COMPARITIVE GENOMICS OF MONGOLIAN PUREBRED AND

CROSSBRED HORSES: CONSERVING AN IMPORTANT BREED

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by

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TABLE OF CONTENTS

Page

ABSTRACT

Comparative Genomics of Mongolian Purebred and Crossbred Horses: Conserving an Important

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The Mongolian horse has been primarily under natural selection since its ancestral time due to

the almost wild existence it lives in Mongolia. This has enabled the Mongolian horse to retain a

high level of ancestral diversity compared to other horse breeds that have been bred selectively

for certain traits. However, their high level of diversity and breed identity is being put in danger

as Mongolian horses are being crossbred to western breeds imported from Europe and Eastern

Russia. In addition to this, a movement has started within Mongolia to create a Mongolian Horse

Breed Registry. Unfortunately there are no precautions currently in place to ensure that only

purebred Mongolian horses are allowed into this new registry. Therefore differences between the

genomes of the Mongolian purebreds and Mongolian crossbreds were examined in order to

develop a test that will identify which individuals should be allowed into the newly proposed

registry. Currently, three microsatellite alleles found on separate loci have been identified in the

crossbreeds but not the purebreds. These three alleles could possibly be used to distinguish

between the purebreds and crossbreds; however more horse samples will have to be processed

before this can be determined. The overall diversity within the Mongolian breed was also analyzed and compared to diversity found within other breeds. This data shows the Mongolian horse to have a higher than average heterozygosity and number of alleles.

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CHAPTER I

INTRODUCTION

The Mongolian horse can be seen as both a culturally and agriculturally important breed not only for Mongolia but for the entire world. It has endured as a symbol of Mongolia; and has continued to be used in everyday Mongolian life, including transportation, breeding, herding, consumption, and racing. Since the Mongolian horse has been used for so many purposes over the past hundreds of years, they have retained a high level of their ancestral diversity and appearance (Hund 2008). In addition, their high genetic diversity could be attributed to caution by the owners to avoid inbreeding and to a strong natural selection from their almost wild existence. Many owners within Mongolia believe that two horses should not be bred unless they are more than seven generations apart (Hund 2008). Instead the owners bring in new breeding stallions from other parts of the country to avoid inbreeding.

Mongolian horses are under a strong natural selection from the environment since they are typically not stabled nor given supplemental feed, and are only caught and used for short periods of time before being rereleased (Hund 2008). There is also very little shoeing of Mongolian horses due to their hooves being hard enough to handle the terrain without such precautions (Jagchild et al 1979). This type of treatment has allowed the Mongolian horse to remain well adapted in surviving the harsh Mongolian weather; something Western breeds are unable to do without additional care. This has been changing, however, due to an effort around the capital city, Ulaanbaatar, in which wealthy individuals are importing western breeds from eastern Russia and Europe to breed to the Mongolian horse in hopes of producing faster race horses. These

mixed breed horses are not built for survival on the steppes, and often need supplemental feeding and stables in order to survive (Hund 2008). This extra care for the crossbreeds requires more money, and is therefore turning racing, a sport once accessible to anyone with a horse, into a sport for the elite (Hund 2008). This practice is also compromising the endurance and robustness of the Mongolian horse in order to create a faster race horse.

Another recent effort by many Mongolians has been to establish an official breed registry for the Mongolian horse. However, if no steps are taken to regulate which horses are allowed into this registry, it is inevitable that the registry will include crossbred horses. This would contribute to the dilution of the Mongolian horse genome and, over enough time, could cause the loss of the Mongolian breed due to continual crossbreeding. However, if genetic markers can be identified and used to distinguish between the Mongolian horses and hybrids, it could be ensured that only pure Mongolian horses are allowed into the registry.

A crucial reason for registering pure Mongolian horses separately from crossbreds is because of their high retainment of ancestral diversity. In a previous study, a population of the Mongolian horse was found to have higher levels of microsatellite diversity than 17 other breeds (Tozaki et al. 2003). This high diversity within the Mongolian breed means their genome could be the best candidate to find diversity that is lacking in other breeds; such as the very low variation that has been observed on the Y chromosome of domestic horses, a chromosome important for stallion fertility (Warmuth et al. 2012). The diversity within the Mongolian horse genome could then be used to help increase diversity within other breeds.

Mongolian history and culture

Mongolia, previously known as Outer Mongolia or Mongolian People's Republic, can be found landlocked between Russia and China. It is a large country at 1,564,100 square km and has a wide variety of landscapes including the southern Gobi desert, the western Altai Mountains, and the central steppes (Sabloff 2001). As a landlocked country it has highly variable weather and temperatures. The summers are agreeable with the average temperature being 18˚C; however the winters are often harsh with the average temperature being close to -14 ˚C (Sabloff 2001).

