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

Kaoru Tsuda [a,1,2], Ryouka Kawahara-Miki [a,2], Satoshi Sano [b], Misaki Imai [a], Tatsuo Noguchi [c], Yousuke Inayoshi [d], Tomohiro Kono [a,e,*]

[a] Genome Research Center, NODAI Research Institute, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan
[b] Ulvaniac Inc., 1-10-7 Higashi-gotanda, Shinagawa-ku, Tokyo 141-0022, Japan
[c] Fuji Experimental Farm, Tokyo University of Agriculture, 422 Fumoto, Fujinomiya-shi, Shizuoka 418-0109, Japan
[d] Livestock Improvement Research Laboratory, Livestock Technology Research Department, Yamaguchi Prefectural Agriculture and Forestry General Technology Center, 1200 Ichoukawara, Mine-shi, Yamaguchi 759-2221, Japan
[e] Department of Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

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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.

* Corresponding author at: Genome Research Center, NODAI Research Institute, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan. Fax: +81 3 5477 2543.
E-mail address: tomohiro@nodai.ac.jp (T. Kono).
[1] Current address: Department of Integrative Genomics, Tohoku Medical Megabank Organization, 2-1 Seiryo-machi, Aoba-ku, Sendai-shi, Miyagi 980-8573, Japan.
[2] These authors contributed equally to this work.

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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

