Asian-Aust. J. Anim. Sci.
Vol. 26, No. 2 : 163-170 February 2013
http://dx.doi.org/10.5713/ajas.2012.12499

www.ajas.info
pISSN 1011-2367 eISSN 1976-5517

mtDNA Diversity and Phylogenetic State of Korean Cattle Breed, Chikso

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ABSTRACT: In order to analyze the genetic diversity and phylogenetic status of the Korean Chikso breed, we determined sequences of mtDNA cytochrome b (cyt *b*) gene and performed phylogenetic analysis using 239 individuals from 5 Chikso populations. Five non-synonymous mutations of a total of 15 polymorphic sites were identified among 239 cyt *b* coding sequences. Thirteen haplotypes were defined, and haplotype diversity was 0.4709 ranging from 0.2577 to 0.6114. Thirty-five haplotypes (C1-C35) were classified among 9 Asia and 3 European breeds. C2 was a major haplotype that contained 206 sequences (64.6%) from all breeds used. C3-C13 haplotypes were Chikso-specific haplotypes. C1 and C2 haplotypes contained 80.5% of cyt *b* sequences of Hanwoo, Yanbian, Zaosheng and JB breeds. In phylogenetic analyses, the Chikso breed was contained into *B. taurus* lineage and was genetically more closely related to two Chinese breeds than to Korean brown cattle, Hanwoo. These results suggest that Chikso and Hanwoo have a genetic difference based on the mtDNA cyt *b* gene as well as their coat color, sufficient for classification as a separate breed. (**Key Words:** Phylogenetic Analysis, Chikso, Haplotype, mtDNA, Cytochrome *b*)

INTRODUCTION

The now-extinct aurochs (*Bos primigenius*), which ranged throughout much of Eurasia and Northern Africa, is widely accepted as the wild ancestor of modern domesticated cattle (MacHugh et al., 1998). Archaeological evidence shows that domestication of this animal occurred independently in the near East and in the Indian subcontinent between 10,000 to 8,000 yrs ago, giving rise to the two taxa of domestic cattle, namely *B. taurus* and *B. indicus* (Helmer et al., 2005; Bradley and Magee, 2006).

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Submitted Sept. 11, 2012; Accepted Oct. 11, 2012; Revised Nov. 19, 2012

Genetic diversity of animal genetic resources (AnGR) compensates for changing environmental conditions, including climate, markets, and disease etc. Because AnGR are likely to continue to change and adapt in the future (Bruford et al., 2003; Toro and Caballero, 2005), the maintaining of their genetic diversity is important and essential for future use. However, during the last century, many indigenous domestic breeds became extinct by replacement or crossbreeding with exotic productive breeds. Therefore, the Food and Agriculture Organization of the United Nations (FAO) has encouraged a series of conservation measures designed to help prevent irreversible loss of domestic animal species (FAO, 2007b). In addition, conservation is one of the four Strategic Priority Areas of the recently adopted Global Plan of Action for AnGR (FAO, 2007a).

Molecular genetic studies within and across breeds are essential for the effective management of AnGR (Hall and Bradley, 1995; Ruane, 2000; Simianer, 2005). Mammalian mitochondrial DNA (mtDNA) shows several special features such as an absence of intron, maternal inheritance, the existence of single copy orthologous genes, lack of recombination events and a high mutation rate (Irwin et al.,

1991; Pesole et al., 1999). Since the complete sequence of bovine mtDNA was published (Anderson et al., 1982), mtDNA have been widely used for genetic diversity and phylogenetic analysis among different cattle breeds (Bradley et al., 1996; Troy et al., 2001; Kikkawa et al., 2003; Kim et al., 2003; Berthouly et al., 2010; Martín-Burriel et al., 2011). Especially mtDNA cytochrome b (cyt *b*) has been broadly determined for the description of phylogenetic states of several species (Lau et al., 1998; Sultana et al., 2003; Souza et al., 2009; Stock et al., 2009; Yap et al., 2010).

