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mtDNA Diversity and Origin of Chinese Mongolian Horses*

Jinlian Li, Youfei Shi[1], Caiyun Fan[2] and Dugarjaviin Manglai**
Department of Biological Science and Technology, Taishan University, Tai'an, 271021, China

ABSTRACT : In order to learn the origin of the Chinese Mongolian horse, we analyzed polymorphisms within the mtDNA D-loop variable region in 305 horses of 6 types of 3 different breeds, including one imported breed, one cultivated breed and 4 types of one local breed. We detected 13 different haplotypes, and subsequent sequence analysis showed that all 6 horse types were genetically diverse. By constructing a cladogram of mtDNA D-loop sequences from the 6 horse types along with homologous sequences from several other horse types obtained from GenBank, we showed that Chinese Mongolian horses have a close genetic relationship with other horse types from Mongolia. We also speculate that several Chinese Mongolian horses descended from Przewalskii horse. Additionally, the 13 haplotypes were dispersed throughout the cladogram, suggesting that Chinese Mongolian horses likely originated from multiple female ancestors. A phylogenetic map of the 6 horse types showed that the genetic relationship between the local Wuzhumuqin and Wushen types were the closest. The Xinihe and Baerhu were also closely related to each other, and slightly more distantly related to the cultivated Sanhe breed. All five of the local Chinese horse types had a much more distant relationship with the imported Thoroughbred breed. (**Key Words :** Horses, mtDNA D-Loop Region, PCR-SSCP, Polymorphism, Origin Evolution)

INTRODUCTION

The Mongolian horse is one of the most ancient breeds in the world. The people in the northern regions of China began to domesticate horses as early as 4,000-5,000 BC. Fossilized bones and teeth of the Pliocene era three-toed horse, and the Pleistocene era Mongolian wild horse have been unearthed in several regions of Inner Mongolia. The existence of the ancient Mongolian horse was known as Przewalskii (Mang, 2002). During the Warring States Period (from the 3rd to 4th century BC) the horse was so common that the Huns Empire was called The Kingdom of the Horse. During the Han dynasty, a large number of Huns horses were imported into China. The distribution of the Mongolian horse throughout the three northeastern provinces occurred during the Song Dynasty, with their population reaching 40,000 throughout the pasturing areas in the north of China by the end of the Qing dynasty (Mang, 2005). After the liberation of Mongolia, horses from the Inner Mongolia area were brought into the neighboring provinces gradually, and at the same time they were improved. These improved horses were in the minority, with the larger population of purebred Mongolian horses remaining in Inner Mongolia.

China has an abundance of horse breeds. The current literature reported sequence variation in the horse mtDNA D-loop region (Wang et al., 1994; Xu et al., 1994; Vila et al., 2001; McGahern et al., 2006a; McGahern et al., 2006b) and genetic relationship among different horses by microsatellite polymorphism (Cho, 2006). but no research as to the origin of Chinese Mongolian horses has been undertaken in China and abroad. In this report, polymorphisms of the mtDNA D-loop variable region were analyzed from 305 horses of 6 types of 3 different breeds including one imported breed, one cultivated breed and 4 types of one local breed. These sequences were compared with mtDNA D-loop sequences from other Mongolian and Arabian species in order to identify the origin of the Chinese Mongolian horses at the molecular level.

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** Corresponding Author: Dugarjaviin Manglai. Dugarjaviin Manglai, College of Animal Science and Veterinary, Inner Mongolia Agricultural University, Hohhot 010018, China. Tel: +86-471-4308299, Fax: +86-471-4313717, E-mail: manglainmg @yahoo.com.cn
[1] College of Animal Science and Veterinary, Shandong Agricultural University, Tai'an, 271018, China.
[2] College of Animal Science, Henan Institute of Science and Technology, Xinxiang, 453003, China.
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Table1. Name, number and collection location of different horse types