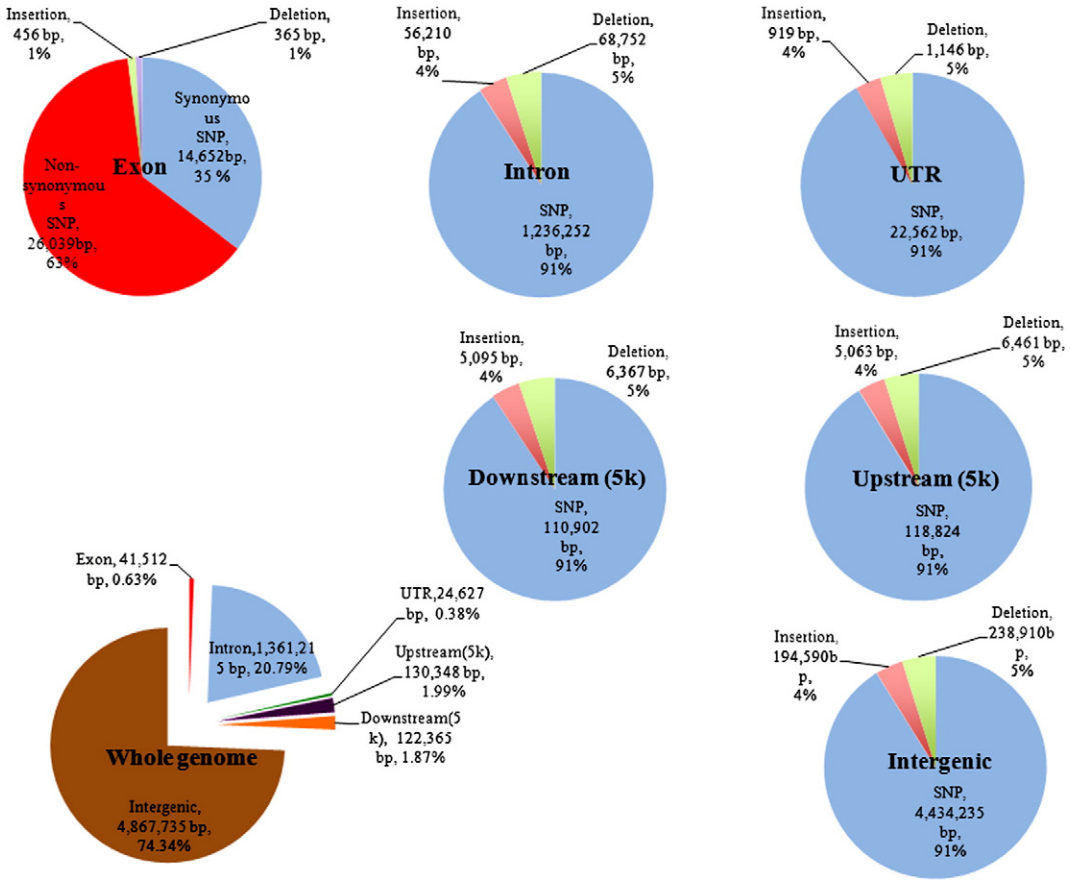

Fig. 1. Average number of detected SNPs and indels in the genome sequences of 8 *Mishima-Ushi* individuals. The genome sequences of 8 *Mishima-Ushi* were compared with the UCSC reference sequence (bosTau7.0). The large circle shows the average number and percentage of SNPs in each genomic region, i.e., exons, introns, UTRs, up- and downstream (5 kbp) regions of genes, and intergenic regions. The 6 small circles show the number of specific kinds of polymorphisms.

was calculated using all the polymorphisms in the whole genome (Table 1). The average number of nsSNPs among *Mishima-Ushi* individuals was 15,183 (range: 13,836–16,242), which was calculated on the basis of the exonic SNPs mapped to the RefSeq database and GenBank.

2.3. Sequence comparison between Mishima-Ushi and Kuchinoshima-Ushi

To identify genetic distance, we calculated the sequence divergence rate by using data mapped to genomic regions. The average number of polymorphisms (SNPs and indels) between *Mishima-Ushi* and *Kuchinoshima-Ushi* was 4,228,798, consisting of 3,830,665 SNPs and 398,133 indels (Table 1). The average sequence divergence rate between the *Mishima-Ushi* and *Kuchinoshima-Ushi* breeds was 0.161%, which was calculated by applying the same position-finding method for SNPs (chromosome number and position in each chromosome) to the whole genome (Table 1). The rate of sequence divergence between the 2 breeds was similar to that among dog breeds (0.2%) [18]. This comparison suggests that *Mishima-Ushi* could be separated from *Kuchinoshima-Ushi* [15], which might have originated from a common ancestor. Further comparative genome analyses may provide additional information for understanding their genetic characteristics.

Table 1
Calculated average sequence divergence rate for the whole-genome sequences of cattle.

Average sequence divergence rate for various comparisons	Average polymorphism number	Average seq. divergence rate	Average number of nsSNPs
In Mishima	3,170,833	0.121%	15,183
Between Mishima and Kuchinoshima	4,228,798	0.161%	14,964
Between Fleckvieh and Hereford	7,310,493	0.279%	27,353
Between native Japanese cattle and domestic European breeds	5,602,636	0.213%	20,107

2.4. Comparison between the SNPs of Mishima-Ushi and polymorphism data from previous studies

In order to gain a better understanding of the association between nsSNPs and phenotypes, we performed gene ontology (GO) analysis for the 313 genes harboring *Mishima-Ushi*-specific nsSNPs by using the web-based tool agriGO [19,20]. We confirmed that 170 genes were associated with molecular functions such as protein binding, enzyme binding, and magnesium ion binding (Additional File 9). The results of GO analysis suggest the possibility that the phenotypes associated with the genes of significantly enriched GO terms may represent specific characteristics of this breed.

We examined genes involved in the marbling of beef, because the meat of *Mishima-Ushi* possesses this feature similar to Japanese Black cattle. Previous studies detected several significant quantitative trait loci (QTL) that are responsible for the production of marbled beef in Japanese Black cattle [http://www.animalgenome.org/cgi-bin/QTLdb/BT/index] [21–24]. Hirano et al. performed a genome scan using a paternal half-sib family of Bull to identify the location of QTLs for intramuscular fat deposition (marbling) in a local population of Japanese Black cattle [21]. They mapped a marbling QTL to the region flanked by DIK0079 (20.7 cM) and TGLA303 (39.3 cM) on bovine chromosome 7.

Some of the genes harboring nsSNPs in *Mishima-Ushi* were found within this marbling-associated region, e.g., *SIM1*, *VNN1*, *SERAC1*, and *PACRG*.