More than 3,000 breeds of cattle are widely distributed throughout the world based on a Domestic Animal Diversity Information System (DAD-IS) from the FAO (http://dad.fao.org/). Four indigenous Korean cattle breeds are listed in the DAD-IS, and of them, the Chikso breed is an endangered breed with a unique brindle coat color. Efforts towards their conservation and proliferation have been made by government, universities and farmers. However, the genetic diversity, origin and evolution of this cattle breed have not been elucidated. The purposes of this investigation are to determine sequences of the mtDNA cyt *b* gene in full length, and to verify the genetic diversity, maternal origins and phylogenetic relationships of the Chikso breed.

MATERIALS AND METHODS

Sample collection and DNA extraction

The national management for the Chikso breed is carried out by 7 local institutions for AnGR management. The 239 blood samples were obtained from 5 institutions (Table 1) and were stored at -70°C until further processing. Genomic DNA was extracted from blood following an established protocol (Miller et al., 1988). The concentration of extracted DNA was measured using NanoDrop ND1000 (Thermo Scientific, USA), and was diluted to use as template for amplification.

Amplification and sequencing

The complete cyt *b* gene was amplified by using forward primer Bcyt-F: 5'-TTCTTACATGGAATCT AACCATGA-3' and reverse primer Bcyt-R: 5'-GGGAGGTTAGTTGTTCTCCTTCTC-3'. The forward and reverse primers were designed from tRNA-Glu and tRNA-Thr sequences of the mtDNA genome (GenBank accession no. V00654). Polymerase chain reaction (PCR) was carried out in a total volume of 25 μl, containing 10 ng of genomic DNA, 2.5 μl of 10×buffer, 0.2 mM of dNTP, 10 pM of each primer and 1.5 units of *Taq* polymerase (TaKaRa, Japan). Thermal cycling was performed on a PTC-200 thermocycler (MJ Research Inc.) under the following conditions; 2 min denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, 60 s at 72°C, and a final 5 min at 72°C before cooling to 4°C for 10 min. The amplified products were separated by electrophoresis on 1.2% agarose gels, and were visualized under UV illumination after staining with ethidium bromide. The PCR products were purified using a QIAquick PCR purification Kit (Qiagen, USA), and were directly sequenced on an ABI 3130xl Genetic Analyzer (PE Applied Biosystems, USA). The boundaries of the cyt *b* gene were determined by comparison with the *B. taurus* mtDNA genome sequence (GenBank accession no. V00654) as reported by Anderson et al. (1982).

Statistical and phylogenetic analyses

The sequences of the cyt *b* gene from different breeds were aligned in CLUSTAL W (Thompson et al., 1994). Numbers of nucleotide polymorphic sites (S) and haplotype (h), nucleotide diversity (Pi), haplotype diversity (Hd) and nucleotide divergence (D*xy*) were performed in DNA sequence polymorphism Version 5.1 (Librado and Rozas, 2009). The Neighbor-joining (NJ) tree (Saitou and Nei, 1987) among haplotypes based on the cyt *b* gene sequences was reconstructed in MEGA 5.05 package (Tamura et al., 2011), with the reliability of the tree topology assessed by 1,000 bootstrap replications (Felsenstein, 1985). The NJ tree among breeds was constructed in MEGA 5.05 package on the basis of D*xy* distances.

RESULTS AND DISCUSSION

Sequence composition and variation of the cyt *b* gene

The full-length coding sequences of the cyt *b* genes in

Table 1. Number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (Hd) and nucleotide diversity (Pi) within 5 Chikso populations

Populations	Institutions	Sample sizes	S	h	Hd	Pi
GAN	Gangwon Provincial Livestock Research Center	54	1	2	0.2577	0.00023
CHU	Chungbuk Institute of Livestock and Veterinary Research	78	7	8	0.6114	0.00067
JEOB	Jeonbuk Institute of Livestock and Veterinary Research	54	7	5	0.3892	0.00066
JEON	Jeonnam Agricultural Research and Extension Services	18	1	2	0.4248	0.00037
GYE	Gyeongbuk Livestock Research Institute	35	6	7	0.5244	0.00057
Total		239	15	13	0.4709	0.00055

239 Chikso individuals were determined. All these sequences spanned 1,140 bp, started with an ATG translational start codon and ended with an AGA stop codon. No insertion/deletion or length variation was detected in these sequences. Compositional frequency of G base was the lowest (13.4%) other 3 bases (A, 31.2%; C, 30.2%; T, 25.2%). These patterns were very similar to those of a previous report which analyzed Chinese cattle breeds (Cai et al., 2007).