Mongolia was first inhabited in prehistoric times by nomadic tribes from the inner Asia area (Sanders 1987). Then in 1206, an individual named Temüjin managed to unite the warring Mongolian tribes and was awarded the title of Chinngis Khan (Sanders 1987). Under his leadership the Mongolians were able to create the Mongolian Empire which, at one point, covered most of Asia and Europe. However, the Mongolian Empire grew too large and soon began to fall to pieces. The ultimate downfall of the Mongolian Empire was the Manchurian Empire which from 1636 to 1691 slowly incorporated the broken pieces of the Mongolian Empire until all of Mongolia was under Manchurian rule (Sabloff 2001). Mongolia then stayed under Chinese rule until December of 1911 when they declared independence, but their independence was short lived when the Soviet Union overtook Mongolia in 1923 and ruled them until 1990 (Andelman 2011). Since 1990 Mongolia has become a Democracy, which has allowed both the country and its economy to have considerable growth.

The Mongolian people have been nomads since before the time of Chinggis Khan (Jagchid et al 1979). Their nomadic lifestyles are reinforced by the large amount of livestock that each Mongolian family keeps. The livestock provides housing, clothing, and food to its owners, making herding one of the main occupations found in Mongolia, (Friters 1949). In order to ensure their animals have enough food, the herder must move his animals, and therefore himself, to new pastures often enough to find areas for grazing. The five main kinds of animal the Mongolian herder is likely to have are horses, camels, sheep, goats, and cattle (Friters 1949).

In order to facilitate this nomadic lifestyle Mongolians live in gers, which are round houses covered in felt and canvas and whose walls consist of folding latticework (Sabloff 2001). These gers allow the Mongolian nomads to quickly break down their homes, transport them to a new location, and rebuild them. The gers' design also works well against the unpredictable weather that is found in Mongolia (Sabloff 2001). The use of gers has begun to diminish in recent times as a development boom has led to more urbanization and people in Mongolian cities. There the living quarters range from gers to apartments and houses. One such city that has experienced a boom over the years is the capital, Ulaanbaatar. People have given up the nomadic lifestyle in the rural areas to move into the cities for many reasons; such as for an education or job opportunities after losing their livestock to harsh Mongolian weather (Andelman 2011; Sabloff 2001). Another reason for the urbanization in Mongolia has been the mining industry. Mines for coal, gold, silver, iron ore, and lead can all be found in Mongolia; these products must then be shipped to factories and processed by workers, creating a need for large cities (Friters 1949).

In the nomadic lifestyle of the Mongolians, the horse has become an indispensable tool. The Mongolian horse allows its owners to travel, hunt, and herd; and once the animals are too old to work they can be slaughtered and used to feed and clothe their owners (Friters 1949). It can easily be argued that the fast and flexible Mongolian horse was paramount to the success of Chinggis Khan's warfare strategies and tactics (Rossabi 1994). The horses allowed his army to avoid confrontational battles and instead use feinting attacks and mobile warfare (Jagchild et al 1979). In addition to their uses as a work animal, the horse plays a very traditional role in Mongolia. This includes participation in one of the biggest traditions of Mongolia, horse racing. Many horse races occur all throughout Mongolia, but the biggest races occur during Naadam, a large festival in Mongolia that occurs every year in July (Sabloff 2001). Many Mongolians will travel long distances in order to attend and participate in a Naadam festival (Peck 1998). This often means there are about a thousand horses in each race, with the races varying from 11 to 50 km, depending upon the age and sex of the horse (Haffner 2004). Furthermore, Mongolian horse racing is different because the owners not only train their own horses, but also use children, usually their own children or grandchildren, as jockeys.

Horse domestication

The original point of evolution for the *Equus* genus was in North America, but during the Pliocene many of the species crossed over to Eurasia (Luis et al 2006). The North and South American species eventually died out, but the horses in Eurasia continued to flourish. It was in Eurasia that one species, *Equus caballus*, became domesticated by humans; although the origin of horse domestication is still inadequately understood. Archeological evidence helps to narrow

the overall timeline by showing that horse domestication did not happen earlier than the Eneolithic period (Achilli et al 2012). Archeological and mitochondrial DNA also provides support for the western Eurasian steppes to be the geographical origin for horse domestication (Warmuth et al 2012). Furthermore, studies on the horse mitochondrial genome show that as the domesticated horses moved across Eurasia, wild mares were brought into the domesticated herds to maintain the herd (Warmuth et al 2012). This is supported by the high diversity of the horse's mitochondrial genome, 18 haplotypes, and the low diversity on the Y chromosome (Achilli et al 2012).

