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Mitochondrial DNA analysis of *Bos taurus* bone collected from ruins of the Joseon Period in a tributary of the *Cheonggyecheon* creek, South Korea

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ABSTRACT

Although genetic information about *Bos taurus* has been successfully revealed by a number of studies, the data on ancient DNA remains insufficient with respect to cattle raised in East Asia during historical times. This is especially so in the case of South Korea, where very little genetic information on cattle DNA has been obtained, notwithstanding the significant changes in the genetic traits of cattle populations that occurred during the country's colonial period. In the present study, we have endeavored to determine the mitochondrial DNA D-loop and coding region sequences obtained from a 15th century cattle bone unearthed amid an archaeological site of the Joseon-period in a tributary of the *Cheonggyecheon* creek, South Korea. The consensus sequence of the cattle's mitotype, established by alignment of the individual clone sequences, was 16138C, 106C, 169G, 221 + C, 587 + C, 2536A, 9682C, 13310C (Haplogroup = T3a). Having blasted it with the sequences available within GenBank, the Joseon cattle mitotype was found to be similar to modern *Bos taurus* sequences from South Korea, Japan, China and even Europe, the Americas and Oceania. By completely sequencing the mtDNA D-loop and coding the regional signature of the bone, we were able to acquire invaluable information enriching our knowledge of the genetic history of the genus *Bos* in South Korea.

1. Introduction

Of the animal species domesticated by human civilizations to date, the domestication of the cattle is one of the most remarkable achievements. Through domestication, cattle became a rich source of meat, dairy and leather products for later prehistoric (Neolithic) peoples (Lin et al., 2016). The development of cattle traction, the mainstay of the Secondary Products Revolution, is another seminal event in human history, in that it harnessed animal energy to facilitate human activities (Sherratt, 1981; Bogucki, 1993; Lin et al., 2016). Indeed, the exploitation of cattle labour in agriculture, transport and trade shaped various human societies (Achilli et al., 2009; Ajmone-Marsan et al., 2010; Lin et al., 2016). Therefore, from an archaeological perspective, information on the timing of the domestication of cattle and its subsequent spread is very important for researchers.

According to previous studies, the domestication of wild ox *Bos primigenius* occurred about 10,000 to 15,000 years ago in very limited areas of the Near East (*Bos taurus*) and Indian sub-continent (*Bos indicus*). Domesticated cattle spread widely thereafter into multiple regions worldwide, replacing the local wild ox (*Bos primigenius*) that became extinct (Mannen et al., 1998; Lai et al., 2006; Jia et al., 2007, 2010; Achilli et al., 2008).

The rapid development of molecular phylogenetics has made a great contribution to a comprehensive understanding of cattle history worldwide. After Anderson et al. (1982) achieved complete sequencing of *B. taurus* mitochondrial DNA (mtDNA, 16,338 bp; V00654.1), many researchers have reported cattle mtDNA sequences in various countries. Phylogenetic analysis has further revealed that most taurine cattle (*B. taurus*) worldwide belong to haplogroup T (subtypes T1 to T5), the descendants of ancient cattle first domesticated in the Near East (Troy et al., 2001; Lai et al., 2006; Achilli et al., 2008, 2009; Chen et al., 2010).

Using molecular biological techniques, the origins of cattle in Korea have also been elucidated. Briefly, Kim et al. (2010) identified the genetic relationship between Korean cattle and the other breeds. In their phylogenic analysis of mtDNA polymorphisms, they found a distinct

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genetic difference between Korean cattle and *B. indicus*. Lee et al. (2012) also analyzed complete mtDNA sequence variation and genetic relationships among different taurine, indicine and *Bison* groups. In a Neighbour-Joining tree analysis, they showed that taurine cattle in different Asian regions, including the Korean peninsula, were very closely related to each other (Lee et al., 2012). A series of studies by Kim et al. (2005, 2013, 2016) has also contributed significantly to our understanding of the genetic traits of Korean cattle. By an analysis of mtDNA diversity, they determined that the Korean Chikso breed can be classified possibly as a separate breed (Kim et al., 2013).

All of these pioneering studies notwithstanding, comprehensive knowledge on the genetic traits of historical Korean cattle have proved elusive. In fact, significant changes are known to have occurred in the genetic traits of Korean cattle during the country's colonial era (Seo, 2014), which means that cattle in pre-colonial periods might have been genetically somewhat different from cattle in 21st century Korea.

However, as for genetic information on ancient cattle raised in Korea, very few studies are currently available, the one exception being the report on ancient Jeju cattle bones collected from an archaeological site dating to the 8th–9th century CE (Kim et al., 2005). In that study, the authors were able to establish that mtDNA sequences of the ancient Jeju cattle were very similar to those of the Black cattle still raised on the same island, thereby exhibiting genetic continuity between them. Although the outcome of this research is notable, Jeju cattle do not represent every historical taurine breed raised on the Korean mainland.

Our present study was thus designed to uncover genetic information on the pre-20th century Joseon cattle raised on the mainland of Korea. Utilizing samples derived from an ancient taurine cattle bone unearthed at an archaeological site of the Joseon Period within Old Seoul City, we attempted to obtain small but significant clues to the genetic traits of historical Korean cattle.

