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Original Article

Comprehensive assessment of genetic diversity, structure, and relationship in four Japanese cattle breeds by Illumina 50K SNP array analysis

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ABSTRACT

There are four unique cattle breeds in Japan: Japanese Black, Japanese Brown, Japanese Polled, and Japanese Shorthorn. The objective of this study was to comprehensively assess the genetic diversity, structure, relationship, and the degree of influence from foreign breeds (Angus, Simmental, Hanwoo, Shorthorn, Ayrshire, Brown Swiss, and Devon) in the Japanese cattle breeds using Illumina 50K SNP array. In principal component analysis, each Japanese breed was separately clustered except for Japanese Shorthorn and Shorthorn. Japanese cattle breeds also showed different genetic components from each other at $K \geq 5$ in population structure analysis. Japanese Shorthorn, on the other hand, had a very similar structure to Shorthorn at $K \leq 9$, and Japanese Polled had a partially similar component with Angus at $K = 3-7$. Such close relationships were also observed in the phylogenetic tree. These findings imply that Japanese cattle breeds share genetic components with European cattle breeds to some extent, while they have been almost differentiated. In population structure analysis, Japanese Black cattle shared little genetic component (3.5%) with European breeds. This is the first study to determine the extent to which European breeds impact Japanese breeds.

Keywords: 50K SNP array, genetic diversity, genetic relationship, genetic structure,

1 *Japanese cattle breeds*

INTRODUCTION

In Japan, there are four unique cattle breeds: Japanese Black, Japanese Brown, Japanese Polled, and Japanese Shorthorn cattle. Due to crossbreeding with several foreign cattle breeds into Japanese indigenous cattle during the dawn of breeding improvement in the early 20th century, they have varied genetically (Gotoh *et al.*, 2014; Mannen *et al.*, 2017; Sasazaki *et al.*, 2006). Some imported European cattle breeds, such as Simmental, Devon, Brown Swiss, and Ayrshire, were temporarily crossbred with Japanese Black cattle. However, because hybrid cattle require more feed than native cattle, the original importation strategy failed, and the Japanese government swiftly removed crossed cattle from herds to re-create totally pure breeds (Mukai *et al.*, 1989; Mannen *et al.*, 1998). Nevertheless, some genetic influence from the European breed may remain. Japanese Brown cattle are categorized into two strains: Kochi-strain and Kumamoto-strain, which are bred in Kochi Prefecture and Kumamoto Prefecture, respectively. These strains were independently established in each prefecture by crossbreeding with Simmental, Devon, and Hanwoo (Honda *et al.*, 2006). As a result, these two strains have different phenotypes in parts. For instance, the Kochi-strain has a brown body with some black parts such as eyelids, horns, and a nose, while the color of the Kumamoto-strain is solid brown. The Japanese Polled and Japanese Shorthorn were established by crossbreeding with

1 Aberdeen Angus and Shorthorn, respectively. These are bred in limited areas, Japanese
2 Polled in Yamaguchi Prefecture (Mannen, 2017) and Japanese Shorthorn in Tohoku and
3 Hokkaido regions (Noda *et al.*, 2018).

4 The genetic structure of Japanese cattle breeds has been studied based on the sequence
5 of the mtDNA D-loop region in previous studies. They discovered that the unique
6 haplogroup T4 was detected in all Japanese cattle breeds, with frequency exceeding half
7 (Mannen *et al.*, 1998, 2004, 2017; Noda *et al.*, 2018; Sasazaki *et al.*, 2006). In addition,
8 Noda *et al.* (2018) discovered that a rare haplogroup P was also detected in Japanese
9 Shorthorn. These results in previous studies revealed that all Japanese cattle breeds may
10 have distinct genetic components and structures from those of foreign cattle breeds.

11 Meanwhile, a few studies were carried out to investigate the genetic relationships
12 between Japanese and foreign cattle breeds using autosomal single nucleotide
13 polymorphisms (SNPs). Using genotyped data of 117 autosomal SNPs, Yonesaka *et al.*
14 (2016) reported that Japanese Polled and Japanese Shorthorn were clustered with
15 European cattle breeds, suggesting strong genetic influences from European breeds
16 during the establishment of these two Japanese breeds. Furthermore, Sharma *et al.* (2016)
17 reported that American Wagyu and Korean cattle were genetically close according to 50K
18 SNP array data analysis. These two previous studies showed the close relationships

1 between Japanese and some foreign cattle breeds, though mitochondrial analysis implied
2 the unique genetic components of Japanese breeds. This suggested that further
3 investigation would be required using enormous SNPs data covering the whole genome,
4 in addition to mtDNA analysis, to elucidate their genetic structures and relationships.

