INTRODUCTION

To produce livestock while conserving genetic diversity, it is important to recognize and understand the genetic and historical background of the stock. Recently, various studies on the genetic relationship among and within breeds in domestic animals have been reported using genetic markers: RFLP and DNA fingerprinting (Mannen et al., 1993b), AFLP (Lee et al., 2000; Sasazaki et al., 2001; Lee et al., 2002), microsatellite (Arranz et al., 1996; Blott et al., 1998) or mtDNA sequence (Mannen et al., 1998). Microsatellite markers have been used as the most powerful tool for estimating genetic relationships (Hanslik et al., 2000) and diversity (Peelman et al., 1998; Martin et al., 1999; Ritz et al., 2000) due to their higher polymorphism, and broad distribution throughout animal genomes. However, there have been only a few studies confirming whether information obtained from DNA polymorphisms accurately reflects pedigree information (Toro et al., 2002).

In the Northern mountainous part of Hyogo prefecture, the Tajima strain, a famous closed strain of Japanese Black cattle, has been maintained for more than 80 years (Mukai et al., 1989; Tsuji et al., 1990). The Tajima cattle are most famous for their ability to produce well-marbled “Kobe beef,” and hence their genes have been spread throughout Japan. This strain is important because of its strong genetic influence on all Japanese Black cattle strains. A highly inbred herd with densely accumulated pedigree records, the Tajima strain is a herd well suited as a test group for evaluating the estimation accuracy of microsatellite genetic relationship analysis (Oyama et al., 2002). Moreover, microsatellite marker genetic structure analysis of this strain will provide useful data, in addition to pedigree information, for maintaining the genetic diversity of the strain while avoiding inbreeding depression.

The aim of this study was to assess the usability of microsatellite markers for estimating genetic relationships in a closed population of cattle. In addition, the genetic structure of the Tajima strain was also evaluated using microsatellite markers.

MATERIALS AND METHODS

Samples

In the Tajima strain, there are three substrains called Nakadoi, Kumanami and Kinosaki substrains. Out of those, we used two main substrains, Nakadoi and Kinosaki. Blood samples were collected from representative 252 dams from...
two substrains, which are kept at Northern Hyogo Prefecture Institute of Agriculture. Two Northern eight animals are from Nakadoi substrain and 44 are from the Kinosaki substrain. We also used 21 animals from other prefectures as an outgroup, which have lower kinship with Tajima strain. Genomic DNA was extracted from blood samples based on standard protocols (Mannen et al., 1993a). The relationship coefficients among individuals were estimated from the pedigree database of Wagyu Registry Association.

### Microsatellite markers

A total of 20 microsatellite markers were studied, including 11 markers (TGLA53, INRA005, INRA063, BM1824, BM1818, ETH225, INRA037, HEL13, TGLA122, SPS115, HAUT24) recommended for diversity studies by the MoDAD program (FAO), five markers (DIK204, DIK102, DIK10, DIK68, DIK97) which are highly polymorphic in Japanese Black cattle developed by Hirano et al. (1996) and the other four markers (CSSM42, TGLA102, TGLA116, MGTG4B).

### PCR reaction

The PCR reactions were performed in 10 µl reaction volumes with 20 ng genomic DNA as a template, 2.0 µl reaction buffer buffer (100 mM Tris-HCl, 15 mM MgCl, 500 mM KCl, pH8.6), 1.6 µl dNTP Mix (2.5 mM), 0.13 µl of each primer (20 mmol/ml) and 1.0 U of EX Taq polymerase (Takara Shuzo Co., Tokyo, Japan). Amplification of PCR products was carried out using a standard PCR program with 5 min denaturation at 94°C, 30 cycles for 1 min at 94°C, 1 min annealing at 55-65°C, 1 min extension at 72°C, and final extension for 7 min at 72°C. Annealing temperatures of each marker were shown at the Cattle Diversity Database (http://www.rri.bbsrc.ac.uk/cdiv_www/) and described by Hirano et al. (1996).

### Data analysis

Measures of genetic variability such as observed and expected heterozygosities, number of alleles per locus and allele frequencies were calculated by POPGENE program version 1.32 (Francis 1999). Polymorphic information content (PIC) and exclusive probabilities (EP) were calculated using the Cervus program (Marshall et al., 1998). Standard genetic distances of Nei (1972) between individuals were also calculated using the microsatellite polymorphic information. This measurement is recommended to construct a phylogenetic tree based on microsatellites (Takezaki and Nei, 1996). Relationship coefficients were calculated from all known pedigree records of the animals. Ancestors could be traced back to those born in 1871. Genetic distance from pedigree records was defined as 1 minus relationship coefficient. Two phylogenetic trees were constructed, one from pedigree record and the other from microsatellite polymorphic information, using the UPGMA method (Sneath and Socol, 1973) by the MEGA program version 2.1 (Kumar et al., 2001). Analysis of molecular variance (AMOVA) was conducted utilizing ARLEQUIN (Excoffier et al., 1992; Schneider et al., 1997). The presence of a genetic bottleneck in the strain was assessed using microsatellite information by BOTTLENECK program (Cornuet et al., 1996) under the two-phase model (TPM) (Di Rienzo et al., 1994). The interpretation of the results was performed by Wilcoxon’s test (Luikart et al., 1997).