Horse types	Numbers	Collection location
Thoroughbred (TB)	52	Huajun breeding horse company, Beijing
Sanhe (SH)	53	Chenbaerhu county, Hulunber, Inner Mongolia Autonomous Region
Wuzhumuqin (WZ)	51	Wuzhumuqin county, Xilingol league, Inner Mongolia Autonomous Region
Xinihe (XN)	54	Ewenke county, Hulunbeier, Inner Mongolia Autonomous Region
Baerhu (BH)	53	Xinbaerhu county, Hulunbeier, Inner Mongolia Autonomous Region
Wushen (WS)	42	Etuoke county, Erdos, Inner Mongolia Autonomous Region

MATERIALS AND METHODS

Sample collection

Samples came from Beijing and different areas of the Inner Mongolia Autonomous Region. Among them, the Wuzhumuqin (WZ) horse originated in the central Inner Mongolia Autonomous Region, its physique was strong, the body size was not bigger but possessed faster speed and stronger endurance. The Xinihe (XN) horse, previously called the Mongolian Buryat horse, originated in the Xini river and Yimin river areas of the eastern part of the Inner Mongolia Autonomous Region. The body was vigorously strong, possessing full muscling and better adaptability. The Baerhu (BH) horse originated in the Chenbaerhu county of the eastern part of the Inner Mongolia Autonomous Region; its famous feature was the faster speed and stronger endurance. The Wushen (WS) horse originated in the Maowusu desert of the western part of the Inner Mongolia Autonomous Region. It had a smaller physique, gentle temperament and strong endurance and was tolerant of the cold summer and adapted to the desert. It inherited the ancestral genetic survival advantage of the desertification. The Sanhe (SH) horse was produced in the three rivers (Root River, Drbour River and Ha Buer River) of the Erguna Right county in the eastern part of the Inner Mongolia Autonomous Region. It belonged to the cultivated breed following the hybridisation of the Zhongya horse and the local Mongolian horse. SH had the taller physique, the faster speed and the stronger endurance. The Thoroughbred (TB) horse was the famous race horse produced in the 17th and 18th century in England. TB was the world's best horses due to the tallest physique, the fastest speed and best physical structure. Information about the different horse types was presented in Table 1.

DNA isolation, primers and PCR amplification

Genomic DNA was extracted from 10 ml of frozen blood using the phenol-chloroform and proteinase K method (Sambrook, 1992).

Primers used to amplify a 444 bp fragment of the horse mtDNA D-loop variable region were generated from horse mtDNA published sequence (Ishida et al., 1994; Xu et al., 1994; Marklund et al., 1995; Kavar et al., 1999). The forward primer was 5' TAT TCC TAG CCA TAC ACT ACA C 3' and the reverse primer was 5' GAA TAA TAC TAG AGT TAG TAG GAG C 3'.

The 25 μl PCR reaction contained 25 μM of each primer, 50 μM dNTP, 50 ng genomic DNA, 1.25 U Taq DNA polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.1% Triton X-100, and 3 mM $MgCl_2$. The reaction parameters were: 5 min denaturation at 95°C, 34 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 10 min. The 444 bp PCR products were fractionated on a 1.2% agarose gel and visualized with ethidium bromide.

SSCP analysis and sequencing

SSCP analysis of the horse mtDNA D-Loop variable region fragments was performed on gels of 10% w/v acrylamide:bis-acrylamide (49:1), 0.5×TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM Na_2-EDTA, pH 8.4), 0.093% w/v ammonium persulfate and 0.08% v/v TEMED. The samples plus loading dye (98% v/v N,N-dimethyl formamide, 10 mmol/L EDTA pH 8.0, 0.025% w/v bromophenol blue, 0.025% w/v xylene cyanol FF) were heated to 98°C for 10 min then placed on ice until loaded. Gels were run in 1×TBE at 8 V/cm for 6 h at room temperature. SSCP patterns were visualized using a silver stain protocol. DNA fragments corresponding to all 13 different haplotypes were purified and sequenced using an ABI PRISM377-96 sequencer (Lianhe Gene Sequencing Company, Shanghai).

Data processing

Sequences of the mtDNA D-loop variable region PCR fragments were aligned using DNASTAR multiple alignment software. The tandem repeat motif CACCTGTG was not included in the analysis because the number of repeats was variable within individuals, indicating a high degree of heteroplasmy (Kim et al., 1999).