5 However, there is no study which investigated on all four Japanese cattle breeds at the
6 same time using 50K SNP array analysis. Furthermore, no study has investigated the
7 degree of genetic influences from foreign cattle breeds on each Japanese breed. Thus, our
8 objective in the current study was to comprehensively assess the genetic diversity,
9 structure, relationship, and the degree of influence from foreign breeds in all four
10 Japanese cattle breeds using 50K SNP array data.

12 **MATERIALS AND METHODS**

13 **Ethics**

14 The experiments in the current study were carried out according to the Kobe University
15 Animal Experimentation Regulations. All samples were collected by veterinarians or
16 individual livestock owners. These treatments were carried out in accordance with
17 Japanese Veterinarians Act (Act No. 186 of 1949).

Collected samples and downloaded data

We collected DNA samples from all Japanese cattle breeds; 48 samples from Japanese Black, 42 samples from Japanese Brown Kochi-strain, 48 samples from Japanese Brown Kumamoto-strain, 48 samples from Japanese Polled, and 48 samples from Japanese Shorthorn (Table S1). The samples of Japanese Black were collected at the 9th National Competitive Exhibition of Wagyu (Zenkoku Wagyu Noryoku Kyoshinkai), which was entered by Japanese Black cattle bred in all over Japan (a total of 34 prefectures). The samples of Japanese Brown Kochi-strain, Japanese Brown Kumamoto-strain, Japanese Polled, and Japanese Shorthorn were collected from animals bred in Kochi, Kumamoto, Yamaguchi, and Iwate Prefecture, respectively. Although animals of these Japanese breeds were somewhat related to each other within each breed, we selected as unrelated samples as we can by random selection. The samples of Japanese Shorthorn were also used in a previous study (Noda *et al.* 2018). DNA was extracted from blood, nasal secretion, or fat tissue. Meanwhile, we downloaded genotyping data of 20 Angus, 20 Simmental, 8 Hanwoo, 17 Shorthorn, 18 Ayrshire, 12 Brown Swiss, and 4 Devon from dryad.org (<https://datadryad.org/stash/dataset/doi:10.5061/dryad.th092>). They were genotyped using BovineSNP50 BeadChip Ver.1 (Illumina, San Diego, CA, USA) in a previous study (Decker *et al.*, 2014).

Genotyping and quality control

All Japanese cattle breed samples were genotyped using each of three versions (*i.e.* Ver.1, Ver.2, and Ver.3) of BovineSNP50 BeadChip (Illumina, San Diego, CA, USA). The Ver.1 was applied for all samples of 48 Japanese Black and 10 Japanese Brown Kochi-strain, the Ver.2 for 48 Japanese Brown Kumamoto-strain and the Ver.3 for 32 Japanese Brown Kochi-strain, 48 Japanese Polled, and 48 Japanese Shorthorn. Common 40,329 autosomal SNPs contained in all versions were selected based on positions on UMD3.0 and SNP_name. Moreover, some allele representations even for the same SNP varied depends on the versions because of different strands. We therefore rewrote alleles of such the SNPs as the consistent allele and excluded SNPs for which the strand of allele was unclear.

One individual of Japanese Brown Kochi-strain was excluded due to its call rate < 0.95 based on the genotyping results. All SNPs subsequently went through quality control using PLINK v.1.9 (Purcell *et al.*, 2007). The exclusion criteria for SNPs were call rate < 0.95 , minor allele frequencies (MAF) < 0.01 , and Hardy-Weinberg equilibrium < 0.001 . A total of 27,007 SNPs was finally used to calculate indices of genetic diversity, perform principal component analysis (PCA), and construct the phylogenetic tree in the current

study.

Statistical analysis

The average of MAF, expected (H_e) and observed (H_o) heterozygosities, and the inbreeding coefficient (F_{is}) for each SNP were calculated in each breed using PLINK v.1.9., followed by the calculation of the average for all SNPs as indices of genetic diversity for each breed. PCA was performed in PLINK v.1.9. from all allele frequencies in each breed.