Mongolian horse

The Mongolian horse is a sturdy breed that is often mistaken for a pony due to its small size. The height, usually 12-14 hands, and body type of the Mongolian horse varies amongst the four main groups: forest, mountain, steppe, and Gobi; and is dependent upon what kind of environment they live in (Pickeral 1999). It is believed by Mongolian herders that this variation within the breed is due to the difference in environmental pressures exerted upon the breed (Hund 2008). This diversity in physical characteristics has allowed the Mongolian horse breed to be used in a variety of ways. Horses from the same Mongolian herd can all be used for many different purposes; fast horses are used for racing, flashy horses are often used for festivals, and other horses are used for work such as transport and herding (Hund 2008).

Thoroughbred

The Thoroughbred is a very elegant horse that is widely considered to be the premier racing breed. They usually stand between 15.2 and 16.3 hands and can be found in any solid color (Pickeral 1999). However, the Thoroughbred suffers from low genetic diversity due to inbreeding. This is because the general stud book for the Thoroughbred was cut off in 1793, and since then only individuals within that studbook, or their descendants, are allowed to breed (Bokor et al 2013). Their diversity also has little variation because the Thoroughbred has been refined for a single purpose over the years, to be a fast racehorse.

Breed registry

Breed registries are tools used by owners to maintain purity within their breed and promote the specific characteristics and traits of that breed. This is done by carefully maintaining the lineages for each horse in the registry, and by only allowing the horses within their registry to participate in breeding and competitions (Ray et al 1991). Breed registries therefore usually provide more benefits to the owners if they have a registered horse versus an unregistered one (Ray et al 1991). It is due to these extra benefits that breed registries are so prevalent in the western world. Almost every western horse breed has its own registry, and in order to be registered the horse's bloodlines and pedigree must be known and tested. In fact the horse's papers and bloodlines are often a big determinate of the horse's worth in the western world (Hund 2008). This is in sharp contrast to the current system in Mongolia that is paperless, and instead relies on the owner's memory for the pedigree of each horse within his herd (Hund 2008).

Research goals

This research will help to ensure that the purity of the Mongolian horse will continue to endure in their newly forming registry. By identifying microsatellite alleles that differentiate between the purebred and crossbred Mongolian horses, an easy test can be developed to test any horses being registered for breed purity, thus ensuring the identity of the Mongolian breed is not lost. This allows the crossbreeding around the Ulaanbaatar to continue without having any major effects on the continuation of the Mongolian breed.

In addition, this research will provide more understanding into the wide range of genetic diversity found within its breed. While previous studies on the Mongolian horses have been done (Tozaki et al 2003, Du et al 2011, Kakoi et al 2007) all of the studies have focused upon other breeds, and have only used the Mongolian horse as a comparison breed. Therefore this research hopes to use microsatellites and the mtDNA control loop sequence in order to describe the genetic diversity concealed within the Mongolian horse.

CHAPTER II

METHODS

Samples

Hair samples were taken, with the owner's permission, by trained a Mongolian veterinarian from sixteen unrelated Mongolian horses located within the Tov aimag. There were four herds with four horses from each herd sampled; one herd was located in the Ulaanbaatar province while the other three herds were located in the Bulgan province. The four horses that were sampled from Ulaanbaatar province were crossbreds, whereas the remaining 12 horses from Bulgan province were purebreds. In addition to the hair samples, additional information on the horse's health and appearance was obtained from the owner by the veterinarian. The DNA was extracted from the samples according to the Qiagen DNA extract kit protocol.

Figure 1. Sampling area for the Mongolian horses

PCR

Both the forward and reverse primers for fifteen autosomal microsatellites; VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB2, HTG10, HTG7, HMS3, HMS2, ASB17, ASB23, LEX33; and one X chromosome microsatellite; LEX3; were obtained (Conant, Juras & Cothran 2011) and used to create a primer mix. Primers to amplify the 360-442 base pair control region of the mtDNA were also obtained (Luis et al 2006). A master mix was made that comprised of 4.96 mM ddH$_2$O, 1.83 mM 10X buffer, 1.83 mM MgCl, 1.83 mM dNTPS, and 0.15mM Taq DNA. Then 10.0 μL of the master mix was combined with 1.2 μL of the DNA (30μg/μL) and 2.5 μL of the primer mix. The samples were then placed into a thermal cycler. The PCR reaction ran at 95˚C for 5 minutes, 85 ˚C for 10 minutes, then repeated cycles of 95 ˚C for 1 minute, 60 ˚C for 30 minutes, 72 ˚C for 30 seconds, and then finished the reaction at 72 ˚C for 10 minutes. After the PCR was complete the samples were stored at 4 ˚C. In order to run samples for the mtDNA, the PCR reaction ran at 95˚C for 5 min, then 30 repeated cycles of 94˚C for 1 minute, 55 ˚C for 30 minutes, 72 ˚C for 30 seconds, with the reaction finishing at 72 ˚C for 10 minutes. After the PCR was done the samples were stored at 4 ˚C. The samples were then run on 2% Agarose gel with Ethidium Bromide to allow for DNA visualization under the UV light.