Fifteen polymorphic sites were identified among 239 cyt *b* coding sequences (Figure 1). All polymorphic sites were transition mutations, but there was no transversion mutation. Among the 15 polymorphic sites, 2 of them (site positions: 329, 643) were variant-singleton variable sites, and the others were two variant parsimony informative sites. In addition, 5 non-synonymous mutations (A232G, T329C, G643A, G736A, and A1114G) were identified, and substitutions of amino acid by these mutations were estimated as I78V, L110P, V215M, A246T, and I372V, respectively.

Thirteen haplotypes were defined by polymorphisms at 15 sites, and the cyt *b* sequences of these haplotypes were submitted to GenBank (Accession no. JX472262-74). The nucleotide diversity (Pi) of all individuals was 0.00055 and ranged from 0.00023 (GAN) to 0.00067 (CHU) (Table 1). Haplotype diversity (Hd) of Chikso was 0.4709 and ranged from 0.2577 (GAN) to 0.6114 (CHU). The Pi and Hd of the Chikso breed is lower than those of Chinese breeds of taurine lineage (Cai et al., 2007).

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                    11
          233566677889901
          326124533152911
          290013456324744
C1        ATGACGCCGGCCTTA
C2        ............C..
C3        .C.............
C4        G...........C..
C5        ........A...C..
C6        .........A..C..
C7        ...G...........
C8        ..........T.C..
C9        ...........TC..
C10       ..A.T.......C.G
C11       ......T.....CC.
C12       .......T....C..
C13       .....A......C..
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Figure 1. Sequence variation of 13 haplotypes (C1-C13) in the mtDNA cyt *b* gene of 239 individuals of the Chikso breed. Mutations are scored relative to the reference sequence (GenBank accession no. DQ186215) of Cai et al. (2007). Dots (.) denote identity with sequence of the C1 haplotype.

Haplotype distribution

For verification of the haplotype distribution of Chikso among Asian and European breeds, we obtained the cyt *b* gene sequences of Korean brown (called Hanwoo), Japanese black (JB), 6 Chinese breeds, and 3 European cattle breeds from GenBank database (Table 2). The distribution of the haplotypes in the Chikso and other breeds is shown in Table 3. Thirty five haplotypes (C1-C35) were classified from 319 cyt *b* gene sequences. C2 was a major haplotype that contained 206 sequences (64.6%) from all breeds used. C1 was the second largest haplotype that included 54 sequences (16.9%). This haplotype did not contain any of the European breeds, nor did it contain 2 of the Chinese breeds (Kazakh and Jinnan). C3-C13 haplotypes were Chikso-specific haplotypes, and there were no other breeds that contained at these haplotypes. C1 and C2 haplotypes contained 80.5% of cyt *b* sequences of Hanwoo, Yanbian, Zaosheng and JB breeds. Especially, all sequences of the Yanbian breed were contained at C1 and C2 haplotypes.

Phylogenetic analysis of the cyt *b* gene

The neighbor-joining (NJ) tree was constructed to verify

Table 2. Information on reference sequences for comparison with Chikso breed

Region	Abbreviation	Breed names	Number of sequences	Accession numbers
Korean	Chi	Chikso	239	JX472262-74 (this study)
	KB	Korean brown	18	AY526085, DQ124371-86, NC_006853
China	Boh	Bohai black	8	AY885297-300, AY952962-3, DQ186227-8
	Yan	Yanbian	6	AY903438, DQ186215-9
	Zao	Zaosheng	7	DQ186220-6
	Kaz	Kazakh	11	DQ186203-13
	Qin	Qinchuan	8	AY885304-6, AY903439, AY952952-3, DQ186241-2
	Jin	Jinnan	8	DQ186229-36
Japan	JB	Japanese Black	7	AB074962-8
European	Ang	Angus	2	AY676857, AY676859
	Cha	Charolais	2	AY676858, AY676861
	Lim	Limousine	3	AY676856, EF693798, JN817331
	Total		319	