Polymorphic site, nucleotide total variating site, point mutable site, base composition, the ratio of transform/ transversion (Ts/Tv), nucleotide and sequence differences were counted using MEGA2.1 software. According to the formula $d = -3/4 \log_e(1-4/3\ p)$, the distances among the different haplotypes were counted using the pairwise distances of the Jukes-Cantor model of the MEGA2.1 software. A phylogenetic tree among the different haplotypes was constructed by Neighbour-Joining (NJ)

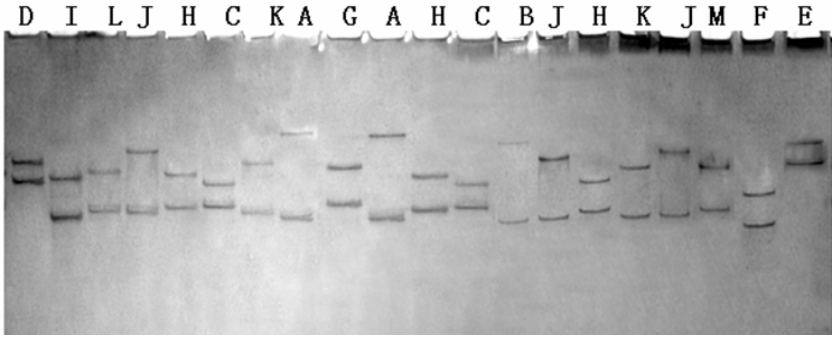

Figure 1. Detection of mtDNA D-loop variable region polymorphisms in different horse types by PCR-SSCP.

using MEGA2.1 software too.

In order to detect the haplotype diversities of the different horse types, the average number of nucleotide differences, nucleotide diversity, net genetic distances, DNA sequence variation within group and among groups, Tajima's D test, Fu and Li's D*, Fu and Li's F* two neutral test were calculated using DnaSP4.10 software.

In order to detect the genetic structure of the different horse types, the variance components among groups and within groups were analyzed and tested for significance using an Analysis of Molecular Variance (AMOVA) program which was included in the Alrequin2.0 package.

According to the net genetic distances (Pnet) among the different horse types, a phylogenetic map among different type horses was clustered using the UPGMA method of PHYLP 3.57c software.

RESULTS AND ANALYSES

Genetic polymorphism analysis of horse mtDNA D-loop variable region

SSCP analysis of the 444 bp PCR fragment of horse mtDNA D-loop variable region resulted in the identification of 13 distinct haplotypes (A-M) in 305 horses (Figure 1).

mtDNA D-loop variable region nucleotide length was 400 bp or 401 bp after 13 haplotypes were sequenced, average ratios of 4 nucleotides was 27.8%, 28.4%, 30.2% and 13.6%, contents of A+T exceeded G+C, and illustrated that AT was richer at the mtDNA D-Loop region. Sequence variable sites observed in 13 haplotypes were shown in Figure 2. Analysis of the mtDNA D-loop variable region showed 34 polymorphic sites, representing 8.5% of the total DNA sequence analyzed (400 bp). All mutable sites were the variation between two nucleotides. Only one of the 34 variable positions represented insertion/deletion of single base pairs. The remaining 33 variable positions were single nucleotide substitutions, indicating a strong transitional bias that was common in mammalian mitochondrial evolution (Kim et al., 1999).