### RESULTS

Table 1 shows the mean number of alleles per locus, the mean observed and expected heterozygosities and PIC values for each population using 20 microsatellite markers. The mean number of alleles per locus ranged from 3 to 9, with an average of 5.8. The mean observed heterozygosity per locus ranged from 0.371 (CSSM42) to 0.827 (DIK24), with an average of 0.623. The mean number of alleles was higher in the outgroup (5.3) than in the Nakadoi (4.5) and Kinosaki (4.2) substrains. The values of both substrains were lower than that of the outgroup in the analyses of mean heterozygosities and PIC values. The result showed that two substrains in Hyogo have lower genetic diversity than the out group. In addition, we also calculated Nei’s Da genetic distance between the Nakadoi and Kinosaki substrains. At 0.0762, the value was quite low and was indicative of the close relationship between two substrains.

Table 1. The number of alleles per locus, heterozygosity and PIC values obtained from 20 microsatellites in Japanese Black cattle population

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Number of alleles per locus</th>
<th>Heterozygosity Observed</th>
<th>Expected</th>
<th>PIC value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyogo</td>
<td>4.5</td>
<td>0.616</td>
<td>0.585</td>
<td>0.517</td>
</tr>
<tr>
<td>Nakadoi</td>
<td>4.2</td>
<td>0.622</td>
<td>0.604</td>
<td>0.544</td>
</tr>
<tr>
<td>Kinosaki</td>
<td>4.2</td>
<td>0.622</td>
<td>0.604</td>
<td>0.544</td>
</tr>
<tr>
<td>Outgroup</td>
<td>5.3</td>
<td>0.705</td>
<td>0.722</td>
<td>0.658</td>
</tr>
<tr>
<td>Average</td>
<td>5.8</td>
<td>0.623</td>
<td>0.618</td>
<td>0.559</td>
</tr>
</tbody>
</table>

Figure 1 illustrates the phylogenetic tree from genetic distance values calculated from the pedigree record. The tree had six main clusters (PC1 to PC6). Clusters PC1 to PC4 consist of all Nakadoi individuals, all Kinosaki individuals were classified into PC5, and all individuals derived from other prefectures, those having low kinship with the Tajima strain, comprised an out group of one independent cluster (PC6). Figure 2 presents the phylogenetic tree constructed using polymorphic information of 20 microsatellite markers. This tree contained nine clusters (MC1 to MC9). The microsatellite
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marker phylogenetic tree corresponded well to that of the pedigree information. All animals of the out group (PC6) were classified into MC9. The percentage of individual accordance (Pa) was 1.00. Most animals of the Kinosaki substrain were classified into two clusters (MC7 and MC8) (Pa=0.682), but 14 animals of the Kinosaki substrain were included in other Nakadoi clusters. In the other clusters, PC1 corresponded to MC3 and MC4 (Pa=0.554), PC2 to MC1 (Pa=0.548), PC3 to MC2 (Pa=0.333), and PC4 to MC5 and MC6 (Pa=0.549). Microsatellite and pedigree information produced a correlation coefficient between genetic distances of 0.686 at a highly significant level (p<0.001). These results suggested that microsatellite information largely corresponded with pedigree information.

In order to estimate the partitioning level between the two substrains, the genetic structure of the two substrains was studied using the analysis of molecular variance (AMOVA) approach (Excoffier et al., 1992). The partitioning level of genetic diversity within and between substrains revealed that 95.8% of the total genetic variance existed within substrains and 4.2% between the Nakadoi and the Kinosaki substrains. AMOVA showed that there was no genetic separation between the Nakadoi and Kinosaki substrains.

The difference between allelic diversity and heterozygosity was used as the basis for statistical tests to detect the presence of a recent genetic bottleneck (Cornuet et al., 1996). The heterozygosity excesses under the TPM were found in 17 out of 20 microsatellite loci for Nakadoi and 16 out of 20 for Kinosaki. Wilcoxon’s test gave highly significant support for the presence of the recent genetic bottleneck in both substrains (p<0.01).

DISCUSSION

In the Tajima strain, inbreeding has continued for a long time using a few artificial insemination (AI) sires per generation without introducing any breeding stock from other areas. The inbreeding coefficient has become unusually high for a commercial herd and has reached an average of about 20% (Honda et al., 2001). In addition, the F-statistics parameters, which estimate the rate of

Figure 1. Genealogical tree from genetic distance values calculated by pedigree information. The symbols indicate animal group. No symbol: Nakadoi, solid diamond: Kinosaki, open square: outgroup.