Table 3. Distribution of mtDNA haplotypes in 12 cattle breeds based on cyt *b* gene sequences

Haplotype	Cattle breed*												Total
	Chi	KB	Boh	Yan	Zao	Kaz	Qin	Jin	JB	Ang	Cha	Lim	
C1	41	4	1	1	1		1		2				51
C2	169	10	2	5	5	1	3	3	4	1	1	2	206
C3	1												1
C4	4												4
C5	2												2
C6	5												5
C7	4												4
C8	2												2
C9	2												2
C10	3												3
C11	3												3
C12	2												2
C13	1												1
C14		1											1
C15		1											1
C16		1											1
C17		1											1
C18			1										1
C19			3			1	2	3					9
C20			1										1
C21					1								1
C22						3							3
C23						1							1
C24						1					1		2
C25						1							1
C26						1							1
C27						1							1
C28						1							1
C29							1						1
C30							1						1
C31								1					1
C32								1					1
C33									1				1
C34										1			1
C35												1	1
Total	239	18	8	6	7	11	8	8	7	2	2	3	319

* All abbreviations are given in Table 2.

the phylogenetic relationship of 35 haplotypes of the cyt *b* gene, on the basis of Kimura two-parameter distances (Figure 2). Four cyt *b* sequences (GenBank Accession no. AF419237, EF061244, GU256940 and JN117614) from Zebu (*B. indicus*) were used together and *B. javanicus* was used as the outgroup (GenBank Accession no. D82889). The reliability of the tree topology was assessed by 1,000 bootstrap replications. The tree was divided into two distinct genetic lineages, *B. taurus* and *B. indicus*. Three (C18, C19 and C25) of 35 haplotypes belonged to *B. indicus* lineage and these haplotypes contained four Chinese breeds (Bohai black, Kazakh, Qinchuan and Jinnan). Cai et al. (2007) reported that 14 Chinese cattle breeds, including these four breeds, were of a mixture of two cattle lineages, *B. taurus* and *B. indicus*. Clade I consisted of haplotypes that derived from all breeds. Clades II-IV were found only in Asian breeds and contained C1 haplotype. Clades III and IV contained Chikso and Chinese breeds. The Chikso breed was classified as being of *B. taurus* lineage and extended to Clade I-III. The existence of a *B. indicus* lineage for Korean cattle breeds has not been found (Kikkawa et al., 2003; Kim et al., 2003; Mannen et al., 2004), and all Chikso individuals used in this study were verified as being of *B. taurus* lineage.

