. The

Table 1. The statistical analysis of Kyrgyz and Korean breeds using 19 MS markers

Marker	No. of Allele	No. of Animal	HObs	HExp	PIC	F_{IS}
HTG4	7	357	0.541	0.747	0.704	0.006
AHT4	11	357	0.824	0.859	0.842	−0.030
TKY297	12	357	0.731	0.837	0.816	0.060
HMS5	6	357	0.616	0.714	0.659	0.061
HTG7	8	357	0.720	0.756	0.714	−0.055
AHT5	9	357	0.782	0.793	0.767	−0.043
VHL20	12	357	0.790	0.842	0.823	−0.007
ASB25	8	357	0.602	0.793	0.764	0.061
LEX3	10	357	0.311	0.861	0.844	0.622
HMS2	11	357	0.697	0.741	0.712	−0.100
ASB23	12	357	0.846	0.846	0.826	−0.067
HMS7	8	357	0.714	0.795	0.766	−0.029
ASB17	18	357	0.801	0.858	0.843	0.035
HMS3	9	357	0.703	0.790	0.762	−0.025
CA425	10	357	0.627	0.674	0.641	−0.017
HTG10	13	357	0.787	0.845	0.827	0.011
HMS1	10	357	0.658	0.786	0.756	0.001
ASB2	14	357	0.793	0.814	0.792	−0.027
HMS6	9	357	0.720	0.736	0.690	−0.045
Mean	10.368	357	0.698	0.794	0.766	0.022

HObs = Observed heterozygosity.
HExp = Expected heterozygosity.
PIC = Polymorphism Information Content.
F_{IS} = Inbreeding coefficient.

multiplex PCR was denatured at 95 °C for 5 min, and the amplification process was 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s repeated 35 times, and an extension step at 72 °C for 5 min. Finally, the PCR reaction was terminated at 8 °C. The PCR products were subjected to electrophoresis using an ABI-3500XL genetic analyzer (Applied Biosystems, USA), sorted by size and type of marker using the Genemapper version 5 (Applied Biosystems, USA) program, and compiled in Microsoft Excel (Microsoft, USA).

2.2.2 Statistical analysis

Utilizing data compiled in Microsoft Excel, basic analyses including allele frequency and distribution, observed heterozygosity (HObs), expected heterozygosity (HExp) and polymorphism indices such as Polymorphism Information Content (PIC) for the markers used in this study were performed using the CERVUS ver. 3.0.7 program was used to convert the input data to perform allele distribution analysis [17]. In addition, to determine the degree of genetic fixation between individuals and populations, estimates of the F-statistic (F_{IS}) proposed by Weir and Hill [18] were estimated using the GENEPOP ver. 4.7.3 program [19]. Factorial correspondence analysis (FCA) using allele frequencies for each marker was performed using the Genetix ver. 4.05 program for the analysis of flexible relationships between Kyrgyzstan and Korean horse breeds [20]. Population structure was analyzed by clustering technique with Bayesian approach using STRUCTURE ver. 2.3.4 [21,22]. Structural analyses were performed with cluster number (K=2~7), burn-in 100,000 times, and Markov chain Monte Carlo (MCMC) 1,000,000 times, with each K repeated 10 times, using independent allele frequencies and admixture models. The program CLUMPP ver. 1.1 was used to align the 1000 repetitions of each K. CLUMPP out files were visualized using DISTRUCT ver. 1.1. Evanno's method [23] was used to identify the optimal K using ΔK based on the log-likelihood rate of change of the data, and the optimal K was set using STRUCTURE HARVESTER [24].

