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Breed Discrimination Using DNA Markers Derived from AFLP in Japanese Beef Cattle

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ABSTRACT : In the meat industry, correct breed information in food labeling is required to assure meat quality. Genetic markers provide corroborating evidence to identify breed. This paper describes the development of DNA markers to discriminate between Japanese Black and F1 (Japanese Black×Holstein) breeds. The amplified fragment length polymorphism method was employed to detect candidate markers absent in Japanese Black but present in Holstein. The 1,754 primer combinations yielded eleven markers that were converted into single nucleotide polymorphism markers for high-throughput genotyping. The allele frequencies in both breeds were investigated for discrimination ability using PCR-RFLP. The probability of identifying F1 was 0.9168 and probability of misjudgment was 0.0066 using four selected markers. The markers could be useful for discriminating between Japanese Black and F1 and would contribute to the prevention of falsified breed labeling of meat. (**Key Words :** AFLP, Breed Discrimination, Beef Cattle, Japanese Black, Holstein)

INTRODUCTION

Japanese Black cattle are the main source of beef, representing more than 90% of beef cattle in Japan. They are famous for the superiority of the meat, especially in marbling and carcass yield. In contrast, most of the dairy cattle are Holstein, which are renowned as dairy cows for their excellent milk output capability throughout the world. However male Holstein has been used as source of domestic beef in Japan, because they don't produce milk. Although the meat quality is inferior to that of Japanese Black, it has been popular due to its lower price.

In the early 90's, inexpensive beef was imported from other counties to Japan in large quantities. The cheaper foreign meat directly competed with Holstein beef in the marketplace, causing a sharp reduction of the demand for Holstein and a rapid fall in its price. As a result, Japan-based beef producers started to create a first filial hybrid (F1), which is a cross of Japanese Black bulls with Holstein cows. However, the problem of misbranded beef has arisen, and it is implied that false sales have occurred. F1 could be misbranded as Japanese Black since the two breeds cannot be easily distinguished by appearance. Additionally, there is very little difference in meat quality between Japanese Black and the high-grade F1. Therefore, an identification system to trace cattle from birth to market has been gradually demanded. It is necessary for preventing false sales and guaranteeing the quality and the safety of meat. As an element of a traceability system, a technology to accurately discriminate between Japanese Black and F1 must also be developed.

Along with significant progress in molecular technology, DNA markers have been used for population discrimination in livestock animals (Alves et al., 2002; Olowofeso et al., 2005). The AFLP (Amplified Fragment Length Polymorphism) method is one of the ways to provide these useful markers (Vos et al., 1995). Since many polymorphic bands can be detected using combinations of selective primers, AFLP is a powerful method for acquiring genome information easily. In our previous study, we attempted to develop six DNA markers derived from AFLP breed-specific bands, which could distinguish between Japanese Black and F1 cattle (Sasazaki et al., 2004). Using these markers, the probability of identifying F1 was 0.882 and probability of misjudgment was 0.0198. They could be useful for discrimination between Japanese Black and F1. However, more effective markers developed by other combinations of AFLP primers will be required to improve the discrimination ability for starting a molecular test and reduction of incorrect labeling of food.

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Table 1. Sequence of AFLP adapters and primers

Name	Sequence (5' →3')
*Eco*RI adapter	CTC GTA GAC TGC GTA CC
	AAT TGG TAC GCA GTC TAC
*Taq*I adapter	GAC GAT GAG TCC TGA C
	CGG TCA GGA CTC AT
EcoRI primer +1	GAC TGC GTA CCA ATT CN
*Taq*I primer +1	GAT GAG TCC TGA CCG AN
EcoRI primer +3	GAC TGC GTA CCA ATT CNN N
*Taq*I primer +3	GAT GAG TCC TGA CCG ANN N

The aim of this study was to develop more effective DNA markers to improve the DNA test ability using additional AFLP primer combinations. For this purpose, we applied the AFLP method to find breed-specific bands, and converted them into SNP (single nucleotide polymorphism) markers for high-throughput genotyping.

MATERIALS AND METHODS

Samples

In this study, two cattle breeds (Japanese Black and Holstein cattle) were collected from diverse areas in Japan. They were selected based on pedigree information and geographic criteria. Genomic DNA was extracted from blood samples according to standard protocols.

AFLP method

The AFLP procedures were performed as described by Vos et al. (1995). Sequences of AFLP adapters and primers are listed in Table 1. Genomic DNA (500 ng) was digested with 5 U of *Taq*I at 65°C for 1 h, followed by second digestion with 5 U of *Eco*RI at 37°C for 1 h. Double-stranded adapters were ligated to the restriction fragments, following addition of 5 pmol *Eco*RI adapter, 50 pmol *Taq*I adapter, 1 mM ATP, and 1 U of T4 DNA ligase at 37°C for 3 h. The ligated DNA fragment solution was then diluted 10-fold with 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and stored at -20°C. Pre-amplification was carried out using 75 ng each of *Eco*RI primer and *Taq*I primer with a single selective nucleotide. After the reactions, the mixtures were diluted 10-fold with 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and stored at -20°C.

Selective amplifications were performed using 5 ng of *Eco*RI primer and 30 ng of *Taq*I primer with three selective nucleotides. PCR products amplified with different primer combinations were loaded onto 5.0% denaturing polyacrylamide gels, and electrophoresed for 2 h and detected by SILVER SEQUENCE™ DNA Staining Reagents (Promega, WI).