Population structure analysis was conducted in ADMIXTURE v1.3.0 to determine the population structure and degree of admixture among the twelve breeds (Alexander *et al.*, 2009). For evaluating the model fitting, the cross-validation (CV) procedure was performed by 5-fold CV. Prior to the analysis, LD pruning was applied for the 27,007 SNPs to exclude SNPs of $r^2 > 0.5$ (Blachburn *et al.* 2017), resulting in 14,355 SNPs. We performed 11 independent runs for each K (from 2 to 12), followed by the visualizing in CLUMPAK server (<http://clumpak.tau.ac.il/>). We additionally applied the population structure analysis for eight cattle breeds including Japanese Black and seven foreign cattle breeds for the estimation of the genetic influence from foreign breeds for Japanese Black cattle in detail. In the additional analysis, the result at $K = 3$ is the most informative to

estimate the genetic influence, and thus, the average of estimated ancestry proportions (%) for each cattle breed were calculated to observe the admixture proportions of foreign cattle breeds for Japanese Black at $K = 3$.

To investigate the phylogenetic relationships among populations, the standard genetic distance of Nei (Nei, 1972) were calculated in RStudio v4.0.2 to construct the phylogenetic tree using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973). In the phylogenetic relationship analysis, we included *Bos indicus* ($n = 10$), which was genotyped using BovineSNP50 BeadChip, as an outgroup.

RESULTS

In all breeds, we determined allele frequencies (Table 1). In Japanese cattle breeds, the mean MAF ranged from 0.196 to 0.213 and the mean H_e and H_o ranged from 0.262 to 0.284 and from 0.283 to 0.299, respectively. In foreign cattle breeds, the mean MAF ranged from 0.197 to 0.234 and the mean H_e and H_o ranged from 0.267 to 0.311 and from 0.269 to 0.322, respectively. The F_{is} ranged from -0.137 to -0.006 among all breeds.

According to PCA, animals of the same breed were nearby plotted and each breed nearly made an independent cluster except for Japanese Shorthorn and Shorthorn (Figure

1, S1). Whereas Asian cattle breeds and European cattle breeds were almost distinguished by PC1 (5.18%), Japanese Polled and Japanese Shorthorn were plotted close to European breeds in the PC1. PC2 (4.27%) was the single factor that distinguished Japanese Polled from other breeds. PC3 (3.34%) significantly distinguished Japanese Black, Japanese Brown Kochi-strain, and Japanese Brown Kumamoto-strain. Japanese Shorthorn was clustered with Shorthorn by any PCs.

The results of population structure analysis were showed in Figure 2. The CV error plot (Figure S2) indicates the higher K values exhibit the lower CV errors at K=1-7, and reaches the plateau at K>7. Japanese Black, Japanese Brown Kochi-strain, and Japanese Brown Kumamoto-strain were clustered at K = 2, and Japanese Polled and European breeds showed similar and admixed patterns. At practically all K's, Japanese Shorthorn was clustered with Shorthorn. Japanese Polled had a nearly solid pattern at K = 3, and its component was admixed in Japanese Black, Hanwoo, and European breeds. Two strains of Japanese Brown were identified at K = 4. At K = 5, Japanese Black made an independent cluster but only Hanwoo has this component. Japanese breeds showed almost independent component at $K \geq 5$, whereas European breeds showed admixed patterns. The genetic component dominant in European breeds were observed at $K \geq 6$. From K = 7 to 9, European breeds were gradually separated. Japanese Shorthorn and Shorthorn were

separated at $K \geq 10$.

An additional population structure analysis was conducted to estimate the degree of genetic influence from foreign cattle breeds on Japanese Black cattle. The result at $K = 3$ would seem to be appropriate to estimate it. According to the result, the mean admixed proportion in Japanese Black cattle was 3.5% (Figure 3, Table S2).

We illustrated the phylogenetic tree using UPGMA method based on Nei's genetic distance (Figure 4). As the result, Japanese Black, Japanese Brown Kochi-strain, Japanese Brown Kumamoto-strain, and Hanwoo were clustered as Asian cattle breeds. Among these Asian breeds, Japanese Black and Hanwoo were nearby located in the cluster. Meanwhile, Japanese Polled was clustered with Angus. Furthermore, similar with the results of the other analyses, Japanese Shorthorn was clustered with Shorthorn.