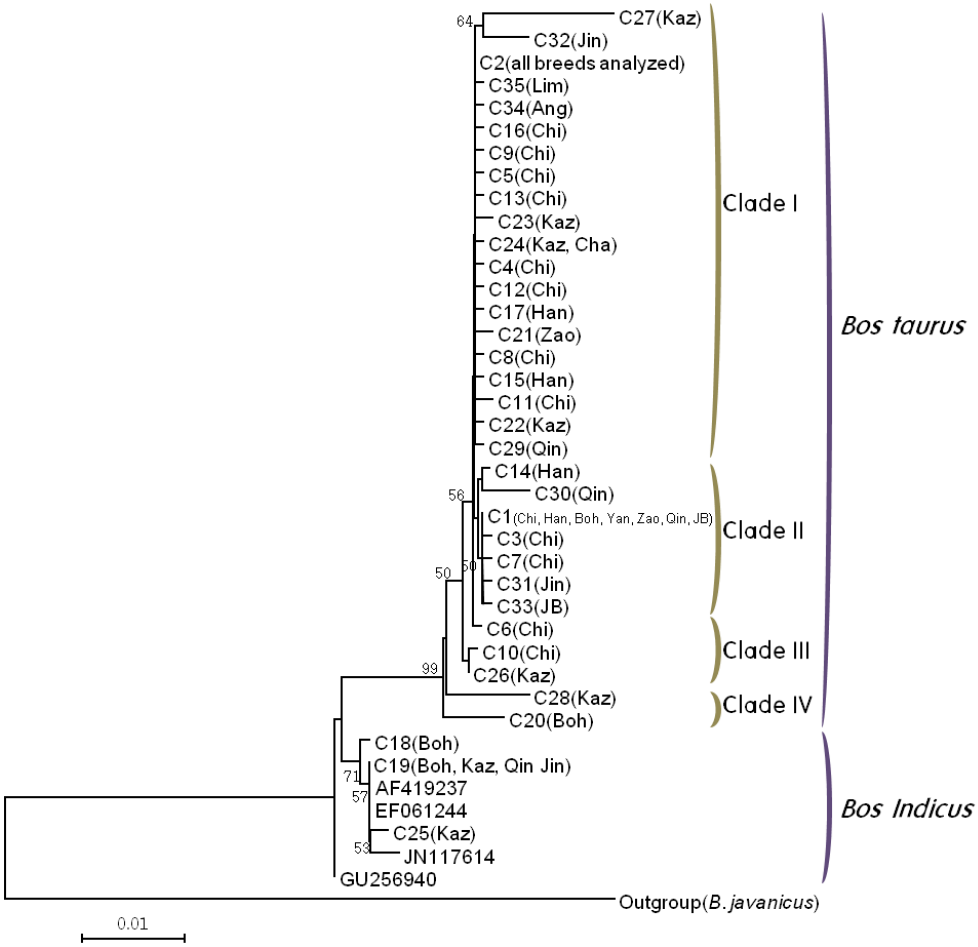

Figure 2. Phylogenetic relationship among 35 haplotypes of the cyt *b* gene from Asian and European cattle breeds. The tree was constructed with the neighbor-joining method (Saitou and Nei, 1987) on the basis of Kimura 2-parameter distances. *B. javanicus* was used as the outgroup (GenBank accession no. D82889). The numbers at branches stand for bootstrap values higher than 50% out of 1,000 replications. The bar scale indicates genetic distance. Abbreviations for population or breed are the same as those in Table 2.

For verification of the phylogenetic status of the Chikso breed, a NJ tree was reconstructed based on D*xy* genetic distances among Asian and European breeds (Figure 3). Four Chinese breeds, Bohai black, Jinnan, Qinchuan, Kazahk, were located in between *B. taurus* and *B. indicus* lineage. In the NJ tree based on 35 haplotypes (Figure 2), these breeds were verified to have two mtDNA maternal lineages, *B. taurus* and *B. indicus*. The phylogenetic location of these breeds was estimated to be due to these characteristics. Asian breeds and European breeds formed two independent groups in *B. taurus* lineage. Korean breeds, Chikso and Korean brown and Japanese black were located to the end of the tree through Chinese and European breeds from *B. indicus*. In the genetic distances estimated for verification of the genetic relationship between Chikso and other breeds, Chikso was more closely related to Yanbian (0.00040) than to Zaosheng (0.00064), Korean brown (0.00066) or JB (0.00066) (Table 4). These two Chinese breeds are distributed in north China (Cai et al., 2007). Yanbian breed samples were collected from Yanji City, which is the closest to the Korean peninsula. These results suggest that Korean cattle breeds might have been introduced from north China. This notion agrees with a report by Jia et al. (2010) that analyzed haplogroup patterns using mtDNA D-loop sequence.

Up until now, Chikso and Korean brown were classified as individual breeds on the sole basis of their coat color, however, there was no molecular genetic evidence. In this study, the Chikso breed was determined to be genetically closer to Yanbian and Zaosheng breeds than to the Korean brown breed in terms of phylogenetic analysis, although genetic distances showed slightly differing results. This result suggests that Chikso and Korean brown have a genetic difference based on their mtDNA cyt *b* gene, as well as based on their coat color for classification as breed.