Jukes-Cantor distances of the 13 haplotypes of the horse

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[                 11111 1111111111 1222222222 2222222333 3333333344 ]
[     5556666 9999900444 5556667778 9000122446 6788889033 6666788900 ]
[  1232341267 1236701034 5671232349 4789056122 8905679007 6789056901 ]
#A TGATCATGT- CACAACCGCG AATCTGATAA CTAAGGATCT CGCGTTATCA CAAACGATGG
#B .........- .........A .......... ...G.A.... .A........ ...G......
#C ...C.G...- ..T.....A. ...TCA.... ..G....... .A......T. .....AG...
#D .........- ....G...TA ...T...... ...G...... TA......T. ..........
#E ...C.G...- ..T......A ....CA.... ..G....... .A......T. .....A....
#F .........- ......T..A ...T....G. T..G.A...C .A........ .....A....
#G .........- .......... ...T...... ...G...... .A...C.... ..........
#H .........- .......... ...T.A.... .........C .A....G.TG ....TA....
#I ........CC .......... ...T.....G ...G....T. .A......T. T....AG...
#J .........- ......T..A .G.T...... T..G.A...C .A........ .....A....
#K .........- .......... .G.T...... ......G..C .A......TG ..G..A....
#L .........- .......... .G.T.A.... ...G.....C .A.A....TG ....TA....
#M .........- .......... .G.T.A.... ......G..C .A......TG ..G..A....
```

Figure 2. Sequence variable sites observed in 13 haplotypes. · Indicates the same base; - Indicates the base deletion.

Table 2. Juker-Cantor distance of 13 haplotypes of mtDNA D-loop variable region

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	***	0.006	0.009	0.007	0.008	0.008	0.005	0.008	0.008	0.008	0.008	0.008	0.008
B	0.013	***	0.009	0.007	0.008	0.007	0.006	0.009	0.008	0.007	0.009	0.009	0.009
C	0.031	0.005	***	0.009	0.004	0.010	0.009	0.008	0.009	0.010	0.009	0.009	0.009
D	0.020	0.018	0.031	***	0.009	0.008	0.006	0.008	0.008	0.008	0.008	0.008	0.009
E	0.025	0.028	0.033	0.031	***	0.010	0.009	0.008	0.010	0.010	0.009	0.009	0.009
F	0.025	0.018	0.036	0.025	0.036	***	0.007	0.008	0.009	0.004	0.008	0.008	0.009
G	0.010	0.013	0.031	0.015	0.031	0.020	***	0.008	0.007	0.007	0.008	0.008	0.008
H	0.023	0.031	0.028	0.028	0.028	0.028	0.023	***	0.008	0.008	0.006	0.005	0.006
I	0.025	0.028	0.031	0.025	0.036	0.031	0.020	0.028	***	0.009	0.008	0.008	0.009
J	0.025	0.018	0.036	0.025	0.036	0.005	0.020	0.028	0.031	***	0.008	0.008	0.008
K	0.023	0.031	0.033	0.028	0.033	0.028	0.023	0.015	0.028	0.023	***	0.006	0.003
L	0.028	0.031	0.033	0.028	0.033	0.028	0.023	0.010	0.028	0.023	0.015	***	0.006
M	0.025	0.033	0.031	0.031	0.031	0.031	0.025	0.013	0.031	0.025	0.003	0.013	***

Below diagonal: Juker-Cantor distances; Above diagonal: standard error.

mtDNA D-loop variable region were presented in Table 2. The distances between haplotypes ranged from 0.003 to 0.036, with an average distance of 0.025.

Frequencies of the 13 different haplotypes of the mtDNA D-loop variable region were shown in Table 3. Haplotype I was common to all 6 horse types, and was detected in 65 of the 305 horses tested. Haplotypes H and J were common to the 4 horse types of the local Mongolian horse breed and the cultivated breed SH, but were absent in TB. Some haplotypes were unique to certain horse breeds. For example, haplotype B was found only in TB at a frequency of 13.46%, haplotype F was unique to BH at a frequency of 26.42% and haplotype M was detected in only WS horses at a frequency of 7.14%.