Sequencing

The PCR samples were cleaned up by running them through a Sephadex column. The mtDNA PCR samples were treated with the Big Dye v. 1.1 kit according to instructions and sequenced with an ABI PRISM 377 sequencer in the Cothran lab (Conant, Juras, Cothran 2011). The sequences were then trimmed of poor quality bases and aligned using ProSeq and Chromas lite

software (Filatov 2009). The resulting halotypes were compared to sequences available in Genbank. The diversity between the Mongolian horses was estimated by observing the nucleotide diversity and halotype diversity with DnaSP 5.10 (Librado et al 2009).

Genotyping

The PCR samples were cleaned up by running them through a Sephadex column. The microsatellite PCR samples were then analyzed by using polyacrylamide gel electrophoresis and automated multicolored fluorescence technology. The ABI PRISM 377 Genetic Analyzer was used to separate the DNA. STRand software was used to analyze the size of the DNA fragments and determine the loci for the microsatellites (Toonen et al 2001). The data resulting from the microsatellite analysis was used to estimate genetic diversity within the breed. This was done by using GeneAlEx software to determine the mean number of alleles, expected and observed heterozygosity, genetic polymorphisms and inbreeding coefficients (Peakall et al 2012). This data was then compared to the data from 40 domestic horse breeds from around the world, previously analyzed in Dr. Cothran's lab, in order to compare the diversity across breeds. The genetic structure was examined by Fst-based estimators and Amova with GeneAlEx. A restricted maximum likelihood phylogenetic tree with 100 bootstraps was built in order to determine the relationship of the Mongolian horse to 66 other breeds with the use of Phylip 3.69 (Felsenstein 2005). The phylogenetic tree was then created with TreeView (Page 1996). Alleles that are unique to only the hybrids were studied with the use of GeneAlEx.

CHAPTER III

RESULTS

In the sample of the Mongolian horses, 10 of the horses were males and 6 of them were females; and the age of the animals sampled ranged from 1 to 14 years, with the average age being 5 years. In comparing the purebreds versus the crossbreds, the average height and weight of the purebreds were 145 cm and 334.2 kg respectively. The average height and weight of the crossbreds were 149 cm and 377.5 kg respectively. The owners of the crossbred horses stated their main causes of death were injury and colic, whereas the owners of purebred horses said the main causes of death were environmental conditions and predatory attacks. All the owners indicated having previous health issues of parasites, open wounds, colic, lameness, and strangles at least once before in their horses; and they stated they wormed their horses twice a year.

SOUM	SEX	AGE	COLOR	WEIGHT (kg)	HEIGHT (cm)	Pure or Crossbred
Ulziit Khoroo	M	7	grey	400	149	Crossbred
Ulziit Khoroo	M	5	bay	401	153	Crossbred
Ulziit Khoroo	F	3	palomino	380	147	Crossbred
Ulziit Khoroo	F	2	palomino	330	147	Crossbred
Dashiuchilen	M	1	grey	180	132	Purebred
Dashiuchilen	F	2	brown	160	141	Purebred
Dashiuchilen	F	5	palomino	350	144	Purebred
Dashiuchilen	M	5	palomino	380	148	Purebred
Dashiuchilen	M	6	palomino	350	146	Purebred
Dashiuchilen	M	4	bay paint	340	142	Purebred
Dashiuchilen	M	7	red paint	350	144	Purebred
Dashiuchilen	M	3	brown	400	148	Purebred
Dashiuchilen	F	4	grey	370	143	Purebred
Dashiuchilen	M	14	dark bay	360	143	Purebred
Dashiuchilen	M	7	grey/white	370	143	Purebred
Dashiuchilen	F	6	gruella	n/a	n/a	Purebred

Table 1. The location and physical characteristics of the Mongolian Horses

The amplification of the 16 microsatellites by Polymerase Chain Reaction was successful. The genotyped results were then used to determine the allele frequencies and heterozygosities for the 15 autosomal microsatellites. The purebred Mongolian horses had a higher mean number of alleles, observed heterozygosity, expected heterozygosity, and unbiased expected heterozygosity than the crossbred horses. The AMOVA test between the purebreds and crossbreds showed that 98% of the variance was within individuals and that 2% was between the purebreds and crossbreds populations. Fst-based tests were also used to examine the differences between the purebreds and crossbreds. The results showed there was inbreeding between the two populations F_{IS}= -0.014 and Fst=0.020.

