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Genetic Diversity of Donkey (*Equus asinus*) by Mitochondrial DNA (mt-DNA) Analysis

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ABSTRACT

Little research has been conducted on domestic donkeys compared with other livestock and companion animals in South Korea. There is no database that records the origins of domestic donkeys in South Korea, although most of the origins of Korean domestic donkeys are known to be in the northern areas (China, Mongolia). The present study conducted research to shed light on the phylogenetic characteristics and migratory route of domestic donkeys. This is the first mitochondrial DNA (mt-DNA) analysis of donkeys in South Korea. Mt-DNA base pairs of five Korean domestic donkeys were analyzed. A total of 16,670 base pairs and 38 related genes were observed in these donkeys. The result of an analysis of haplotype in CytB domain, revealed that there were 5 polymorphisms, 3 haplotypes, a haplotype diversity value of 0.800 and nucleotide diversity value of 0.00211 in domestic donkeys. As a result of analyzing the phylogenetic analysis based on these results, it is assumed that domestic donkeys were introduced to South Korea via China originated from the Somali wild ass breeds of northern region.

Key words: Donkey, mt-DNA, Haplotype, Phylogenetic Analysis, Somali wild ass, Korea

INTRODUCTION

Donkeys are one of the largest livestock that do not even study the most, although economically important. The process of domestication of the donkey is particularly interesting because the donkey has been widely used by humans a means of transport, are reported to have been domesticated approximately 5,000~6,000 years ago (Clutton-Brock 1992; Blench 2004; Smith and Pearson 2005; Rossel et al. 2008; Davis 2019). The domestication of the donkey dramatically changed the ancient transportation systems in Africa and Asia, indicates a major cultural move away from settlement and an agrarian society life-style towards more relocation and trade (Beja-Pereira et al. 2004; Rosenbom et al. 2015).

There are approximately 1,000 donkeys bred in South Korea, mainly for tourists and meat, but there is little research on them, especially when it comes to their origins (Yun et al. 2018). The equine genome contains nearly 3,000 megabases (Mb) of DNA, similar to other mammals. The DNA is distributed in 31 autosomes as well as the X and Y heterosomes. A horse has 50,000 to 70,000 genes, which consist of repeated bases. These repeated bases have various forms such as minisatellite, microsatellite, α-satellite, SINE, and LINE. Repeated bases are very useful in genetic studies because they enable the differentiation of individual identification, parentage verification, gene mapping, and determination of breeds (Binns et al. 2000).

The horse mitochondrial genome is composed of approximately 16,660 nucleotides. Due to the strict maternal inheritance of mt-DNA, individuals within a maternal family line share the same mt-DNA haplotypes, thereby allowing the maternal line assignment accuracy to be evaluated (Wan et al. 2004). Several researchers have shown that using two or more mt-DNA markers might be more powerful for genetic diversity analysis (Pedrosa et al. 2005). The mt-DNA D-loop does not contain genetic information, which is usually utilized as a very useful utensil in phylogenetic analysis (Groeneveld et al. 2010; Han et al. 2014). Genetic studies based on mt-DNA pointed out that African wild donkeys are the most likely ancestors of domestic donkeys, but questions about their origins remain unresolved (Rosenbom et al. 2015).

In *Equus caballus*, the mt-DNA sequence was decided by Xu and Arnason (1994) and researched by Oakenfull et al. (2000) and Vilstrup et al. (2013). Analysis of mt-DNA

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genome from a modern horse appeared that there were 18 haplogroups, and the various diverse genetics of wild horses were mixed into domestic species during the domestication process (Lippold et al. 2011; Achilli et al. 2012).

The Equidae family includes a single genus Equus which is classified into four subgenera having eight species. Donkeys are classified in the equine family, which also includes horses, zebras, and mules (Wilson 1990).

For centuries, donkeys have played an important role in agriculture and transport (Xie 1987; Seyiti and Kelimu 2021). Currently, donkeys are useful not only for meat, but also for Ejiao (a kind of Chinese medicine made by donkey skin), milk, and cosmetics manufacturing raw materials (Kumeta et al. 2014; Davis 2019). Also, donkeys are used as a working power in several world regions (Cozzi et al. 2018).