Analysis of haplotype' and nucleotide polymorphisms of the mtDNA D-loop variable region within different horse types was shown in Table 4. The average number of nucleotide differences (K) within the 6 horse types were all large, and the values of haplotype diversity (H) and nucleotide diversity were high, thus mtDNA from the different horse types was genetically diverse.

The differences between all horses were not significant by Tajima's D test ($p>0.10$). However, TB and SH were significantly different, and 4 different horse types of local Mongolian horse breed had no significant difference, consistent with neutral mutation by Fu and Li's test.

The average number of pairwise differences (PiXY) between different horse types were significant ($p<0.01$) (Table 5), indicating genetic diversity among the different horse types. The greatest net genetic distance was between TB and WS, and the smallest distance was between WZ and WS, with XN and BH having a small net distance as well.

Analysis of molecular variance (AMOVA) of the mtDNA D-loop variable region fragments of the 6 horse types was shown in Table 6. The frequency of variation among types (Va) was 8.32%, while the frequency of variations between types (Vb) was 91.68%. The fixation index (Fst) was equal to 0.08316 ($p<0.01$), indicating that most genetic differentiation occurred between types.

Molecular phylogenetic analysis of different horse types using mtDNA D-loop variable region sequences

Net distances between the 6 different horse types were used for clustering analysis using the UPGMA method of

Table 3. Different haplotypes and frequencies of mtDNA D-loop variable region within different horse types

Haplotypes	TB	SH	WZ	XN	BH	WS	Total
A	2/0.03846		9/0.1765			6/0.1429	15/0.04918
B	7/0.1346						7/0.02295
C	14/0.2692		6/0.1176	3/0.05555	3/0.05660		26/0.08525
D	5/0.09615	7/0.1321	1/0.01961	1/0.01852	1/0.01887		15/0.04918
E		3/0.05660		9/0.01667			12/0.03934
F					14/0.2642		14/0.04590
G		4/0.07547	1/0.01961		12/0.2264		17/0.05574
H		10/0.1887	6/0.1176	3/0.05555	3/0.05660	8/0.1905	30/0.09836
I	8/0.1538	10/0.1887	2/0.03922	20/0.3704	13/0.2453	12/0.2857	65/0.2131
J		8/0.1509	11/0.2157	9/0.01667	7/0.1321	9/0.2143	44/0.1443
K	13/0.2500	11/0.2075	8/0.1568	2/0.03704		4/0.09524	38/0.1246
L	3/0.05769		7/0.1373	7/0.1296			17/0.05574
M						3/0.07143	3/0.009836
Total	52/1.0000	53/1.0000	51/1.0000	54/1.0000	53/1.0000	42/1.0000	305/1.0000

Table 4. Analysis of haplotype and nucleotide polymorphism of mtDNA D-loop variable region within different horse types

	TB	SH	WZ	XN	BH	WS
Numbers	52	53	51	54	53	42
Haplotype sequence	7	7	9	8	7	6
Numbers of polymorphic sites	29	30	32	30	28	22
Numbers of parsimony informative sites	29	30	28	26	25	22
Average number of nucleotide differences	9.384	8.628	8.565	9.226	7.652	8.258
Haplotype diversity (H)	0.825	0.853	0.866	0.797	0.810	0.821
	±0.056	±0.025	±0.017	±0.035	±0.023	±0.025
Nucleotide diversity (%)	2.346	2.157	2.141	2.306	1.913	2.064
	±0.044	±0.079	±0.090	±0.087	±0.104	±0.045
Tajima's D	1.525	1.007	0.681	1.321	0.787	1.040
Fu and Li's D*	1.827**	1.842**	0.833	0.745	0.961	0.700
Fu and Li's F*	2.045**	1.837*	0.927	1.133	1.062	1.149

** Indicates significant notable difference at significance level of 0.01.
* Indicates notable difference at significance level of 0.05.

Table 5. Average number of pair-wise differences between different horse types (PiXY)

	TB	SH	WZ	XN	BH	WS
TB	***	9.0726	9.8326	9.0492	8.8026	9.6365