In addition, 3 genes harboring *Mishima-Ushi*-specific nsSNPs are known to be involved in meat quality, i.e., calpastatin (*CAST*), cystic fibrosis transmembrane conductance regulator (*CFTR*), and glucosidase, alpha acid (*GAA*); however, their positions were not the same as those reported previously [25–31] (Table 2). Among them, we found that *CAST*, which is known to be involved in meat tenderness, had 2 reported nsSNPs [26–28] (Table 2). We suspect that these 2 variations might be conserved and associated with the meat tenderness of *Mishima-Ushi*, while additional studies are required to examine the association of identified nsSNPs with phenotypic traits in this population and to determine the functional consequences of these nsSNPs. We did not identify four reported sequence variations in titin (*TTN*), ribosomal protein L27a (*RPL27A*), akirin 2 (*AKIRIN2*), and endothelial differentiation sphingolipid G-protein-coupled receptor 1 (*EDG1*), which are thought to be associated with the marbling of meat [25,32,33].

We searched for other genes connected with the constitution of cattle. We found that there was an amino acid variation in the non-SMC condensin I complex, subunit G (*NCAPG*) gene, which was lysine to threonine (amino acid number 818) and sequence divergence of C to G, between two native Japanese cattle (*Mishima-Ushi* and *Kuchinoshima-Ushi*) and European breeds (Fleckvieh and Holstein). The *NCAPG* gene contains the candidate causative variation underlying CW-2 [34,35], which encodes a protein of the mammalian condensin I complex that has an important function in the regulation of mitotic cell division [36]. Setoguchi et al. [37] found that there was an amino acid variation in the *NCAPG* gene, which was isoleucine to methionine (amino acid number 442) and sequence divergence from T to G (sequence number 1326), in cattle with a large body size. They showed that sequence variation of *NCAPG* is associated with body frame size, and they observed that its associations with length- and height-associated traits temporally preceded those of thickness- and width-associated traits during adolescence, suggesting that the primary effect of CW-2 may be exerted on skeletal growth [37]. *Mishima-Ushi* is characterized as being lean with a small body size, wide breast, and narrow waist, similar to the Japanese native cattle *Kuchinoshima-Ushi*, while the marbling of fat in the meat of *Mishima-Ushi* is prominent as observed in Japanese Black cattle (Additional File 1). Although the reported positions of the variations were different to our result for *NCAPG*, our identification of SNPs specific to native Japanese cattle might support their hypothesis for the effects of sequence variation in the *NCAPG* gene.

We also examined the nsSNPs of each *Mishima-Ushi* individual. On average, 26,033 nsSNPs in 7581 genes were identified in each individual. Among them, 1230 were previously reported nsSNPs associated with meat traits in other cattle breeds (Additional File 10). In particular, 99 nsSNPs in 65 genes have been reported as nsSNPs of *Kuchinoshima-Ushi* (Additional File 10) [15]. Although these nsSNPs were not *Mishima-Ushi*-specific since they were not shared in all of the individuals, they provide additional information for further analysis of *Kuchinoshima-Ushi*.

2.5. Construction of a phylogenetic tree and a hypothesis for the migration of ancestral native Japanese cattle

To confirm the phylogenetic position of *Mishima-Ushi*, a consensus phylogenetic tree was constructed using fragmented sequences of exons and introns of 10 genes (21,865 bp) (Fig. 2A), which showed similarity with our previous consensus tree built for *Kuchinoshima-Ushi* [15].

The 2 native Japanese cattle breeds were completely separated from the European Hereford and Holstein strains. In addition, they were grouped in the same clade of the consensus phylogenetic tree when using the entire mtDNA sequence (16,335 bp) (Fig. 2B). In the phylogenetic tree constructed using complete mtDNA sequences, *Mishima-Ushi* and *Kuchinoshima-Ushi* were positioned very close to each other and were clearly separated from the European strain Hereford as well as from two strains of *B. indicus* (Fig. 2B).

The migration of ancestral cattle into Japan is thought to have started approximately 1500–2000 years ago [6–8]. According to previous analysis on mtDNA haplotypes, the primary population that migrated into Japan was perhaps a North Asian strain with low levels of genetic polymorphisms [6–8]. There is a possibility that this low level of genetic diversity resulted in a bottleneck in ancestral Japanese cattle. However, because of advanced cross mating among these strains, genetic variation has increased in native Japanese cattle [8]. In the phylogenetic tree computed using mtDNA sequences, both breeds were not clearly separated, suggesting that a common ancestor could have been involved in their establishment (Fig. 2B).