In this study, we determined the full-length coding sequences of mtDNA cyt *b* gene and analyzed the genetic relationship and phylogenetic status of the Chikso breed. The Chikso breed was classified into *B. taurus* lineage and was found to be genetically more closely related to two

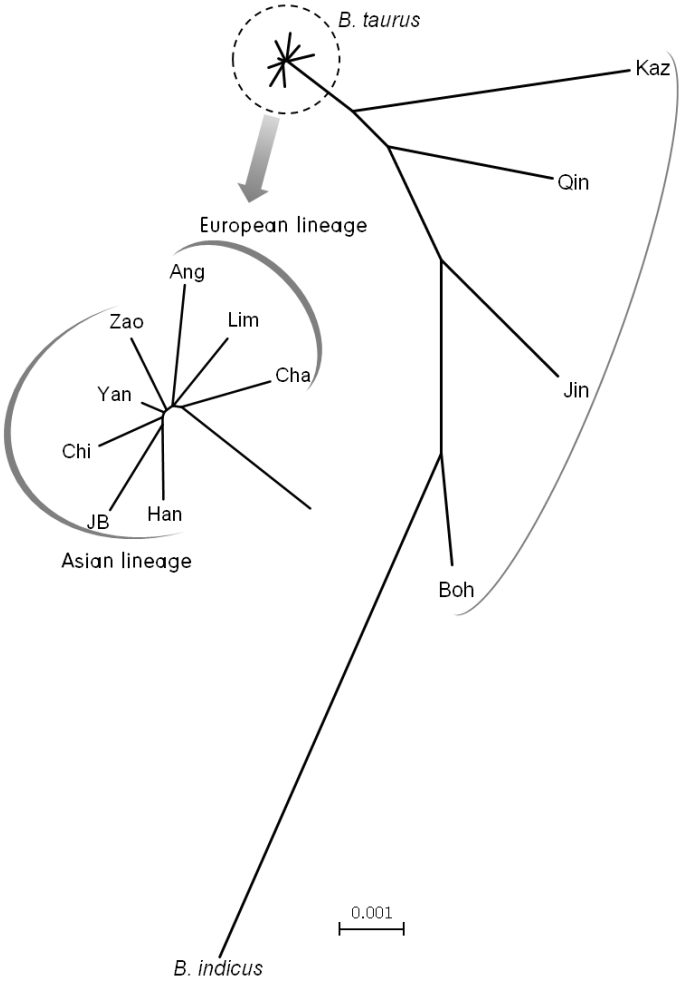

Figure 3. Phylogenetic relationship among 12 cattle breeds of *B. taurus* lineage and a breed of *B. indicus* lineage (GenBank accession no. JN117614-5). The tree was constructed with the neighbor-joining method (Saitou and Nei, 1987) on the basis of D*xy* distances. The bar scale indicates genetic distance. Abbreviations for population or breed are the same as those in Table 2.

Chinese breeds than to the Korean brown. For more detailed phylogenetic analysis and genetic characterization of the Chikso breed, additional genetic markers (e.g., mtDNA control region, Y chromosome and microsatellites) are necessary, as well as sample collection through the whole of Korea.

Table 4. D*xy* genetic distances among 12 cattle breeds and based on mtDNA cyt *b* gene sequences

	Chi	Han	Boh	Yan	Zao	Kaz	Qin	Jin	JB	Ang	Cha	Lim	Zebu
Chi	-												
Han	0.00065	-											
Boh	0.00889	0.00904	-										
Yan	0.00040	0.00050	0.00877	-									
Zao	0.00064	0.00074	0.00901	0.00048	-								
Kaz	0.00595	0.00609	0.01099	0.00581	0.00604	-							
Qin	0.00485	0.00497	0.00938	0.00471	0.00495	0.00865	-						
Jin	0.00671	0.00685	0.00935	0.00658	0.00681	0.00972	0.00825	-					
JB	0.00066	0.00073	0.00907	0.00052	0.00077	0.00616	0.00501	0.00688	-				
Ang	0.00075	0.00088	0.00910	0.00058	0.00081	0.00610	0.00504	0.00691	0.00094	-			
Cha	0.00075	0.00088	0.00910	0.00058	0.00081	0.00602	0.00504	0.00691	0.00094	0.00088	-		
Lim	0.00060	0.00073	0.00895	0.00044	0.00067	0.00595	0.00490	0.00676	0.00079	0.00073	0.00073	-	
Zebu	0.01673	0.01693	0.01033	0.01664	0.01687	0.01596	0.01390	0.01217	0.01699	0.01693	0.01693	0.01678	-

* All abbreviations are given in Table 2.

ACKNOWLEDGEMENTS

This work received grant support from the Agenda Program (no. PJ008431012012), Rural Development Administration, Republic of Korea.

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