Microsatellite Allele Frequencies

	Na			Ho			He			uHe		
	Pure	Ref.	Cross	Pure	Ref.	Cross	Pure	Ref.	Cross	Pure	Ref.	Cross
VHL20	7.000	10.000	4.000	0.833	0.929	1.000	0.813	0.872	0.656	0.848	0.905	0.750
HTG4	4.000	6.000	2.000	0.750	0.643	0.250	0.688	0.513	0.469	0.717	0.532	0.536
AHT4	6.000	9.000	3.000	0.750	1.000	0.750	0.764	0.809	0.594	0.797	0.839	0.679
HMS7	8.000	6.000	4.000	1.000	0.429	0.750	0.743	0.676	0.656	0.775	0.701	0.750
HTG6	6.000	6.000	3.000	0.583	0.643	0.750	0.632	0.559	0.531	0.659	0.579	0.607
AHT5	5.000	6.000	5.000	0.833	0.786	1.000	0.788	0.783	0.781	0.822	0.812	0.893
HMS6	6.000	5.000	3.000	0.667	1.000	1.000	0.757	0.763	0.594	0.790	0.791	0.679
ASB2	9.000	7.000	6.000	0.833	0.643	1.000	0.840	0.816	0.781	0.877	0.847	0.893
HTG10	7.000	7.000	6.000	0.833	0.929	0.750	0.753	0.781	0.813	0.786	0.810	0.929
HTG7	5.000	4.000	2.000	0.583	0.500	0.750	0.726	0.684	0.469	0.757	0.709	0.536
HMS3	7.000	6.000	4.000	1.000	0.714	1.000	0.781	0.704	0.688	0.815	0.730	0.786
HMS2	8.000	5.000	4.000	0.833	0.714	0.500	0.799	0.689	0.719	0.833	0.714	0.821
ASB17	12.00	13.00	4.000	0.833	0.857	0.500	0.861	0.883	0.688	0.899	0.915	0.786
ASB23	9.000	10.00	6.000	1.000	0.714	1.000	0.819	0.839	0.781	0.855	0.870	0.893
LEX33	7.000	8.000	5.000	0.833	0.786	0.750	0.792	0.819	0.750	0.826	0.849	0.857
Mean	7.067	7.200	4.067	0.811	0.752	0.783	0.770	0.746	0.665	0.804	0.774	0.760
SE	0.511	0.619	0.345	0.034	0.044	0.059	0.015	0.028	0.029	0.016	0.029	0.033

Table 2. A comparison of the number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), and unbiased expected heterozygosity (uHe) for the purebreds, crossbreds, and a reference group of Mongolian horses previously sampled.

The purebred and crossbred horses were also compared for private alleles. During this comparison, three alleles were found in the crossbred horses that were not found in the purebreds. Each of these alleles occurred only once, in separate horses, and they were all from different loci. The alleles were: the 162 allele at the HMS3 locus, the 185 allele at the ASB23 locus, and the 209 allele at the LEX33 locus.

 The mean number of alleles for the purebred population was 7.067; this is higher than the average mean number of alleles of 6.585 for 40 other breeds. In addition the Mongolian horse had a higher observed and expected heterozygosity, 0.811 and 0.770 respectively, than the average observed and expected heterozygosity for the other 40 horse breeds, 0.714 and 0.720. In fact, the Mongolian horse had the highest observed heterozygosity out of all of the horse breeds compared (Table 3). Additionally, the Mongolian horses have shown to be 100% polymorphic for the 15 autosomal microsatellite loci tested.

Phylip 3.69 was used to generate a restricted maximum likelihood phylogenetic tree from the microsatellite data made with 100 RML bootstraps. The tree consisted of the Mongolian horse and 66 other horse breeds with the Przewalski's horse as the out group. In this tree the Mongolian horse was grouped closely with the Kyrgyzstan horse, Yakut, and Altai native horse. All three of these horse breeds are inner Asian breeds. The bootstrap values for the tree were low, less than 90%, even though the overall clades remained constant; however this is common for trees made with horse breeds since they all belong to the same species.