3. Conclusion

In the current study, we performed whole-genome sequencing of 8 individuals of the native Japanese cattle species *Mishima-Ushi* to gain a better understanding of the genomic features of this species. The results showed that each individual *Mishima-Ushi* had a large number of polymorphisms, that is, approximately 6.54 million SNPs, including 400 nsSNPs in 313 genes that were specific to each of them. Although we could not find any genetic factors connected with their meat quality, our data are in agreement with a report in which *NCAPG* was associated with the body frame size of cattle. Furthermore, phylogenetic analysis showed the independent phylogenetic position of *Mishima-Ushi*, highlighting the pure breeding of this strain. Our results provide a framework for further genetic studies in the *Mishima-Ushi* population and research on the function of SNP-containing genes.

4. Materials and methods

4.1. Samples and their breeding conditions

We got permission from four breeders of *Mishima-Ushi*, the Mishima-Cattle Conservation Association of Mishima Island, and the mayor of Hagi-City, Yamaguchi Prefecture, Agency for Cultural Affairs

Table 2
List of genes harboring *Mishima-Ushi* specific nsSNPs that were previously reported to have an association with the marbling of meat in cattle.

Gene symbol	Gene ID (mRNA)	Description	Chromosome	Genome position of reported SNPs	Reference sequence/ variant sequence in *Mishima-Ushi*	Function	dbSNP ID	Reference
CFTR	NM_174018	Cystic fibrosis transmembrane conductance regulator	4	52,558,356	C/A	CFTR leads to fatty acid composition and metabolism	Not registered	[29–30]
CAST	NM_001030318	Calpastatin	7	98,435,972 98,436,005	A/G G/A	Higher CAST expression in longissimus dorsi muscle has a direct role for calpastatin in meat tenderization	rs210072660 rs384020496	[26–28]
GAA	NM_173913	Glucosidase, and alpha acid	19	53,598,234	C/G	Two mutations in GAA lead to generalized glycogenosis	Not registered	[31]

