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Abundant sequence divergence in the native Japanese cattle *Mishima-Ushi* (*Bos taurus*) detected using whole-genome sequencing ☆

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ABSTRACT

The native Japanese cattle *Mishima-Ushi*, a designated national natural treasure, are bred on a remote island, which has resulted in the conservation of their genealogy. We examined the genetic characteristics of 8 *Mishima-Ushi* individuals by using single nucleotide polymorphisms (SNPs), insertions, and deletions obtained by whole-genome sequencing. Mapping analysis with various criteria showed that predicted heterozygous SNPs were more prevalent than predicted homozygous SNPs in the exonic region, especially non-synonymous SNPs. From the identified 6.54 million polymorphisms, we found 400 non-synonymous SNPs in 313 genes specific to each of the 8 *Mishima-Ushi* individuals. Additionally, 3,170,833 polymorphisms were found between the 8 *Mishima-Ushi* individuals. Phylogenetic analysis confirmed that the *Mishima-Ushi* population diverged from another strain of Japanese cattle. This study provides a framework for further genetic studies of *Mishima-Ushi* and research on the function of SNP-containing genes as well as understanding the genetic relationship between the domestic and native Japanese cattle breeds.

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1. Introduction

Cattle domestication is thought to have originated via multiple independent events in the Fertile Crescent and the Indian subcontinent, which have resulted in the taurine (*Bos taurus*) and indicine (*Bos indicus*) lines of cattle, respectively [1–5]. Previous reports using mitochondrial DNA (mtDNA) polymorphisms have shown that cattle strains from northeast Asia (Japan, Korea, Mongolia, and China) had northeast Asian-specific mtDNA haplotypes (T4 type) [6–9], as well as many mtDNA haplotypes common to domestic European cattle. Historical records indicate that the cause of these mixed mtDNA haplotypes in northeast Asian cattle could be the result of trade between Europe and the Far East through the Silk Road. The mixed ancestral Northeast Asian cattle then migrated to Japan through the Korean peninsula [6–8].

The improvement and breeding of cattle for meat purposes by crossing with European cattle breeds became popular in Japan during the 20th century. After World War II, 4 cattle breeds, i.e., Japanese Black, Japanese Red, Japanese Shorthorn, and Japanese Polled, were established by mating native Japanese cattle to various domestic European breeds using strict selection criteria based on phenotypic quality. Although Japanese Black cattle were selected due to the marbling of their meat caused by intramuscular fat deposition, in recent years, this severe selection process has caused a serious situation in which the genetic variation of Japanese Black cattle has decreased. Thus, it is essential to investigate the genetic background of native Japanese cattle.

In 2009, the genome sequence of Hereford cattle was determined by the international bovine whole-genome analysis project through a combination of the shotgun sequencing method and the use of bacterial artificial chromosomes (BACs) [10]. Using a next-generation sequencer, Van Tassell et al. [11] identified over 23,000 single nucleotide

Abbreviations: mtDNA, mitochondrial DNA; BACs, bacterial artificial chromosomes; SNPs, single-nucleotide polymorphisms; indels, insertions and deletions; UTRs, untranslated regions; PCR, polymerase chain reaction; nsSNPs, non-synonymous SNPs; GO, gene ontology; QTL, quantitative trait locus; CAST, calpastatin; CFTR, cystic fibrosis transmembrane conductance regulator; GAA, glucosidase, and alpha acid; NCAPG, non-SMC condensing I complex, subunit G; NJ, neighbor-joining; MEGA, Molecular Evolutionary Genetics Analysis.

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polymorphisms (SNPs) by using 8 major strains of domestic cattle, including Holstein, and deposited these SNPs in the bovine dbSNP database. Eck et al. [12] generated 24 gigabases of sequence with an average sequence depth of 7.4-fold from a single Fleckvieh bull, mapped it to the reference genome sequence of Hereford cattle by using the UCSC genome database, and identified more than 2 million new SNPs and 115,000 insertions and deletions (indels). Stohard et al. [13] sequenced Black Angus and Holstein using the ABI SOLiD system and generated approximately 7 million SNPs, 24% of which were identified in both animals. Although bovine genome studies and the international HapMap project are progressing at an increasing pace by using large-scale genome analysis [14], there is no sequence information for consideration of the detailed genetic features of Japanese breeds. Thus, this study was designed to generate useful new sequence information by the analysis of native Japanese cattle.

In a previous study, we found that the native Japanese cattle *Kuchinoshima-Ushi* have numerous unique SNPs, including non-synonymous SNPs (nsSNPs), that clearly distinguish them from European cattle species [15]. Another kind of native Japanese cattle is *Mishima-Ushi*, which was designated as a national natural treasure in 1928 (Additional File 1). A previous report [16] and historical documents suggest that *Mishima-Ushi* individuals were isolated on Mishima Island at least 200–300 years ago and that they are still breeding as a closed colony on the island today. Therefore, these native cattle are expected to have conserved various polymorphisms in their genome. Interestingly, this breed has marbled meat, which is similar to the meat of Japanese Black cattle, suggesting that the *Mishima-Ushi* species might have contributed to the establishment of the present Japanese bovine breeds. However, only 90 individuals of the strain are closely bred on the island. Due to their considerable isolation, there is a risk of genetic inbreeding, resulting in reduced genetic diversity; therefore, we believe that acquiring genomic information for *Mishima-Ushi* will be important for this strain.

In the current study, we used a next-generation sequencer to perform whole-genome analysis of 8 *Mishima-Ushi* individuals to understand their precise genetic features. The results clearly show that these native cattle possess numerous unique and valuable genetic variations in their genome, providing important information for understanding their genetic characteristics.