As a result of investigating the morphological characteristics of donkeys raised in South Korea, the ear length (17-28cm, average 23cm), body height (90-135cm, average 118.3cm), and body length (109-150cm, average 131.2cm) were confirmed. Coat colors was distributed white, chestnut, gray, black, and brown (Yun et al. 2018). Most of the domestic donkeys are believed to have come from northern areas such as China, but there is no accurate literature in South Korea. The present study was conducted to examine the phylogenetic characteristics and route of introduction of domestically bred donkeys.

MATERIALS AND METHODS

DNA Extraction, PCR Amplification and Sequencing

The mt-DNA was extracted from whole blood samples (3-5mL) of 5 donkeys using the QIAamp DNA Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions. The DNA was quantified using NanoDrop™ 1000 Spectrophotometer. In order to analyze of mt-DNA sequence, two pairs of primer (F-CCCCAAGGACTA TCAA, R-GTTCTTTCTTCAGGGCCATT, GenBank X97337) were designed. For PCR, 2μL template DNA, 2μL of both 10pmol forward and reverse primers, and 2μL sterile distilled water were mixed in PCR Premix buffer (QIAgen, Hilden, Germany), adjusted to a total volume of 15μL, and then amplified by the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). The PCR amplification was as follows; initial denaturation for 5min at 94℃, followed by 35 cycles (denaturation at 94℃ for 30s, annealing at various temperatures for 30s and extension at 72℃ for 60~120s). An extension process at 72℃ for 7 min was added after the final cycle.

PCR products were purified using Millipore plate MSNU030 (Millipore SAS, Molsheim, France), then Sanger-sequenced with the BigDye terminator v3.1 sequencing kit and a ABI 3730xl Automated Sequencer (Applied Biosystems, Foster City, USA).

Nucleotide sequences were determined on both strands of PCR amplification products at Macrogen Sequencing Facility (Macrogen Inc., Seoul, Korea). Nucleotide sequence data were analyzed with Variant Reporter Computer Software Version 1.1 (Applied Biosystems, Foster City, USA). BioEdit v7.2.5 (Hall 2004) Sequence Alignment Editor Software was used to prove and correct individual electropherograms of the sequences. All sequence alignments were performed using a general reference sequence (GenBank).

Data Analysis

DNA sequence polymorphism software (DNASP ver. 5.1) was used to explore the variation in regions, estimate hereditary variation, and determine the haplotypes (Librado and Rozas 2009). For an analysis of the cousin relationships of haplotypes and the hereditary relationship between groups, Network Software Ver. 4.5 was used (www.fluxus-technology.com) based on the result of donkey base sequence registered 70 world donkeys in the NCBI database (Rozas et al. 2003).

RESULTS

The results of the mt-DNA genome analysis are presented in Table 1 and Fig. 1. A total of 16,670 bases were observed in this study. Also, a total of 38 related genes were observed including 22 genes that indicate tRNA, 2 genes that indicate rRNA, 13 genes that provide information to produce proteins, and 1 control region.

The analysis of haplotypes in the CytB domain is shown in Table 2. Data on CytB in donkey stored in the NCBI revealed that the number of polymorphic sites was 89; the number of haploids, 68; the diversity of haploids, 0.997; and the diversity of acid, 0.00911. The result of an analysis of 5 domestic donkeys showed that the region of polymorphisms was 5; the number of haploids, 3; the diversity of haploids, 0.800; and nucleotide diversity, 0.00211, which was relatively less than that of donkeys registered with NCBI.

The result of a network analysis using the median joining method based on the 68 haplotypes observed is shown in Fig. 2. As shown in Fig. 2, the 68 haplotypes of 75 individuals including 5 domestic donkeys were classified into Clades A (left, Somali *E. asinus*), B (top center, Chinese *E. asinus*), C (bottom center, Chinese *E. asinus*), and D (right, domestic *E. asinus*) through 15 Median Vectors (MVs).