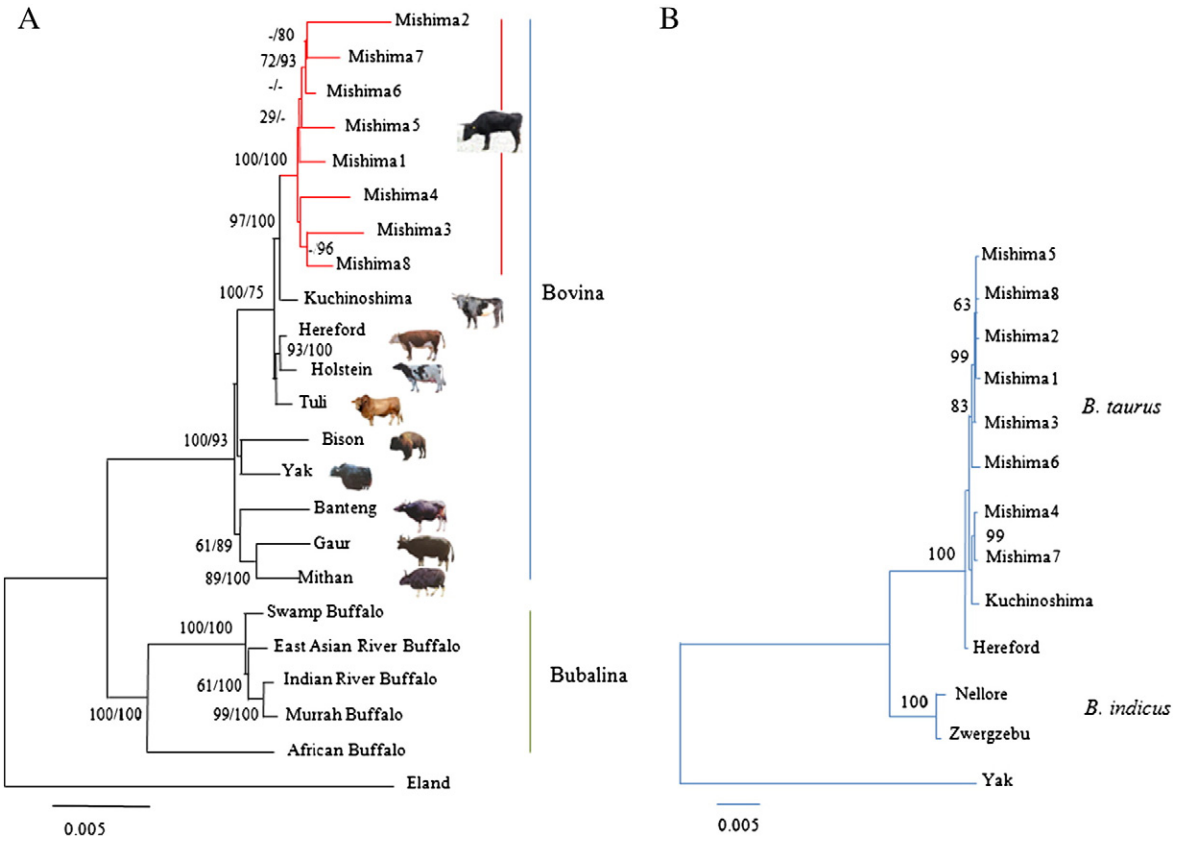

Fig. 2. Consensus phylogenetic trees of bovine-related species. A. NJ tree of 16 bovine-related strains using fragment sequences of nuclear genes. The position of the *Mishima-Ushi* strain is marked as a red branch. The numbers beside the internal branches indicate bootstrap values from 10,000 replicates (left) and Bayesian posterior probabilities from 5,000,000 replications (right) (shown as percentages). "—" indicates a node not recovered in the Bayesian analysis or 50% of bootstrap values. Eland (*Taurotragus oryx*), a species from Strepsicerotini, was used as an outgroup. "Mithan," also called "gayal," is a domesticated gaur. B. Consensus phylogenetic tree using the entire mtDNA sequence. Expected phylogenetic tree with the mtDNA sequences of 6 strains (entire sequence data) for the proposed migration roots of ancestral Japanese cattle. The numbers beside the internal branches indicate the bootstrap value from 10,000 replicates using the MEGA 5.05 program.

in Japan, for collecting blood samples from *Mishima-Ushi* and carrying out this project. We collected blood samples from 8 *Mishima-Ushi* (Mishima1–8) cattle, aged 2–8 years (7 females and 1 male) (Additional Files 1 and 2). Several breeders are breeding these cattle in both separate and closed fields on Mishima Island in Yamaguchi Prefecture. These 8 individuals came from 4 maternal ancestors. The number of individuals of this strain, which was bred for farm work until around 1945, decreased due to the popularization and automation of agricultural machines. These cattle are in a severe breeding condition because breeders have to maintain their genuine genetic phenotype, which is designated as a national natural treasure in Japan, while increasing the number of animals in order to avoid inbreeding.

4.2. DNA library construction and sequencing

Genomic DNA was extracted from 4 mL blood by using standard phenol/chloroform extraction methods [38]. Libraries were prepared using a Paired-End DNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. Briefly, 5 μg DNA were fragmented to a median fragment size of 300 bp by using Adaptive Focused Acoustics (Covaris, Inc., Woburn, MA, USA). The products were end-repaired, adenosines ("A" bases) were added to the 3′-end of the fragments, and paired-end adapters were ligated. After size selection on a 2% agarose gel, the DNA fragments were enriched by 12 cycles of the polymerase chain reaction (PCR). The derived fragment DNA libraries were used to generate clusters on an Illumina cBOT by using either a TrueSeq PE Cluster Kit v2-cBot-HS (Illumina) and sequenced on a HiSeq2000 or by using a TrueSeq SBS Kit-HS (200 cycles; Illumina) following the manufacturer's instructions.