3. Results and Discussion

One KH (117 horses) and three Korean horses (40 JH, 100 JC and 100 TB) were subjected to polymorphism analysis using 19 MS markers, and the total number of alleles, HObs and HExp, PIC, and Inbreeding coefficient (F_{IS}) were calculated for each marker (Table 1). A total of 197 alleles were found in the 19 MS loci, with a mean value of 10.368 for all alleles, indicating that all markers were polymorphic with more than 6 alleles. The number of alleles varied from 6 (HMS5) to 18 (ASB17). The HObs and HExp ranged from 0.311 to 0.846 (mean 0.689) and from 0.674 to 0.861 (mean 0.794), respectively. The values between 0.3-0.8 mentioned in the study by Takezaki & Nei are suitable to be used as useful markers [11]. For all markers except the

Table 2. The mean basic statistics for Kyrgyz and Korean Horse breeds

Marker	No. of Allele	No. of Animal	HObs	HExp	PIC	F_{IS}
KH	9.474	117	0.727	0.791	0.761	0.081
JC	6.737	100	0.714	0.693	0.641	-0.031
JH	4.737	40	0.675	0.637	0.578	-0.060
TB	5.947	100	0.658	0.688	0.640	0.044
Mean	6.724	89.25	0.693	0.702	0.655	0.009

KH = Kyrgyz native horse, JC = Jeju crossbred, JH = Jeju horse, TB = Thoroughbred

ASB23 marker, the HExp was higher than the HObs. This suggests a loss of heterozygotes as a result of decreased genetic diversity and increased inbreeding. PIC values ranged from 0.641 to 0.844 (mean 0.766), which is a criterion for genetic diversity. Among these markers, AHT4, TKY297, VHL20, LEX3, ASB17 and HTG10 loci had relatively high PIC values ($>$ 0.8). To determine the degree of genetic fixation and inbreeding in this population, F-statistics analysis was performed to determine the value of F_{IS}. The mean value of F_{IS} was found to be 0.022.
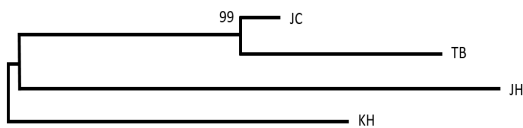

Fig. 1. A neighbor joining tree of genetic relationship among the 4 group using DA genetic distance estimated from 19 microsatellite loci.

The mean basic statistics of HObs, HExp, PIC, and F_{IS} of the four horse breeds were calculated (Table 2). The number of alleles is required to be at least four to reduce errors when estimating genetic distances between populations using MS markers. The results of this study confirm that there is no problem in estimating genetic distances with alleles ranging from 4.737 to 9.474 for all four populations [25]. The average basic statistics of the KH showed that the distribution of alleles, HObs, HExp, PIC, and F_{IS} values were relatively high compared to the other lines with 9.474, 0.727, 0.791, 0.761, and 0.081, respectively, indicating high genetic diversity. This can be assumed to be due to the fact that the Korean horse breed is raised in Jeju Island, which is limited compared to Kyrgyzstan, and the population size is relatively small. This is consistent with the results by Schmid [26], who reported that the population size of the individuals used in the analysis affects the number of alleles. For JC and JH, the HObs is

higher than the HExp, and for KH and TB, the HExp is higher than the HObs. In the case of JC, the HObs is considered to be the result of introducing genes from other breeds to develop this breed, and in the case of JH, it is considered to be the result of breeding management of local populations to reduce inbreeding [27]. Also, the F_{IS} values (-0.031 and -0.060) were consistent with these results, with values less than zero. KH and TB, which showed high HExp, had F_{IS} values of 0.081 and 0.044, respectively, which are higher than zero, indicating less genetic variation and more inbreeding.

To investigate the phylogenetic relationship between each breed, a phylogenetic tree was constructed using the Neighbor-Joining method (Fig. 1). The tree of the four breeds shows two major clustering. JC, TB, and JH form one branch and are separated from KH. The tree clearly separates KH from the rest of the breeds, indicating the differentiation of the breeds.
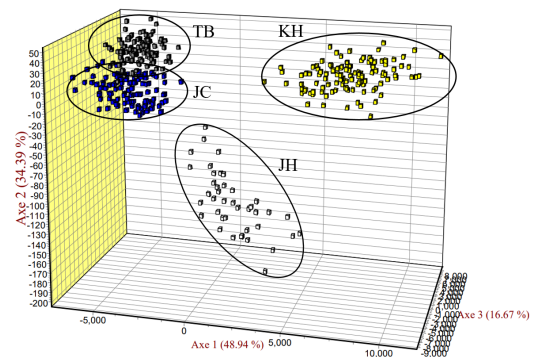

Fig. 2. Results of Factorial Correspondence Analysis (FCA) analysis. This plot shows a tendency to cluster by each breed.