DISCUSSION

In contrast with genomic DNA, mt-DNA nucleotide sequences are a recognized utensil for resolving phylogenetic relationships at different evolutionary level of its special properties (Sun et al. 2016). Also, mt-DNA is very useful for relationship studies, which compare close species of animals and plants (Tanaka et al. 2014; Iorizzo et al. 2012).

In the present study, an analysis of the mt-DNA genome showed a total of 16,670 bases. This was shorter than the 16,813 bp reported by Sun et al. (2016), but showed similar results. Also, the results of cytB analysis of domestic donkeys presented relatively small value, indicating that there are few breeding individuals and little blending within the population of domestic donkeys in South Korea. Cozzi et al. (2018) studies 6 Italian donkeys breeds using mt-DNA D-loop, and found 15 haplotypes, grouped in three haplogroups and Xia et al. (2019) reported that the haplotype diversity of Egyptian donkeys and Brazilian donkeys was 0.910±0.032, 0.879±0.060, respectively, showing slight differences from the results of this study. Therefore, research on more donkey breeds samples is required in the future.

Table 1: Sequence characterization of donkey mitochondrial genome in South Korea

Sequence (Start/End)	Locus name	Length (nt)	Haplotype	Haplotype Diversity	Nucleotide diversity	InDel sites	Total No. of mutations	Singleton variable sites	Parsimony informative sites
1-71	tRNA-Phe	71	1	0	0	0	0	0	0
72-1045	12SrRNA	974	1	0	0	0	0	0	0
1046-1112	tRNA-Val	67	1	0	0	0	0	0	0
1113-2692	16S rRNA	1340	4	0.9	0.00164	1	5	4	1
2693-2766	tRNA-Leu (UUR)	75	2	0.4	0.00533	0	1	0	0
2769-3725	NADG S1	957	1	0	0	0	0	0	0
3725-3793	tRNA-lle	69	1	0	0	0	0	0	0
3791-3863	tRNA-Gin	73	1	0	0	0	0	0	0
3866-3934	tRNA-Met	69	1	0	0	0	0	0	0
3935-4975	NADG S2	1026	4	0.9	0.00214	13	5	4	1
4974-5042	tRNA-Trp	69	1	0	0	1	0	0	0
5048-5116	tRNA-Ala	69	1	0	0	0	0	0	0
5118-5190	tRNA-Asn	73	1	0	0	0	0	0	0
5191-5225	L-stran	35	1	0	0	0	0	0	0
5223-5288	tRNA-Cys	66	1	0	0	0	0	0	0
5289-5355	tRNA-Tyr	67	1	0	0	0	0	0	0
5357-6901	CytC S1	1545	4	0.9	0.00129	0	4	2	2
6899-6967	tRNA-Ser (UCN)	68	1	0	0	0	0	0	0
6976-7042	tRNA-Asp	67	2	0.4	0.01791	0	3	2	1
7043-7726	CytC S2	684	5	1	0.0114	0	18	15	3
7730-7798	tRNA-Lys	68	1	0	0	0	0	0	0
7800-8003	ATP S8	208	2	0.4	0.00196	0	0	1	0
7961-8641	ATP S6	680	5	1	0.00709	0	11	9	2
8641-9424	CytC S3	809	1	0	0	0	0	0	0
9425-9494	tRNA-Gly	71	1	0	0	0	0	0	0
9495-9839	NADH S3	334	1	0	0	0	0	0	0
9842_9910	tRNA-Arg	69	1	0	0	0	0	0	0
9912-10208	NADH S4L	298	2	0.6	0.00606	1	3	0	3
10202-11579	NADH S4	1377	4	0.9	0.00116	0	3	1	2
11580-11648	tRNA-His	69	1	0	0	0	0	0	0
11649-11708	tRNA-Ser	60	1	0	0	0	0	0	0
11710-11779	tRNA-Leu	70	1	0	0	0	0	0	0
11780-13600	NADH S5	1821	3	0.7	0.00132	0	6	6	0
13584-14111	NADH S6	529	5	1	0.00606	1	7	5	2
14112_14180	tRNA-Glu	69	1	0	0	0	0	0	0
14185..15324	Cyt_B	1140	5	1	0.0093	0	23	16	7
15325..15396	tRNA-Thr	72	1	0	0	0	0	0	0
15398..15463	tRNA-Pro	67	1	0	0	0	0	0	0
15464-16670	Control	1281	3	0.8	0.00182	18	3	0	3