Breed	n	He	sd	Ho	sd	Fis	Mean number of alleles	SE
Turkoman	113	0.77	0.08	0.75	0.08	0.034	8.733	2.081
Venezuelan Criollo	213	0.78	0.06	0.76	0.06	0.026	8.400	2.471
Colombian Paso Fino	88	0.75	0.07	0.75	0.11	0.006	7.800	2.104
Shetland pony	97	0.75	0.10	0.72	0.08	0.038	7.800	2.688
Mangalrga Marchador	111	0.74	0.07	0.70	0.08	0.053	7.600	1.925
Garrano	37	0.79	0.06	0.75	0.13	0.042	7.533	1.857
Irish Draught	47	0.78	0.06	0.78	0.07	0.003	7.533	1.746
Morgan Horse	59	0.76	0.08	0.71	0.10	0.060	7.533	1.962
Caspian pony	87	0.74	0.12	0.74	0.14	-0.001	7.400	1.818
Peruvian Paso	42	0.75	0.11	0.76	0.10	-0.010	7.333	1.619
Brazilian Criollo	50	0.74	0.08	0.76	0.10	-0.027	7.200	1.558
American Saddlebred	174	0.71	0.14	0.70	0.16	0.019	7.133	1.996
Quarter horse	40	0.76	0.08	0.74	0.09	0.033	7.067	1.436
Rocky Mountain Horse	45	0.74	0.07	0.72	0.07	0.035	7.067	1.289
Argentine Criollo	25	0.73	0.07	0.74	0.09	0.013	6.870	1.461
Campolino	30	0.74	0.11	0.71	0.16	0.034	6.867	1.500
Arabian	89	0.70	0.11	0.64	0.14	0.091	6.867	2.029
Connemara Pony	69	0.75	0.08	0.77	0.08	-0.017	6.800	1.833
Hannoverian	34	0.77	0.06	0.75	0.13	0.035	6.733	1.436
Pantaneiro	25	0.75	0.09	0.77	0.12	-0.025	6.667	1.350
Puerto Rican Paso Fino	60	0.68	0.08	0.70	0.07	-0.027	6.667	1.075
Tennessee Walker	49	0.68	0.12	0.72	0.17	-0.052	6.400	1.818
Sella Francis	41	0.74	0.09	0.73	0.13	0.010	6.267	1.526
Dales pony	86	0.69	0.13	0.71	0.12	-0.031	6.200	1.327
Trakehner	64	0.73	0.10	0.76	0.09	-0.042	6.067	2.144
Luistano	62	0.72	0.13	0.68	0.14	0.058	6.067	1.569
Welsh Pony	31	0.75	0.07	0.74	0.11	-0.021	6.000	1.707
Andulusian	33	0.73	0.10	0.70	0.08	0.047	5.933	1.181
Fell Pony	55	0.72	0.08	0.72	0.12	0.002	5.933	1.340
Chilean Crillo	30	0.72	0.08	0.69	0.12	0.038	5.933	1.181
American Standardbred	33	0.69	0.10	0.67	0.12	0.032	5.867	1.500
Holsteiner	36	0.74	0.07	0.72	0.16	0.027	5.800	1.424
Thoroughbred	139	0.71	0.08	0.69	0.10	0.026	5.667	1.300
Halfinger	46	0.67	0.18	0.66	0.19	0.015	5.667	1.491
Suffolk Punch	50	0.71	0.12	0.73	0.14	-0.033	5.467	1.543
Shire	32	0.66	0.11	0.69	0.14	-0.032	5.400	1.083
Ahal Teke	29	0.65	0.18	0.73	0.23	-0.111	5.400	1.405
Highland Pony	28	0.67	0.08	0.68	0.08	-0.013	5.333	1.193
Exmoor pony	46	0.63	0.10	0.60	0.11	0.052	5.267	1.181
Friesian	134	0.52	0.20	0.50	0.19	0.041	5.133	2.093
Mongolian horse (reference)	14	0.75	0.03	0.75	0.04	-0.013	7.200	0.619
Mongolian horse	12	0.77	0.02	0.81	0.03	-0.051	7.067	0.511

Table 3. Observed and expected heterozygosity (Ho and He), Fis, and mean number of alleles for the Mongolian horse, a reference group of Mongolian Horses previously sampled, and 40 horse breeds