2. Results and discussion

2.1. Summary of sequencing and mapping

The main advantage of whole-genome sequence analysis performed using a next-generation sequencer is that researcher prejudice can be avoided by using randomly extracted SNPs throughout the genome. For the first set of experiments, we conducted a whole-genome sequencing study of multiple *Mishima-Ushi* individuals from the same strain (Additional File 2). The results showed that a number of polymorphisms indicated that the *Mishima-Ushi* population had rich genetic variation and could be distinguished from other breeds.

After confirming that sufficient data had been acquired, further sequence analysis of 8 *Mishima-Ushi* individuals was carried out (Additional File 3). The average number of reads for each individual was 206,886,029 (approximately 20.6 gigabases). The number of unique reads identified by mapping the sequenced fragments to the reference genome sequence was 184,039,298 (approximately 18.4 gigabases) in *Mishima-Ushi*. The average number of multi-mapped reads was 19,842,426 (approximately 1.9 gigabases) and that of unmapped reads was 3,004,305 (approximately 3 gigabases). The sequence depth of the uniquely mapped sequence reads was 8.4 (minimum: 6.14 in Mishima2; maximum: 10.39 in Mishima6) (Additional File 3). The average sequence depth of 8.4 in this study is sufficient for further informatics analysis because the average depth was supported by a number of individuals. We also extract polymorphisms from the merged data of the 8 *Mishima-Ushi*, which had a sequence depth of 62.93, to confirm the difference between the average data from the 8 *Mishima-Ushi* and their merged data. In this comparison, we did not find a remarkable difference (Additional File 4).

We separated all extracted polymorphisms into 6 categories according to their location; exons, introns, untranslated regions (UTRs), upstream and downstream regions (5 kbp) of genes, and intergenic regions. Exonic polymorphisms were divided into synonymous SNPs and nsSNPs. As preliminary analysis, we compared the number of polymorphisms generated using the different criteria for calling polymorphisms, i.e., percent aligned reads calling the SNP (30, 60, and 100%), and read depth (3, 6, and 9) (Additional File 5). When the percent aligned reads calling the SNP was increased, the number of SNPs at each depth was decreased. When the read depth was increased, the number of SNPs was decreased, while the degree of reduction was the same between the read depths. The difference in the number of SNPs between the depths could mean that erroneous SNPs might be picked up at a low depth or true SNPs might be dismissed at a high depth. From the results of the preliminary analysis using various criteria for calling SNPs, the similar degree of reduction of called SNPs at all depths suggests that erroneous SNPs were not picked up even when they were extracted at 3 depths and true SNPs might be dismissed at a high depth. The degree of reduction was different between the regions, e.g., exons and introns (Additional File 5), and in particular, exonic nsSNPs were dramatically decreased at 60 and 100% of aligned reads calling the SNP; thus, the ratio of nsSNPs/synonymous SNPs was reversed between 30% and 60/100%. Such a decrease of exonic nsSNPs may be caused by the existence of heterozygous nsSNPs detected at a low percentage of aligned SNP calling reads, which are harmful if they are homozygous. On the basis of these results, we counted SNPs in at least 3 depths and at least 30% polymorphic to identify as many SNPs as possible for further detailed analysis of the variations of *Mishima-Ushi* individuals.

Of the polymorphisms detected 0.63% were present in exons, 20.79% in introns, 0.38% in UTRs, 1.99% in the upstream region of genes, 1.87% in the downstream region of genes, and 74.34% in intergenic regions (Fig. 1). An average of 7594 genes had nsSNPs (range, 7114–8026) (Additional File 6). Furthermore, we mapped and compared the coverage rate of sequence reads with other whole genome data, i.e., Fleckvieh [12], Holstein, Black Angus [13], and *Kuchinoshima-Ushi* [15]. As these sequence data were mapped to the old version of the reference genome, we collected these sequence data and mapped them to bosTau7.0. After confirming the coverage rate at a depth of 1, 3, and 5 (Additional File 7), we decided to use 2 sets of sequence data from *Kuchinoshima-Ushi* and Fleckvieh. We compared sequence data between the 8 *Mishima-Ushi* individuals and the whole genome sequence of 2 other strains, i.e., Fleckvieh [12] and *Kuchinoshima-Ushi* [15]. As a result, we found 400 *Mishima-Ushi*-specific nsSNPs that were shared by all 8 *Mishima-Ushi* individuals and not shared by the other breeds examined (Hereford, Fleckvieh, and *Kuchinoshima-Ushi*). These nsSNPs were included in 313 genes.

The sequence data were deposited in a database [DDBJ Read Archive: DRA000497]. In addition to submitting the data to the standard repositories, the positions of the SNPs for *Mishima-Ushi* can be viewed in GBrowse [17], along with supporting evidence (number of reads for each allele and density of SNPs) [URL: http://www.nodai-genome.org/cgi-bin/gb2u/gbrowse/NGRC_Ushi-DB/].

2.2. Sequence comparison between Mishima-Ushi individuals

To identify the genetic distance between *Mishima-Ushi* individuals, we performed a detailed comparison of all the detected polymorphisms, both SNPs (90%) and indels (10%), from the mapped data. The average number of polymorphisms in *Mishima-Ushi* individuals was 3,170,833, which consisted of 2,843,047 SNPs and 327,786 indels, ranging from 2,480,292 between Mishima1 and Mishima3 to 3,483,350 between Mishima2 and Mishima8 (Additional File 8). The average sequence-divergence rate between *Mishima-Ushi* individuals was 0.12%, which

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