Table 2: Number of polymorphic sites, number of mt-DNA haplotypes, haplotype diversity and nucleotide diversity of donkeys

Population	No. of samples	No. of Polymorphic sites	No. of haplotypes	Haplotype diversity	Nucleotide diversity
World donkey (NCBI)	70	89	68	0.997	0.00911
Domestic donkey	5	5	3	0.800	0.00211

Five domestic donkeys were analyzed and 70 world donkeys were registered with NCBI for the cousin relationship of haplotypes and the hereditary relationship in this study. According to the medican network profile of mt-DNA haplotypes observed in this study, domestic donkeys raised in South Korea may be originated from *E. asinus saliqualensis* (Somali wild ass) via China.

Beja-Pereira et al. (2004) suggested that donkeys have African maternal origins and exclude the possibility that their ancestors were Asian wild animals. It has also been inferred that the Chinese donkey breed originated from African wild donkey. Ultimately, results of this study were consistent with the results of Sun et al. (2016), who reported thorough mt-DNA analysis of Chinese Dezhou donkeys that Chinese donkey breeds were originated from the *E. asinus somaliensis* (Somali wild ass), not the Asian wild ass. Chen et al. (2006) and Zhang et al. (2010) have reported that modern Chinese donkeys originated from African maternal origins.

Due to modern industrialization, the number of donkeys has decreased significantly, though the ways they are used have greatly diversified (Xie 1987; Xu et al. 2012). Recently, there has been an increase in raising donkeys used as meat and a component of traditional Chinese medicine (Lei et al. 2007; Kumeta et al. 2014).

Donkeys raised in South Korea are mainly bred for tourism and meat, but systematic population management and disease management on donkey have been less than that on other animals. In Korea, donkeys are expected to get a lot of attention in the future as a high value-added genetic resources. In some way, it might be disguised turning into a horse meat. To aim this purpose, it is necessary to protect the breeding farms of donkey and its meat product systems.

In the future, in order to meet the needs of the domestic donkey markets and produce better quality meat, it is necessary to develop and improve donkey breeding technology. Further research is also needed to manage the