The sequences for the mtDNA were only acquired for the 12 purebred Mongolian horses. All sequences were originally around 480 nucleotides long, but were shortened to 404 or 403 nucleotides with Chromas lite to get rid of poor nucleotide readings. The mtDNA was then blasted with Blastn on GenBank to determine the closest breeds. Of the 12 individuals that were sequenced, 3 of them had completely unique mtDNA sequences, with their closest matches being 99%. The remaining 9 horses had a 100% match to various breeds. The specific breeds that were hit more than once in the GenBank were the Kathiawari, Arabian, Maremmano, Vladimir Heavy Draught, and Manipuri Pony. DnaSP was used to determine that there was 10 mtDNA haplotypes out of the 12 horses sequenced, giving an overall haplotype diversity of 0.9697. The nucleotide diversity was determined by looking at individual sites, or column by column so that all 404 nucleotides sites would be analyzed since once sequence contained a deletion. This gave 21 polymorphic sites with an overall nucleotide diversity of 0.01905, and the average number of differences being 7.697.
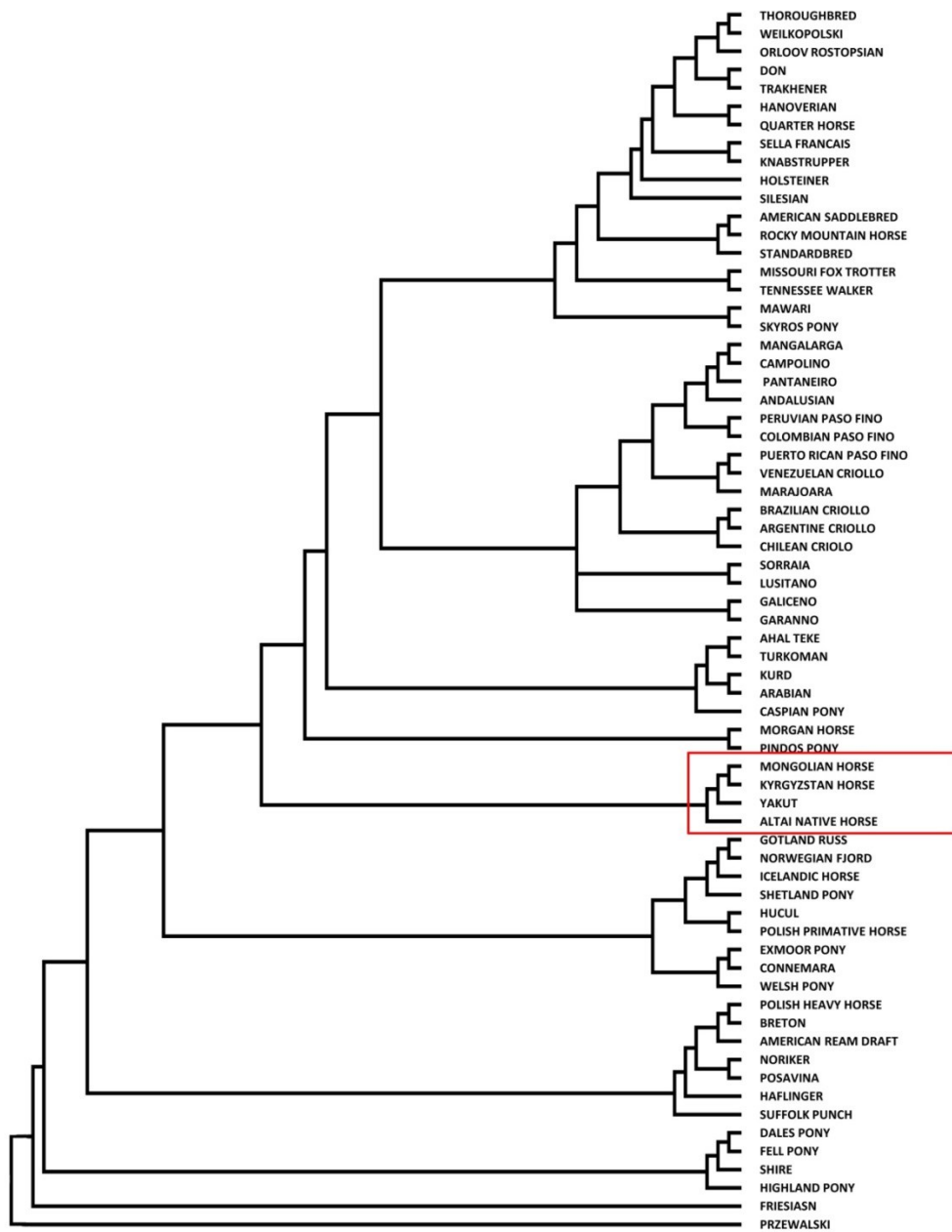
Figure 2. Maximum likelihood phylogenetic tree with 100 bootstrap replicates consisting of the Mongolian horse and 66 other breeds. All bootstrap values were lower than 90% although the overall clades remained constant.

CHAPTER IV

DISCUSSION

The Mongolian purebreds vary from the crossbreds in both phenotypic and genetic aspects. Phenotypically, the Mongolian purebred is a smaller horse. Both the average height and weight of the purebreds was lower than the average height and weight of the crossbreds. This was to be expected since the mixed parentage of the crossbreeds consists of Thoroughbreds and Mongolian horses; and the average height and weight of the Thoroughbred is larger than that of the Mongolian horse. In addition, the crossbreeds' appearance is more refined than the sturdy Mongolian horse. This lack of sturdiness is evident in the variation in the main causes of death for the two groups of horses. The sturdier purebreds had less health issues and more problems with the environment, such as harsh weather and predator attacks; whereas the crossbreds had more health problems, such as colic and injury.