DISCUSSION

In the current study, we investigated on genetic diversities, structures, and relationships in Japanese cattle breeds using 50K SNP array data. Although they have been reported in prior research, based on sequences of mtDNA D-loop region, SNPs on Y chromosome, and several hundred autosomal SNPs (Mannen *et al.*, 1998, 2004, 2006, 2017; Noda *et al.*, 2018; Sasazaki *et al.*, 2006; Yonesaka *et al.* 2016), this is the first study to

comprehensively investigate using 50K SNP array data in all four Japanese cattle breeds. We also used 50K SNP datasets of foreign cattle breeds downloaded from a database to compare the genetic diversity and survey the genetic relationship between Japanese and foreign breeds. Although the number of datasets for the foreign breeds was relatively small, we were able to assess the broad relationships as with previous studies (Alam *et al.*, 2021; Decker *et al.*, 2014; Strucken *et al.*, 2021). However, the number of datasets of Devon was significantly small, and therefore, we paid attention considering the small number in assessment of the relationships between Devon and the other breeds in this study.

The indicators of genetic diversity (the mean MAF, H_e , and H_o) in Japanese cattle breeds (MAF: 0.196–0.213, H_e : 0.262–0.284, H_o : 0.283–0.299) were relatively lower than those in the foreign breeds (MAF: 0.197–0.234, H_e : 0.259–0.311, H_o : 0.269–0.322). This was consistent with some previous studies showing that Japanese cattle breeds had low genetic diversity than European and Korean cattle breeds based on sequences of mtDNA D-loop region and autosomal SNPs (Mannen *et al.*, 2004; Sharma *et al.*, 2016; Yonesaka *et al.*, 2016). In Japanese Black, the inbreeding coefficient has recently increased mainly due to the intensive use of a few sires for the improvement of beef quantity and quality, and therefore its genetic diversity has decreased (Honda *et al.*, 2001).

1 Meanwhile, the genetic diversity of the Japanese Brown Kochi-strain, Japanese Brown
2 Kumamoto-strain, Japanese Shorthorn, and Japanese Polled was minimal, owing to the
3 limited size of the populations (Nagamine *et al.*, 1997; Honda *et al.*, 2006; Mannen, 2017).
4 For example, the Japanese Brown Kochi-strain has been maintained on less than 500
5 individuals (Honda *et al.*, 2006). Since the genetic diversity in the Japanese breeds is
6 going to decrease, we would be required to constantly observe the genetic diversity to
7 avoid the decrease.

8 In PCA, almost all breeds were separately clustered. Although Japanese Black and
9 Japanese Brown (Kochi-strain and Kumamoto-strain) were nearly plotted in PC1 and PC2,
10 they were completely separated in PC3. We assumed that these Japanese cattle breeds
11 would not have such strong relationships because PC1, PC2, and PC3 explained similar
12 proportions (5.18%, 4.27%, and 3.34%, respectively) and we observed similar results in
13 PC4 (3.00%, data not shown) and PC5 (2.54%, data not shown) with the result in PC3.
14 Furthermore, the Japanese cattle breed clusters were wider apart than the foreign cattle
15 breed clusters. In addition to the PCA, population structure analysis revealed that the
16 Japanese breeds have the different genetic structures from each other at $K \geq 5$, while the
17 foreign breeds showed the similar genetic structures. These results suggested that all
18 Japanese cattle breeds have distinct genetic characteristics.

1 We detected a unique genetic structure in Japanese Polled by PCA and population
2 structure analyses. This breed has been maintained as small population (< 200) in a
3 limited area of Yamaguchi Prefecture, Japan (Mannen *et al.*, 2017). According to a
4 previous study, the genetic drift happens faster in small population than larger one (Masel,
5 2011). The small population size might cause the great genetic drift in Japanese Polled,
6 and therefore the breed has been differentiated from the other cattle breeds. However, the
7 population structure analysis at $K = 6$ and 7 found a dominating genetic component in
8 Japanese Polled in Angus, and these two breeds were clustered in the phylogenetic tree
9 (Figure 4). Japanese Polled was established by crossbreeding with imported Angus to
10 improve its carcass traits (Mannen, 2017). Based on the breeding history and the results
11 in the current study, this breed shares some genetic components with Angus, while it has
12 been differentiated by genetic drift.