SH	0.8518	***	9.0673	8.3197	8.1545	8.3837
WZ	1.1683	0.6528	***	8.9619	8.9342	8.5892
XN	0.8345	0.3548	0.5535	***	8.0598	8.3201
BH	0.7770	0.3787	0.7149	0.2901	***	8.4888
WS	1.3675	0.3645	0.1265	0.3070	0.6648	***

Above diagonal: Average number of pair-wise differences between populations (PiXY).
Below diagonal: Number of net nucleotide substitute per site between populations (Pnet).

Table 6. Percentage of variation between different horse types

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation index (Fst)
Among types	5	106.047	0.37009 Va	8.02	0.08021
Within types	300	1232.329	4.08056 Vb	91.98	
Total	305	1338.376	4.45065		

PHYLP 3.57c software (Figure 3). The results showed that the 4 different Mongolian horse types clustered with the cultivating breed, SH, illustrating that the different horse types of Inner Mongolia were more closely related to each other than to TB. Further analysis showed that WZ was most closely related to WS. Among the remaining three horse types, XN and BH were more closely related to each other than to SH.

Phylogenetic analysis of the mtDNA D-loop variable region was performed using MEGA 2.1 software. Sequences including the 13 haplotypes in this study and homologous sequences of different horse types from GenBank including two Przewalskii, one Tsushima, two Cheju, four Mongolian, two Arabian, two Thoroughbred, one Kazakh, two Akhal-Teke, two Guan-shan, one Guanzhong and two Yunnan horses were constructed using neighbor-joining methods. The donkey sequence was used as an outgroup. The neighbor-joining tree showed two large groups of related horses (Figure 4). The first group included the 13 different haplotype sequences of the Chinese Mongolian horses as well as with other Chinese horse types, including Przewalskii and other Mongolian horses. The second group contained haplotypes B and C as well as Thoroughbred and Arabian horse types.

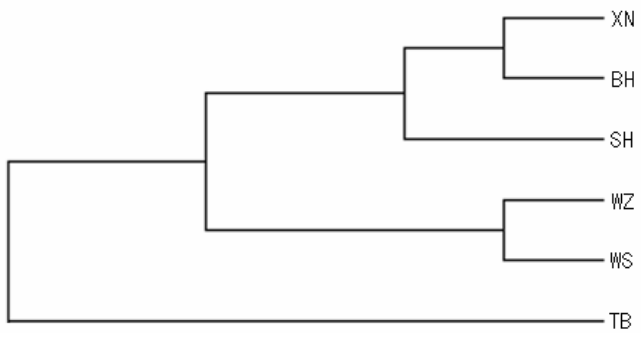

Figure 3. Phylogenetic map among different type horses.

DISCUSSION

The evolutionary relationship between the 13 haplotypes of the mtDNA D-loop variable region and other Mongolian and Arabian horse types was determined. The

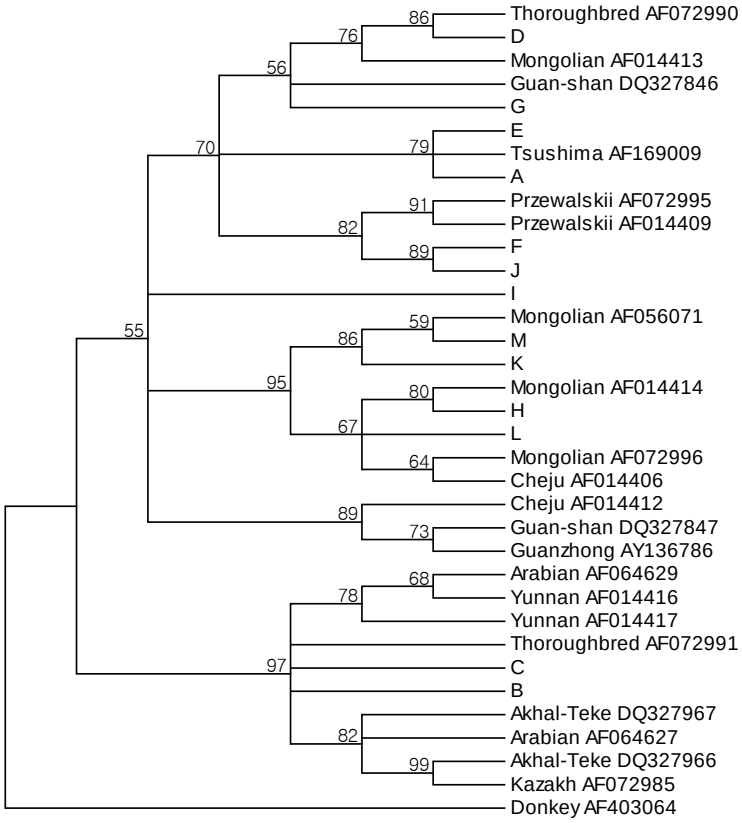

Figure 4. Neighbour-Joining tree of mtDNA D-loop variable region from different haplotypes.

resulting cladogram showed that haplotypes J and F of the Inner Mongolian horses and the two sequences of Przewalskii were clustered on the same branch. Haplotype J was common to all five of the Inner Mongolian horse types researched, and haplotype F was unique to BH. Thus, we can conclude that certain Chinese Mongolian horses had an intimate kinship with, and possibly decended from Przewalskii.

Recent studies had been undertaken to determine the relationship between Mongolian wild horses and domesticated horses at the molecular level. Zhang reported that the two metacentric chromosomes of the domestic horse were formed through Robertsonian translocations between the 4 acrocentric chromosomes of Przewalskii, leading to the domesticated horse having two less chromosomes than Przewalskii (Zhang, 2000). Myka et al. in 2003, formed a comparative genetic map of Mongolian wild and domestic horses by FISH, which reached the same conclusions found by the G-band staining method. It was also shown that acrocentic chromosomes 23 and 24 of Przewalskii were homologous to the metacentric chromosome 5 of the domesticated horse (Myka et al., 2003). Another study looking at unique nucleotide sequences of the Y chromosome suggested a taxonomic relationship between Przewalskii and the domesticated horse, and that Przewalskii's haplotype was ancestral (Wallner et al., 2003).