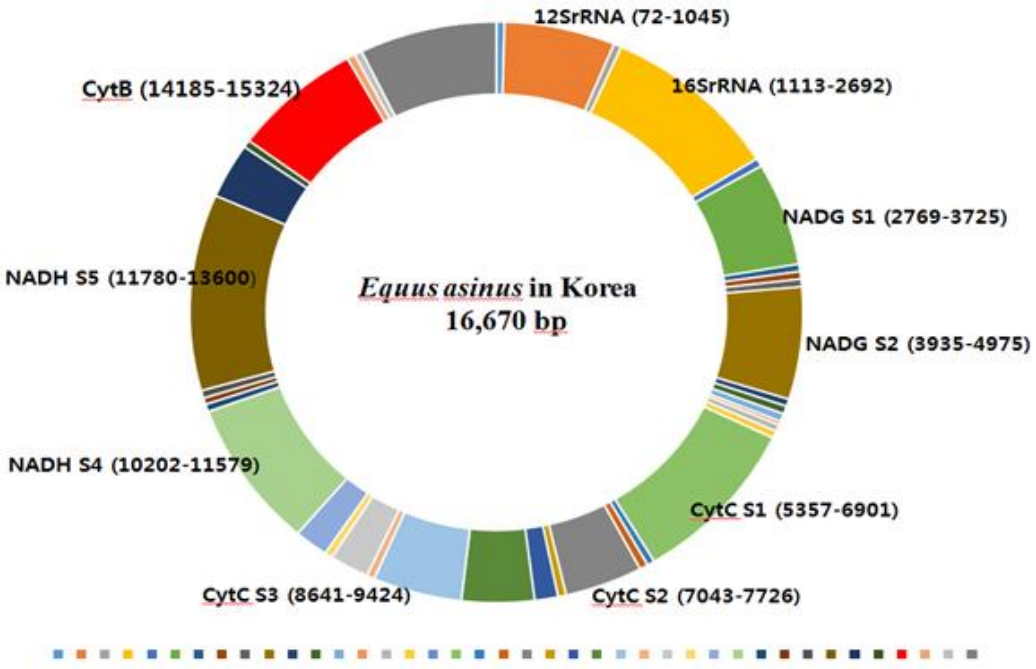

Fig. 1: Map of the donkey mitochondrial genome in South Korea.

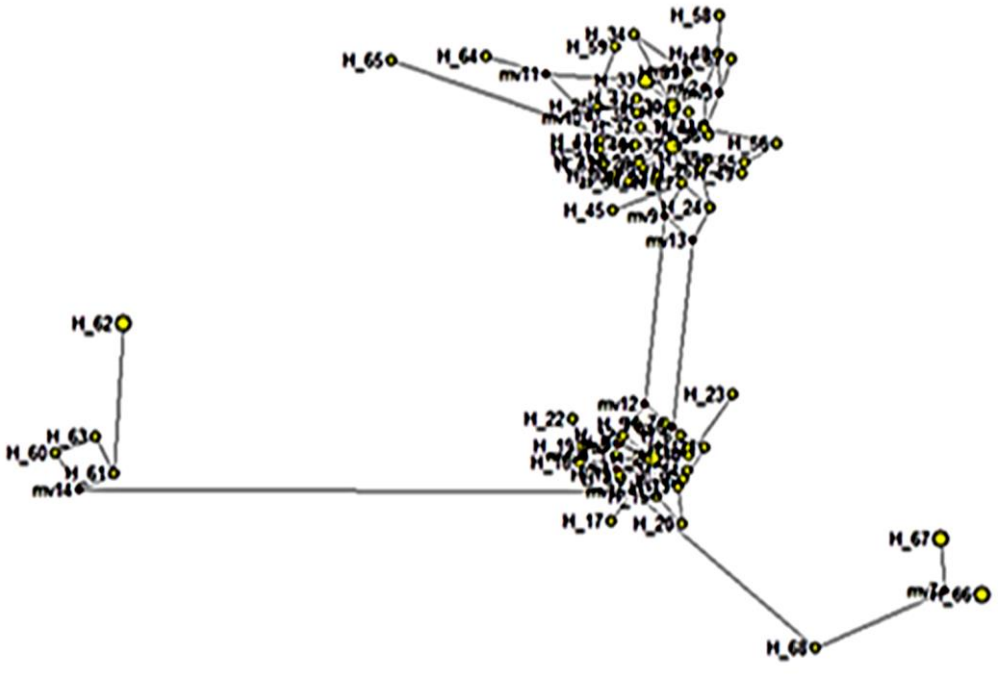

Fig. 2: Median network profile of mt-DNA haplotypes observed in this study. Red circles are median vectors used in connecting in directly related haplotypes. Clades A (left, Somali *E. asinus*), B (top center, Chinese *E. asinus*), C (bottom center, Chinese *E. asinus*), and D (right, domestic *E. asinus*).

lineage of domestic donkeys. And, additional research is required to promote welfare, such as the management of donkey hooves and disease management.

Conclusion

Most of the donkeys raised in South Korea are known to have come from northern regions (China, Mongolia), but there is no accurate literature in Korea. As a result of this study, it was confirmed that these donkeys originated from the Somali wild donkey species among the Somali donkeys and the Nubian donkeys raised in China.

Author's Contribution

All research protocols and animal experiments in this study designed, conducted experiments by SW Yun and contributed to data acquisition. GJ Cho contributed to the interpretation of the experimental results and the writing of the manuscript.

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