13 Japanese Shorthorn showed the quite similar genetic structure with Shorthorn in PCA,
14 population structure analysis, and the phylogenetic tree, while Japanese Shorthorn had
15 distinct mitochondrial haplogroups P (Noda *et al.*, 2018). This difference was due to
16 genetic markers used in each study, *i.e.* autosomal markers in the current study and
17 mitochondrial markers in the previous study. Whereas mitochondrial markers would
18 useful for estimation of maternal origin, autosomal markers are important to investigate

1 genetic structure and relationships within and among breeds. Japanese Shorthorn was
2 established by crossbreeding of indigenous cattle “Nambu” bred in Tohoku region in
3 Japan with Shorthorn imported from America (Noda *et al.*, 2018). The results in the
4 current study suggested that this breed would have the strong genetic influence of
5 Shorthorn. Furthermore, in terms of origin, the ancestor of the Japanese Shorthorn
6 “Nambu” is thought to be distinct from the common ancestors of the other Japanese cattle
7 breeds (Yamada, 1922; Noda *et al.*, 2018). This historical context could also explain the
8 apparent genetic differences between the various Japanese cattle breeds.

9 In population structure analysis, the two strains of Japanese Brown and Japanese Black
10 cattle showed almost solid patterns at $K \geq 5$, indicating that these breeds are not
11 influenced by foreign cattle breeds. With these Japanese breeds, only Hanwoo showed
12 the same genetic components. In particular, Japanese Black and Hanwoo were observed
13 in almost the same cluster in the PCA and phylogenetic tree. This result was consistent
14 with some previous studies (Sharma *et al.*, 2016; Lin *et al.*, 2010; Lee *et al.*, 2014),
15 implying that despite no crossbreeding, these two cattle breeds would have similar genetic
16 constructions. Furthermore, the result in population structure analysis showed that they
17 shared about 50% of their genetic constructions (Figure 3, Table S2). Based on these
18 results, Japanese Black and Hanwoo may share a genetic component acquired from a

common ancestor during the migration period across the Korean Peninsula to Japan. Meanwhile, population structure analysis revealed that Japanese Black shared 3.5% of their genetic constructions with European cattle breeds. Though the value might not be so accurate, the result suggested that the influence of crossbreeding is little for Japanese Black cattle. The recent breeding system, which is fully isolated to other cattle breeds, has reduced genetic impacts from European cattle breeds, resulting in a small genetic component.

As for sub-structures within Japanese Black, Japanese Brown Kochi-strain and Japanese polled, they show the disappearance and reappearance at $K \geq 9$. The CV error plot (Figure S2) suggests the model fitting improved in ascending order of K (up to 7), and fittings reach plateau at $K > 7$. Therefore, the instable appearance/ disappearance of sub-structures within Japanese breeds are not due to model misspecification caused by the parameter-overfitting. It is noteworthy that the sub-structures within Japanese breeds are fundamentally descended from unique sources, and the reappeared sub-structures are essentially consistent with the ones before disappearances. These observations suggest the hierarchical nested relationships in the population structure are stable. Accordingly, the instability may not be due to the instable inferences of genetic structure itself, but the instable exposure of the “hidden” genetic structure consists of the multiple sub-structures

1 with similar levels of genetic differentiation ($F_{st} = 0.079\sim 0.109$). Japanese cattle breeds
2 have been isolated from other breeds and maintained by a limited number of sires since
3 the breed establishment, leading to the lower genetic diversity. Such the genetic
4 background would cause the sub-structures in each breed.

5 The genetic relationships illustrated in the current study were almost consistent with
6 the results based on 117 autosomal SNPs (Yonesaka *et al.*, 2016). Accordingly, we might
7 not need so abundant SNPs data to broadly assess the relationships. On the other hand,
8 we should use much genetic information to estimate the accurate degree of influences and
9 admixtures from other breeds. Further investigation using whole-genome sequences
10 might provide more accurate evaluation.

11 The results in the current study revealed that all the Japanese cattle breeds have distinct
12 genetic structure from each other, implying that the Japanese cattle breeds have been
13 almost differentiated from indigenous cattle in Japan. On the other hand, they shared a
14 part of genetic component with the foreign cattle breeds which were established from the
15 same ancestor or crossbred with the Japanese cattle breeds. In addition, for the first time,
16 the degree of influence from foreign breeds was measured, particularly in Japanese Black
17 cattle. As the result, Japanese Black showed little influence, whereas Japanese Shorthorn
18 was strongly influenced by Shorthorn. Further research into the genetic structure of each

- 1 breed would provide further knowledge useful for improving breeding and maintaining
- 2 genetic diversity.