The neighbor-network analysis of the 4 breeds confirmed the results of FCA which horse breeds are segregated in a similar pattern of Fig. 1 (Fig. 2). The individuals were separated into four different clusters corresponding to the four populations. The axis 1 (48.94% of the genetic variation) discriminated individuals to KH, JH and the other breeds. The second axis (34.39% of

the genetic variation) discriminated from JC and TB along with KH and JH breed. It is noticeable that JC and TB which are genetically close, leading to a genetic isolation by distance pattern. From the Factorized variance, it was considered that KH is separated independently from Korean horse breeds, and JC is located between them due to the genetic introduction of JH and TB, and is particularly genetically adjacent to TB.

To analyze the genetic structure of the four breed populations, the structure analysis by population is shown in Fig. 3, and the optimal number of clusters (K) is shown in Table 3. The structure analysis was used to compare the log-likelihood values of the data by fixing the value of K=2~7 without using the cluster information in advance. As a result of the structural analysis according to Evanno's method [23], the optimal number of clusters (ΔK) was found to be four (K=4), and the genetic structure of each breed was different. At K=2 and K=3, the genetic structure of JC and TB breeds was found to be similar. However, at the optimal K=4, the genetic structure was found to be different, which was considered to be the result of genetic mixing of TB and JB to develop the JC breed [28].
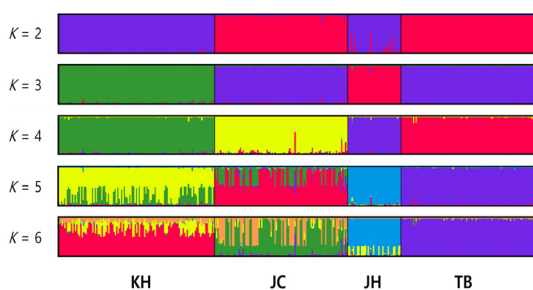

Fig. 3. The Admixture result is analyzed using the STRUCTURE. The optimal value of K is 4, and each line shows an independent genetic structure.

DNA markers are being used as an important tool for livestock breed conservation and management with the development of molecular genetic technologies. According to the international community requires molecular biological characterization data as evidence of each country's unique breeds, various DNA marker studies are being conducted to establish breeds and recognize them as unique breeds. Many studies on the origin and breed formation of horses have been conducted using methods such as mtDNA and MS markers [12-15]. In particular, MS markers have been widely used for genetic characterization of species and quantitative trait loci studies. The KH, which has high economic and cultural value in Kyrgyzstan, has been studied using MS markers to identify unique genetic traits [29,30].

The objective of this study was to determine the genetic diversity, structure, and characteristics of KH through comparative analysis with Korean horse breeds. Based on the results of this study, it is considered that it is necessary to maintain and preserve the KH through planned breeding considering inbreeding, and based on this results, it is considered that the KH can be utilized as a basis for future genetic resource maintenance and conservation planning.

Table 3. Estimated ΔK values by Evanno method

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	ΔK
2	10	-22528.20	0.249	–	–	–
3	10	-21550.93	165.161	977.27	237.15	1.436
4	10	-20810.81	0.723	740.12	602.42	832.830
5	10	-20673.11	75.337	137.70	5.32	0.071
6	10	-20530.09	116.900	143.02	6.54	0.056
7	10	-20393.61	149.505	136.48	–	–

4. Conclusion

Securing the genetic diversity of livestock has important value as a genetic resource that can compensate for genetic losses that may occur due to the intensification of uniform breeding in the livestock industry, and is considered the

fastest way to secure competitiveness for sustainable livestock development. Therefore, in order to establish and develop conventional resources as breeds, it is necessary to conduct phenotyping studies, molecular biological analysis studies for characterization, and comparative analysis studies between genomes.