Figure 2. Genealogical tree constructed by polymorphic information using 20 microsatellites markers. The symbols indicate animal group. No symbol: Nakadoi, solid diamond: Kinosaki, open square: outgroup.
inbreeding, were calculated for the Hyogo populations in this study. $F_{IT}$ ($=15.05\%$) and $F_{ST}$ ($=15.1\%$) values were about three times higher than all registered Japanese Black cattle in Japan (Nomura et al., 2001). Recently, several new genetic disorders originating from the strain have been observed. Two substrains, Nakadoi and Kinosaki, were isolated until the 1960s due to traffic difficulty between the producing regions. However, since then the two subgroups were gradually mixed by the adoption of AI systems and by improvements of transportation. Identification of the two subgroups in the Tajima strain is an important matter for breeding of the strain without increasing the inbreeding coefficient.

The results of allele numbers, PIC values and the observed and expected heterozygosity from microsatellites analysis suggest that the Tajima strain tends to have the lowest genetic diversity in Japanese Black cattle. The low diversity of the Tajima strains suggests the population bottleneck after the dramatic reduction of the population size because of the closed system and limited number of sires. The existence of the recent bottleneck effect was supported by analysis using the BOTTLENECK program (Cornuet et al., 1996) in this study.

AMOVA showed no separation of Nakadoi and Kinosaki substrains due to low genetic variance ($4.2\%$) between the two substrains. This could be explained by possible gene flow between the two substrains. In contrast, two phylogenetic trees built from the pedigree information and microsatellite markers clearly separated the Nakadoi and the Kinosaki substrains, as shown in Figure 1 and 2. The results of phylogenetic analyses demonstrated that the two substrains have still been separated and classified genetically. This could be the result of efforts to preserve substrains as unique genetic sources, in spite of the substrains being mixed due to AI and transportation improvements. Continuous effort would be required in order to maintain the substrains.

Comparison of the pedigree information and microsatellite markers of phylogenetic trees revealed that most of animals among two substrains and out group were separated (Figures 1 and 2). And the correlation coefficient was 0.686 with a high level of significance ($p<0.001$), even though both strains are maintained as closed systems and have high relationship coefficients among individuals. Therefore, we concluded that the two trees were consistent with each other. Toro et al. (2002) investigated the relationship between pedigree and information from 49 microsatellites by using two strains of Iberian pigs. They showed that the correlation was very high when two strains were mixed together ($r=0.93$), while lower correlations were observed when the two strains were observed separately ($r=0.63$ and 0.39). Our result revealed a coefficient similar to one of the results analyzed for each strain. This result indicated that analysis of microsatellite markers had good potential for determining the genetic relationship between populations or individuals. In addition, some specific bands of each substrain were detected in allele frequencies, and microsatellite marker DIK024 exhibited especially substantial frequency differences between substrains (Table 2). These strain-specific bands would be useful as genetic markers to distinguish and classify the two substrains.

However, 14 animals of the Kinosaki substrain appeared in the Nakadoi cluster in the microsatellite tree with markers as shown in Figure 2, While pedigree information completely separated the two substrains (Figure 1). The reasons for the out-positioning in these animals are as follows. (1) Although the relationship coefficients of two strains were estimated by the pedigree information, the part of DNA polymorphisms before pedigree recording could be affected on a different level for each individual. This unknown relationship before pedigree recording may give some bias. (2) Both strains have been maintained as a closed system and have quite high inbreeding coefficients. In addition, heterozygosity excess indicated the existence of the recent genetic bottleneck. In this case, some microsatellite loci or genomic segments may have genetic drift and may have been lost. As a consequence, some individuals may be estimated to be in an unexpected position. (3) Stochastic errors might be introduced by incomplete sampling of the genome with 20 markers. Ajmone Marsan et al. (2002) demonstrated that the standard deviation of the genetic distance values affect the correlation of additional relationships and genetic distances calculated from AFLP band sharing.

Two substrains, Nakadoi and Kinosaki, were clearly distinguished within the Tajima strain using 20 microsatellite markers and pedigree records. We can thus use this information to make breeding plan to maintain the strain considering the performance characteristics. This result demonstrated that DNA polymorphic information from microsatellite markers clearly reflects the pedigree record, even in a very highly inbred herd of cattle, suggesting that analysis using microsatellite markers can be a useful tool for determining the genetic structure without pedigree information.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Alleles 1</th>
<th>Alleles 2</th>
<th>Alleles 3</th>
<th>Alleles 4</th>
<th>Alleles 5</th>
<th>Alleles 6</th>
<th>Alleles 7</th>
<th>Alleles 8</th>
<th>Alleles 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nakadoi</td>
<td>0.017</td>
<td>0.169</td>
<td>0.237</td>
<td>0.254</td>
<td>0.000</td>
<td>0.000</td>
<td>0.295</td>
<td>0.002</td>
<td>0.027</td>
</tr>
<tr>
<td>Kinosaki</td>
<td>0.011</td>
<td>0.125</td>
<td>0.000</td>
<td>0.125</td>
<td>0.250</td>
<td>0.000</td>
<td>0.350</td>
<td>0.011</td>
<td>0.148</td>
</tr>
<tr>
<td>Outgroup</td>
<td>0.071</td>
<td>0.167</td>
<td>0.000</td>
<td>0.262</td>
<td>0.310</td>
<td>0.024</td>
<td>0.167</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>
REFERENCES


Kumar, S., K. Tamura, I. B. Jakobsen and M. Nei. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, Arizona, USA.