1 **ACKNOWLEDGMENTS**

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3

4 **CONFLICT OF INTEREST**

5 There is no conflict of interest relevant to the contents in the current study.

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Table 1 Indices of genetic diversity within each cattle breed

Breed	n	MAF	H_e	H_o	F_{is}
JB	48	0.207	0.278	0.287	-0.034
JBR-Ko	42	0.196	0.262	0.283	-0.080
JBR-Ku	48	0.213	0.284	0.299	-0.055
JP	48	0.203	0.269	0.291	-0.082
JSH	48	0.209	0.280	0.290	-0.035
HNW	8	0.212	0.282	0.307	-0.088
AN	20	0.234	0.311	0.322	-0.033
SIM	20	0.228	0.305	0.319	-0.049
SH	17	0.198	0.267	0.269	-0.006
AYR	18	0.230	0.306	0.321	-0.049
BSW	12	0.208	0.278	0.303	-0.092
DEV	4	0.197	0.259	0.294	-0.137

MAF: mean minor allele frequencies, H_o : observed heterozygosity, H_e : expected heterozygosity, F_{is} : inbreeding coefficient, JB: Japanese Black, JBR-Ko: Japanese Brown Kochi-strain, JBR-Ku: Japanese Brown Kumamoto-strain, JP: Japanese Polled, JSH: Japanese Shorthorn, HNW: Hanwoo, AN: Angus, SIM: Simmental, SH: Shorthorn, AYR: Ayrshire, BSW: Brown Swiss, DEV: Devon.

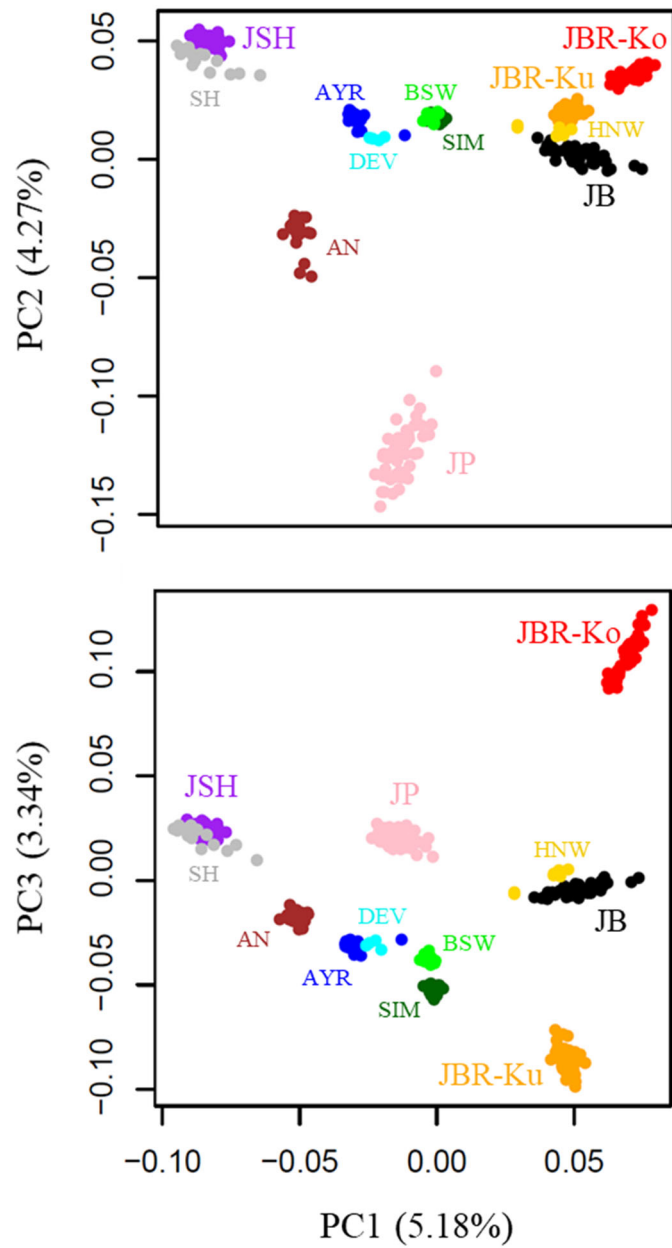


Figure 1 Principal component analysis (PCA) in Japanese and foreign cattle breeds
 JB: Japanese Black, JBR-Ko: Japanese Brown Kochi-strain, JBR-Ku: Japanese Brown Kumamoto-strain, JP: Japanese Polled, JSH: Japanese Shorthorn, HNW: Hanwoo, AN: Angus, SIM: Simmental, SH: Shorthorn, AYR: Ayrshire, BSW: Brown Swiss, DEV: Devon.