We detected polymorphisms within the mtDNA D-loop variable region through PCR-SSCP and found 12 haplotypes in 4 different types of local Mongolian horses and one captive breed. A thirteenth haplotype was found that was unique to the imported TB breed. The cladogram constructed of the 13 haplotypes and homologous mtDNA sequences from other horse types showed that the 13 haplotypes were dispersed among several small clades, suggesting that the Chinese Mongolian horse originated from multiple maternal lines. The suggestion that modern horses had multiple origins had been made by many other scholars as well (Kim et al., 1999; Hill et al., 2002; Mirol et al., 2002; Cozzi et al., 2004; Lopes et al., 2005).

One evolutionary branch of the Neighbor-Joining cladogram of the mtDNA D-loop variable region sequences clusters the Chinese Mongolian horse haplotypes H, L, M, and K, three Mongolia Mongolian horse sequences and a Korean Cheju horse sequence from GenBank (Figure 4). The findings show that the Chinese Mongolian horse and Mongolia Mongolian horse had a close genetic relationship, and may had the same ancestor. The Inner Mongolia Autonomous Region and Mongolia are neighbors and only within the last 100 years had become separated. Prior to this,

horse populations in this region intermingled, which may be one reason for their close genetic relationship.

Chinese Mongolian horses were widely distributed and had a complex evolution, but there had been no exploration of the evolutionary relationships between all categories of Mongolian horse in China. This research showed that the relationship between WZ and WS was closest. XN and BH also shared a close relationship, with the SH horse being more distantly related. TB was a distant relative to all 5 of the Inner Mongolian horses. SH, BH and XN came from three counties in the Hulunbeier Inner Mongolian Autonomous Region. Because of their close proximity, there may be gene exchanging, causing them to be related to each other. However, SH was originally a hybrid resulting from crosses between the tsarist Russia western horse stock, Japanese horse stock and the local Mongolian horse, explaining the more distant relationship of SH to XN and BH. WZ was produced in Wuzhumuqin county, Xilinguole league and was known as an excellent Mongolian horse type due to long-time selection by the local herdsmen. There was seldom gene exchange with the other types because of the geographical isolation, however it was found to have a close kinship to WS, which is produced in Etuoke county, Erdos. There was a great geographical distance between these locations, so it was difficult to explain their close kinship. Perhaps there was communication between these two regions historically, allowing genetic similarities to arise.

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ACCESSION NUMBERS

GenBank accession numbers for the Chinese Mongolian horse mtDNA D-Loop of haplotype A, B, D-M are from DQ297622 to DQ297633.

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