Microsatellites are short tandem repeats that occur in a large percent of genomes (Selkoe 2006). Microsatellites have a high mutation rate and therefore a large allelic variation that makes them ideal for analyzing genetic variation and distances (Bordin 2011). In addition, microsatellites follow an easy and stable inheritance pattern when being passed down from the parent to the offspring (Tozaki et al 2001). It is due to their wide variability but stable inheritance that microsatellites were used to identify genetic differences between the purebreds and crossbreds. The main purpose was to determine allelic differences between the purebreds and crossbreds by finding private alleles for each group. This resulted in the discovery of three possible alleles at

22

three different loci that were found only in the crossbreds. These alleles could possibly be used to distinguish between the Mongolian purebreds and crossbreds. However, since our original sample size consists of only 16 horses, more samples of both the purebreds and crossbreds must be collected and genotyped before anything can be concluded.

In comparing the microsatellite diversity between the Mongolian purebreds and hybrids, the purebreds had consistently higher mean number of alleles, observed heterozygosity, expected heterozygosity, and unbiased heterozygosity. However, these results could change once larger sample sizes for the two populations are obtained. The AMOVA test showed high variance within the individuals and low variance between the purebred and crossbred populations. The Fst tests showed crossbreeding between the purebreds and crossbreds, as was to be expected. Overall, it can be concluded that there is a genetic difference between the purebreds and crossbreds.

Despite the small sample size of the Mongolian purebreds, they showed to have higher than average heterozygosity and mean number of alleles, and the highest overall observed heterozygosity for the 15 microsatellites (as shown in Table 4). This high diversity within the Mongolian horses is most likely because their breed has been under natural selection compared to the artificial selection of the horse breeds they were compared to. In addition, the phylogenetic tree, shown in Figure 2, shows the Mongolian purebred to be closely related to the other inner Asian horse breeds (Kyrgyzstan horse, Yakut, and Altai native horse) but not any of the common Western breeds. This is due to the Mongolian breed having founders' that were not

Thoroughbreds, Arabian, or Barbs, which have been the founders for many of the Western breeds.

The 360 to 442 bp control region of the mitochondrial DNA in horses is very important in determining both their origin and diversity because of the wide-ranging variation found both within and among the breeds (Luis et al 2006). Therefore the control region of the mtDNA was sequenced for the 12 purebred horses and shortened to 404 nucleotides for all but one sequence that was shortened to 403 nucleotides due to a deletion within that sequence. Of these 12 sequences, there were 10 distinct haplotypes and 3 completely unique sequences. This showed wide haplotype diversity for the mtDNA of the Mongolian horses since there are only 18 major haplotypes for the domestic horse (Achilli et al 2012).

The ultimate goal of the project is to create an easy genetic test to determine if the horse is a Mongolian purebred or crossbred. In order to accomplish this we will continue to acquire more Mongolian horse hair samples and genotype them for the 15 autosomal microsatellites. This will allow us to positively identify specific microsatellite alleles that are present only in crossbreds, and will ultimately result in being able to limit the horses allowed into the Mongolian Horse Registry to purebred Mongolian horses only.

Conclusion

The analysis of the microsatellites for the Mongolian purebreds and hybrids identified three possible alleles at three separate loci; ASB23, HMS3, LEX33; that could be used to distinguish between the two groups of horses. We plan on continuing to genotype more Mongolian purebreds and hybrids at all 15 autosomal microsatellites in order to identify more possible microsatellite alleles and, to ensure that the three microsatellite alleles that we have identified could be used to distinguish between the two populations. Both of these tasks will be possible with a larger sample population. We also plan on continuing to search the Mongolian horse genome for diversity lacking within other breeds by genotyping and sequencing more Mongolian horses and comparing their data to other breeds.

The Mongolian horse has remained essentially the same since the time of Chinngis Khan. However, with the increased crossbreeding occurring around the capital, the integrity of this robust breed is being put in danger. This will particularly be a problem if the crossbred horses are allowed into the newly forming registry; which would cause the Mongolian horse genome to become diluted and eventually the Mongolian horse breed would be lost. If we can identify microsatellites that distinguish between the Mongolian purebreds and hybrids, we can ensure that only purebred Mongolian horses would be allowed into the registry. This in turn safeguards the Mongolian breed from being lost due to continual outbreeding. This is a vital task since the Mongolian horse is an important breed, whose full diversity has not yet been discovered.

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