In this study, a comparative analysis was conducted with three Korean horse breeds to determine the genetic diversity of KH as a conventional breed using the diversity and frequency of alleles with MS markers. Genotyping of 19 MS markers was performed for comparison among breeds, and the basic statistical values were relatively high compared to other breeds, indicating good genetic conservation. In addition, FCA and structural analysis showed that KH have a different genetic structure from Korean horse breeds, with very low levels of gene exchange and gene introgression. Therefore, KH has been maintained as a unique conventional resource in Kyrgyzstan and needs to be managed as a locally adapted breed through continuous conservation and management.

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Seungchang Kim   [Regular member]

• Feb. 1999 : M. A., Biology, Chonnam National University
• Feb. 2009 : Ph. D., Biology, Chonnam National University
• Feb. 2018 ~ present : Researcher, NIAS, RDA

〈Research Interests〉
Genetic breeding, Genetic resource management

Bolot Toktosunov [Regular member]

- May, 1992 : Kyrgyz Agricultural Institute after K.I Skryabin
- May, 2014 : Ph. D., Kyrgyz Agricultural Institute after K.I Skryabin
- Jun. 2019 ~ present : Leading Researcher, IB NAS KR

〈Research Interests〉
Animal Genomics, Molecular Physiology

HuiMang Song [Regular member]

- Aug. 2021 : M. Agriculture, Chonnam National University
- Jan. 2023 ~ present : Researcher, NIAS, RDA

〈Research Interests〉
Genetic resource management, Pig production

Myrzakul Israilovl [Regular member]

- May, 2015 ; Jalal-Abad State University, the Kyrgyz Republic
- May, 2015 ~ present : Junior Researcher, IB NAS KR

〈Research Interests〉
Animal Genomics, Molecular Physiology

Jae-Yeong Lee [Regular member]

- Feb. 2017 : B. Agriculture, Konkuk University
- Feb. 2023 : M. A., Pusan National University
- Sep. 2019 ~ Present : Researcher, NIAS, RDA

〈Research Interests〉
Applied Life Science, Animal Reproduction

Daehyeok Jin [Regular member]

- Feb. 2018 : B. Agriculture, Jeonbuk National University
- Sep. 2020 ~ present : Researcher, NIAS, RDA

〈Research Interests〉
Genetic breeding, Genetic resource management

Sangrae Cho [Regular member]

- Feb. 2000 : M. A., Gyeongsang National University
- Aug. 2003 : Ph. D., College of Applied Life Sciences Gyeongsang National University
- Jan. 2008 ~ Present : Senior Researcher. NIAS. RDA

〈Research Interests〉
Applied Life Science, Genetic Engineering Embryo

Bong-Hwan Choi   [Regular member]

- Aug. 2000 : Ph. D., Chonnam National University
- Jun. 2002 : Researcher, NIAS, RDA
- Mar. 2021 : Senior Researcher, NIAS, RDA

⟨Research Interests⟩
Animal Genomics, Animal Molecular Physiology

Sungsil Moon   [Regular member]

- Feb. 1988 : Professional Bachelor of Science. Seoyoung University.
- Sep. 2004 ~ Sep. 2019 : Researcher, NIAS, RDA
- Jun. 2021 ~ present : Researcher, NIAS, RDA

⟨Research Interests⟩
Animal Genomics, Genetic resource management

Jae-Young Choi   [Regular member]

- Feb. 2015 : M. A., Pusan National University
- Aug. 2022 : Ph. D., Animal Science, Pusan National University
- Feb. 2019 ~ present : Researcher, NIAS, RDA

⟨Research Interests⟩
Animal genetics, molecular biology

Asankadyr Zhunushov   [Regular member]

- May, 1972 ; Kyrgyz Agricultural Institute after K.I Skryabin
- May, 1976 ; Ph. D., Kyrgyz Agricultural Institute after K.I Skryabin
- Jun. 1993 ; Professor, IB NAS KR
- 2000 ~ present : Director, IB NAS KR

⟨Research Interests⟩
Animal Genomics, Molecular Physiology