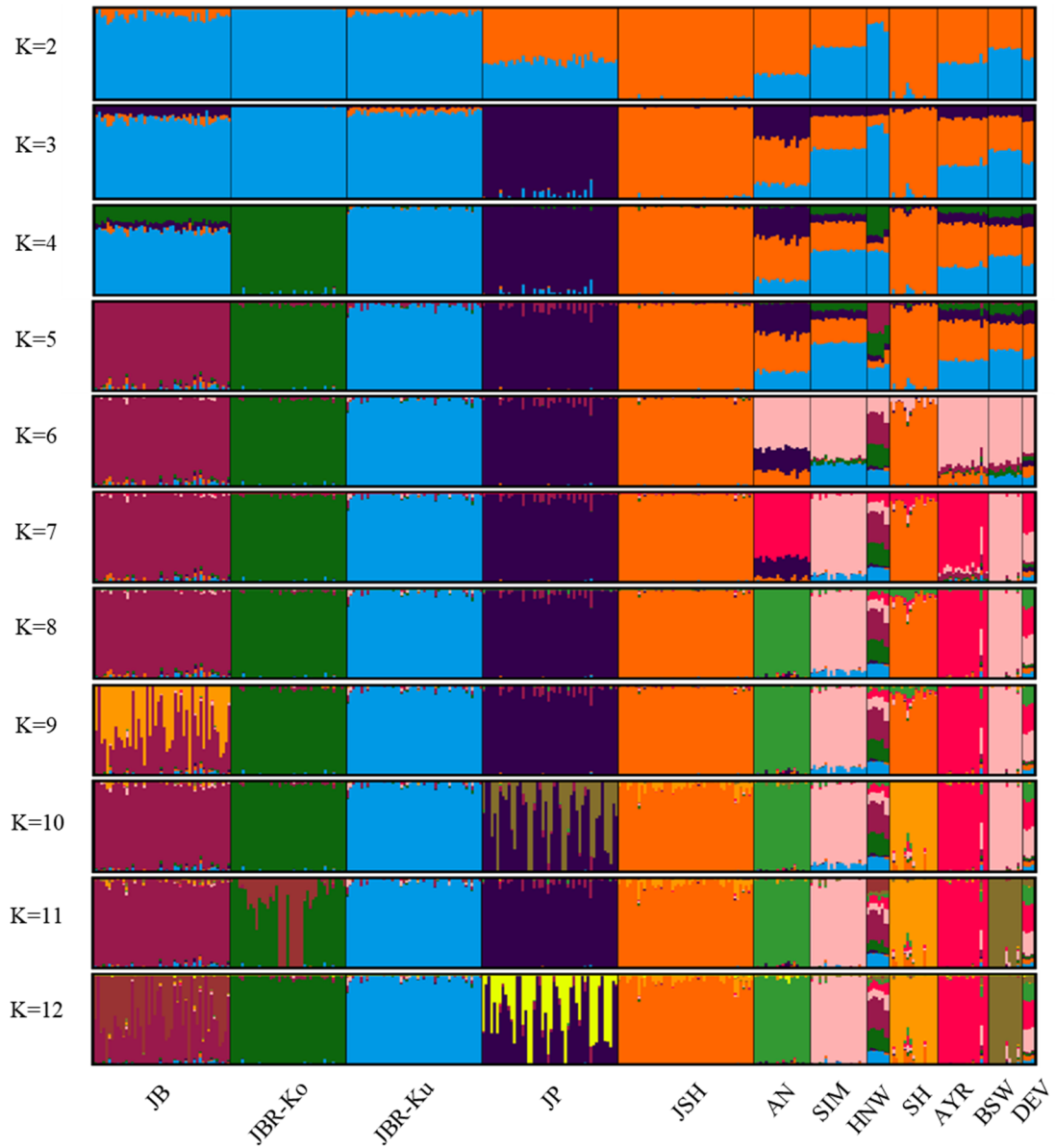


Figure 2 Genetic structure of Japanese and foreign cattle breeds at K = 2 - 12

JB: Japanese Black, JBR-Ko: Japanese Brown Kochi-strain, JBR-Ku: Japanese Brown Kumamoto-strain, JP: Japanese Polled, JSH: Japanese Shorthorn, HNW: Hanwoo, AN: Angus, SIM: Simmental, SH: Shorthorn, AYR: Ayrshire, BSW: Brown Swiss, DEV: Devon.