2. Site background and sample context

Jongmyo was a royal shrine where the Joseon kings performed religious ceremonies. The shrine is located at the centre of Old Seoul City, where many private houses and offices were also located during the Joseon Period (Seoul Museum of History, 2012; Shin et al., 2013). In 2009, a cattle femur (*B. taurus*; #D-157) was obtained from an archaeological site along a tributary of the Cheonggyecheon creek (*Hoedong/Jesengdongcheon*) in front of the *Jongmyo* shrine. According to historical records, this site included a marketplace that was first opened in 1413 CE. The records' regional layouts and descriptions, indicating an alley, gutter, private house and streambed, were confirmed by archaeological investigations (Seoul Museum of History, 2012). In the course of the excavation, the cattle bone was found in the lowest layer of the site. By archaeological and AMS radiocarbon dating, it was judged to belong to the period between 1410 and 1470 CE (Seoul Museum of History, 2012). A zoological examination for species discrimination, performed by one of the authors (T-S Cho), confirmed it as a femur bone of *B. taurus*.

3. Materials and methods

To guarantee our aDNA work's authenticity, we followed recommendations about strict laboratory protocols (Hofreiter et al., 2001; Ho Simon and Gilbert, 2010). Briefly, the specialized facilities and strict laboratory protocols recommended for authentic aDNA analysis (Ho Simon and Gilbert, 2010) were used in this study. To meet the criteria for keeping modern DNA contamination to a minimum, every research participant wore sterilized gowns, head caps, gloves, and masks. Every tool used in this study was sterilized before use. The obtained samples were stored in sterilized containers. Nobody was permitted to contact with the samples without permission.

Following the previous DNA extraction method of Kim et al. (2011), the surface of the femur was abraded and was exposed to UV light for 20 min. After the sample was treated with 5.4% sodium hypochlorite, it was powdered by SPEX 6750 Freezer / Mill (SPEX SamplePrep, Metuchen, NJ). The sample (0.5 g) was incubated in 10 ml of lysis buffer (pH 8.0; including 50 mM of EDTA; 1 mg/ml of proteinase K; 1% SDS; 0.1 M DTT) at 56 °C for 48 h.

Total DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Purification of extracted DNA was made with QIAmp PCR purification kit (QIAGEN, Hilden, Germany). PCR amplification was done with pre-designed primer sets (for mtDNA control region, BRS 15773-352; for mtDNA coding region, 567-658; 2509-2595; 9637-9699; 12,101-12,192; 13,272-13,363), on a PTC-200 DNA Engine (Bio-Rad Laboratories, Hercules, CA) (Table 1). The PCR conditions used in this study were as follows: pre-denaturation at 94 °C for 10 min; 42 cycles of denaturation at 94 °C for 30 s; annealing at 55–62 °C for 30 s; extension at 72 °C for 30 s; final extension at 72 °C for 10 min.

The PCR products were separated on 2.5% agarose gel (Invitrogen, USA), and then stained with ethidium bromide. They were photographed using a Vilber Lourmat ETX-20.M equipped with Biocapt software (Vilber Lourmat, France). The PCR amplicons were isolated using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Bacterial transformation was performed with the pGEM-T Easy Vector system (Promega Corporation, Madison, USA). Transformed bacteria were grown in an agar plate containing ampicillin (50 μg/ml), 0.5 mM IPTG, and X-GAL (40 μg/μl) for the next 14 h. After selected colonies were grown once again in LB media for 12 h, the purification of cultured bacteria was performed by a QIAprep® Spin Miniprep kit (Qiagen, Hilden, Germany).

Sequencing of each strand was done usingan ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) on the 3730xl Automated Sequencer (Applied Biosystems, Foster City, CA). The haplogroup of the current *Cheonggyecheon* was determined by consensus sequences of mtDNA control and coding regions, based on the methods in Mannen et al. (2004), Achilli et al. (2009) and Jia et al. (2010).

By use of NCBI/BLAST tools, the *Cheonggyecheon* cattle's consensus sequence was compared to those available within GenBank. Web browser module and Alignment Explorer implemented in MEGA6 (Hall, 2011) were used for retrieving the sequences of interest from the GenBank database of National Center for Biotechnology Information (NCBI). Retrieved sequences are *B. taurus* mtDNA control region sequences (BRS 15773-352) from South Korea (n = 64), Japan (n = 30) and China (n = 24) (Fig. 1; Supplementary Data 1). Sequences of *B. indicus* (n = 3), *B. primigenius* (n = 1) and the cattle from European countries (n = 6) were also retrieved as outgroups. Among retrieved mtDNA of South Korean cattle breeds, Heugu, Jeju Black, and Chikso were reported by Kim et al. (2016); and Hanwoo were reported in the work of Jia et al. (2010). By Clustal W implemented in MEGA6, multiple sequence alignments were done with the *Cheonggyecheon* and its homologous mtDNA sequences from GenBank (Thompson et al., 1994; Hall, 2011).

To estimate the evolutionary divergence between cattle mtDNA sequences, the numbers of base substitutions per site between sequences were counted. Pairwise distances between sequences were calculated using the maximum composite likelihood model implemented in MEGA6 (Hall, 2013). The calculation setting was d: transitions + transversions for substitutions to include, uniform rates for rates among site, homogeneous(same) for pattern among lineages, complete deletion for Gaps/Missing data treatment.

The evolutionary relationship of *Cheonggyecheon* and the other taxa from NCBI GenBank was inferred by the Maximum Likelihood (ML) method in MEGA6 (Hall, 2011). The tree model was based upon Hasegawa-Kishino-Yano's method. Other settings were gamma distributed for rates among sites; 5 for Number of Discrete Gamma Categories; Nearest-Neighbor-Interchange for HL Heuristic Method; and Complete deletion for gaps/missing data treatment. To estimate the reliability of