4.3. Software for data analysis

As a reference sequence, we used the Oct. 2011 bosTau7.0 assembly (UCSC Genome Bioinformatics) [http://genome.ucsc.edu/cgi-bin/hgGateway], excluding scaffolds not yet assigned to specific chromosomes. Image analysis and ELAND alignment were performed using Illumina's Pipeline Analysis software ver. 1.7.0 (Illumina). Sequences passing the standard Illumina GA pipeline filters (i.e., clusters with intensities >0.6 times the average of the highest intensity and the sum of the 2 highest intensities for the first 25 cycles) were retained.

For short-read alignment and consensus assembly, we used the recently developed Burrows–Wheeler algorithm (BWA ver. 0.6.2) [39]. The BWA default values for mapping were as follows: maximum edit distance (maxDiff) = 0.04; maximum number of gap opens (maxGapO) = 1; maximum number of gap extensions (maxGapE) = −1; disallow a long deletion within bp (nDelTail) = 10; disallow an indel within bp (nIndelEnd) = 5; take the first subsequence as seed (seedLen) = infinite maximum edit distance in the seed (maxSeedDiff) = 2; number of threads (nThrds) = 1; mismatch penalty (misMsc) = 3; gap open penalty (gapOsc) = 11; gap extension penalty (gapEsc) = 4; and parameter for read trimming (trimQual) = 0. After read mapping, we deleted multi-mapped reads and unmapped reads. Uniquely mapped reads were used for further analyses.

SAMtools (ver. 0.1.16) was used to identify any base that deviated from the reference base (SNP calling) [40]. After the preliminary analysis using various filters (minimum read depth = 3, 6, 9; cutoff of percent aligned reads of SNPs per total mapped reads: 30%, 60%, 100%) and comparing the results (Additional File 5), the following filters were applied: minimum read depth = 3; minimum SNP = 2; and a

30% cutoff of percent aligned reads identifying the number of SNPs per total mapped reads at the SNP sites. We also used BWA to estimate the sequence read depth, which represents the reliability of continuous sequences in the same region. After SNP calling by using BWA, we annotated the identified SNPs by using GenBank and RefSeq gene sets (14,382 genes; the gene set is available on the UCSC download site [ftp://hgdownload.cse.ucsc.edu/goldenPath/bosTau7]).

4.4. Sorting of analysis data

We identified SNPs by comparing the mapped sequence data with sufficient sequence depth to those of the reference sequence. The location of the detected SNPs and indels are given in terms of the chromosome number and the genomic position of the reference sequence, according to the mapped fragment sequence.

After separating all SNPs and indels by coding regions and intergenic regions, they were further classified into exons, introns, UTRs, upstream (5 kbp) region, and downstream (5 kbp) region in the coding region and in the intergenic region. In addition, all of the exonic SNPs were separated into synonymous and nsSNPs. Comparison among individuals or between different strains was performed using all SNP and indel information. We also mapped all genomic sequence data of *Kuchinoshima-Ushi* and Fleckvieh to bosTau7.0 for this analysis. Sequence divergence rates were calculated by comparison with the reference sequence. We calculated the detection rates for common nsSNPs, indels, and genes with nsSNPs specific to all *Mishima-Ushi* samples. Annotation of all genes with common nsSNPs specific to the *Mishima-Ushi* strain was executed for comparison with another native Japanese cattle (*Kuchinoshima-Ushi*) or domestic European breeds (Fleckvieh and Hereford).

4.5. Phylogenetic analysis

We used 16 species from the Bovinae subfamily (10 species from the subtribe Bovina, 5 species from the subtribe Bubalina, and 1 species from the tribe Strepsicerotini as an outgroup) for the phylogenetic analysis. On the basis of previous studies [15,41], we used 10 nuclear genes (21,865 bp) of which the sequence data have been determined and published, except for *Mishima-Ushi*. We concatenated newly determined sequences of *Mishima-Ushi* and the published sequences and aligned them for the phylogenetic analyses. Phylogenetic trees were obtained by applying the neighbor-joining (NJ) method [42] by using Molecular Evolutionary Genetics Analysis (MEGA) software (version 5.05) [43]. In addition, we applied the Bayesian method using MrBayes (ver. 3.1.2) [44] for calculating the posterior probability. Phylogenetic trees with complete mtDNA sequences were estimated by using the NJ method [42] with MEGA [43].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2013.08.002.

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