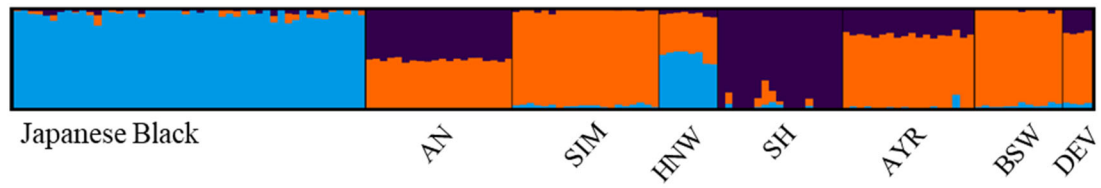


Figure 3 Genetic structure of Japanese Black and 7 foreign cattle breeds at $K = 3$
 AN: Angus, SIM: Simmental, HNW: Hanwoo, SH: Shorthorn, AYR: Ayrshire, BSW: Brown Swiss,
 DEV: Devon.

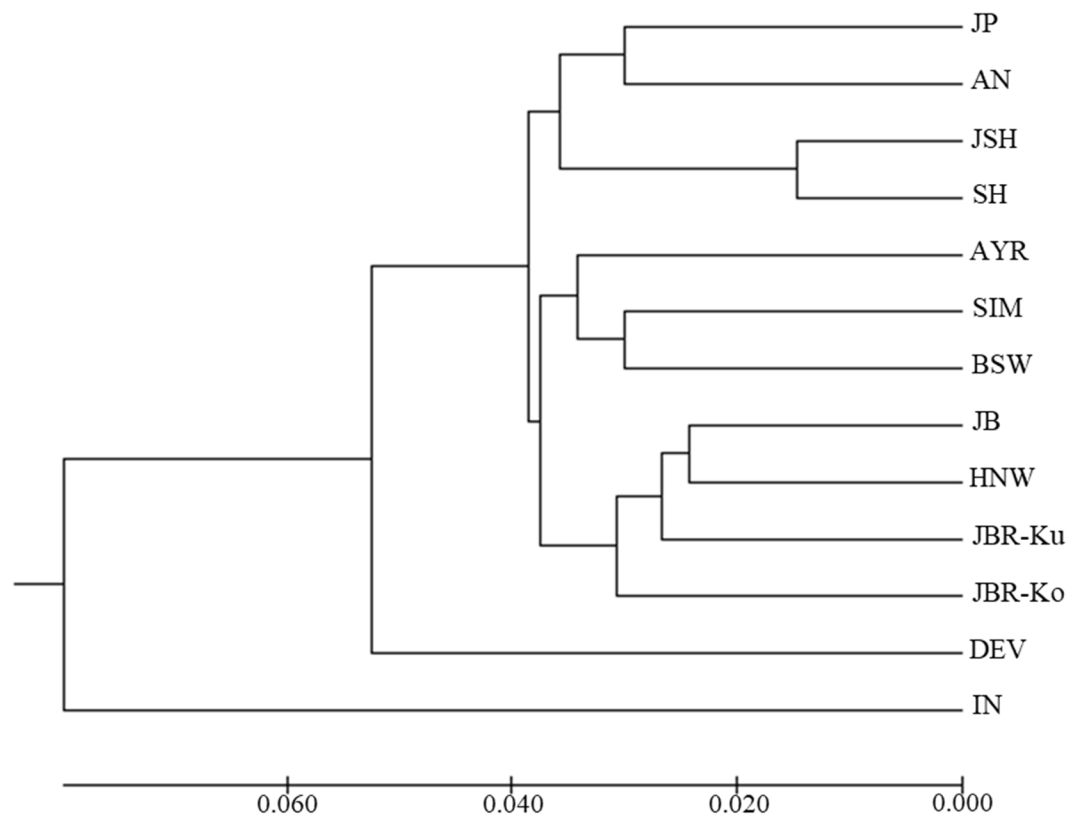


Figure 4 Phylogenetic tree using UPGMA method based on Nei's genetic distance
 JB: Japanese Black, JBR-Ko: Japanese Brown Kochi-strain, JBR-Ku: Japanese Brown Kumamoto-strain, JP: Japanese Polled, JSH: Japanese Shorthorn, HNW: Hanwoo, AN: Angus, SIM: Simmental, SH: Shorthorn, AYR: Ayrshire, BSW: Brown Swiss, DEV: Devon, IN: *Bos indicus* (out group).