Cloning and sequencing of AFLP fragments

Selected bands were excised from the gels, and soaked in 20 μl of Tris/EDTA buffer. DNA was eluted by overnight incubation at -20°C. Samples were centrifuged, and 5.0 μl of supernatant was used for PCR under standard conditions using 60°C of annealing temperature and the primers used in the selective amplification of AFLP assays. The amplified PCR products were cloned by the pGEM-T Easy Vector System II (Promega, WI) according to the manufacturer's instruction. After checking the product size of the original DNA fragment, white positive colonies were picked up and cultured overnight in Luria-Bertani medium and plasmids were isolated. The sequence of the plasmid was determined by the Sequi Therm EXCEL™ II DNA sequencing Kits-LC (EPICENTRE Technologies, WI) with IRD800 labeled M13 forward (5'-CAC GAC GTT GTA AAA CGA C) and reverse primers (5'-GGA TAA CAA TTT CAC ACA GG). All sequences were analyzed for homology to database using the website of the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) running the Blast programs (Altschul et al., 1990).

PCR-walking

Both flanking sites of the AFLP fragment were determined using the PCR-Walking method (Devic et al., 1997) according to the Universal GenomeWalker™ Kit (BD Biosciences, CA). At first, four kinds of genomic library were constructed from Japanese Black cattle. Genomic DNA was digested with four restriction enzymes (*Dra* I, *Eco*R V, *Pvu* II and *Stu* I) and ligased with each Genome-Walker adaptor (5'-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT and 5'-PO_4-ACCAGCCC-N_2H). Then PCR reactions were performed with each adapter-specific primer (5'-GTA ATA CGA CTC ACT ATA GGG C and 5'- ACT ATA GGG CAC GCG TGG T) and AFLP fragment specific primers designed using OLIGO 4.0 software. PCR products were cloned and sequenced using the same procedures described previously.

We designed the primers on the flanking site of AFLP fragments based on the sequences of PCR-Walking. The region including an AFLP fragment was amplified with genomic DNA of Japanese Back and Holstein cattle. PCR products were sequenced using SILVER SEQUENCE™ DNA Sequencing Reagents (Promega, WI) and the mutation identified by comparing between the two breeds.

PCR-RFLP

To test the power of DNA markers for discriminating between the two breeds, the genotype frequencies of the subject animals were investigated. PCR-RFLP method was performed with the primers designed for amplifying the genomic DNA segment containing the mutation site, and digested with adequate restriction endonuclease (Table 2).

Table 2. Marker information for PCR-RFLP

Marker	Forward primer (5'→3') Reverse primer (5'→3')	Annealing temperature (°C)	Product size (bp)	Mutations
BIMA 1	GAGTGTAGTTGATTTATTTTTATTTGT GAGTACTGACGCAGCACACCTACAGCC	65	177/174	3 bp insertion/deletion
BIMA 2	GTAAAACAACTTAGTGGTGAATTCGGG TCGGATTGCTTACGTGCCTTTCTGGAGAC	65	305	SNP at *Eco*R I site (G→A)
BIMA 3	CCTTTGTCTTCCACTGCCCACCTGTCA CACATCTCTTTAGCACTCTCGTTCTGGT	65	170/179	9bp insertion/deletion
BIMA 4	TAGGGAAGATACCACAATAAGTAAAG GTAAAGATAAACATGTAAAGATATAGCACAGCATCGACC	65	249	SNP at *Taq* I site (C→G)
BIMA 5	TGTTACAACGCAAGGCTGGGAAACTG GAGAGTGGAGAGAATAGCGGATGCCTCGACCTGACTTTC	65	223	SNP at *Taq* I site (C→G)
BIMA 6	CGGGCTGGTCTGAGAAAAGTCAAGTCAC CAGTCAATGAAGAGCCGAGTAGAAGAAC	65	638/639	SNP at *Eco*R I site (A→×)
BIMA 7	TCTTGGTCACCTGCTGCTTCCTGTCCTG CGTATCCGTAGTATAGTAGTATGGTG	63	562	SNP at *Taq* I site (T→C)
BIMA 8	ATTCTATCAACAGCAAAAACCAAGCATT AAATGGCAGGAAGGAAGGCTATAGATGG	62	365	SNP at *Taq* I site (C→T)
BIMA 9	CCCAAGGTCTAAGAGCCAGGGTACTGATGC TCTGTAAAGACAAAGTGAATCTCTAAGG	59	366	SNP at *Taq* I site (A→G)
BIMA 10	ACCCCCGTCCTTCTTCCCCATCACAGCC GCAGACAACAGGAAGACCCGTAAGTTTC	65	243	SNP at *Eco*R I site (A→G)
BIMA 11	CACATGATACAGCAAAAGGAGTTC CCCAATGTTCTGACGTCTTCCGA	65	136/133	3 bp insertion/deletion

Statistical analysis

Analyses of Hardy-Weinberg equilibrium (HWE) and likelihood ratio test of linkage disequilibrium were performed using the program ARLEQUIN Ver 2.000 (Excoffier et al., 1992; Schneider et al., 1997; Slatkin and Excoffier, 1996).

RESULTS AND DISCUSSION

Detection of breed specific AFLP bands

The aim of this study was to detect specific markers that can discriminate between Japanese Black and F1. For this purpose, Holstein-specific markers were required since F1 (the first filial hybrid from Japanese Black bulls and Holstein cows) has 50% of nuclear genetic material from Holstein. Initially, 10 animals from each breed were investigated, considering experimental efficiency.

512 primer combinations were already tested in our previous study. In this study, an additional 1,242 primer combinations were used to searching for more effective markers. A total of 1,754 primer combinations yielded a total of approximately 245,000 bands, with an average of 140 amplified fragments per primer combination. The AFLP bands, which were present in less than 10% of Japanese Black and more than 70% of Holstein, were considered as candidate markers. Using this criterion, we selected a total of 58 bands. These possible markers were tested with 50 samples from each breed at a second screening. Out of those, eleven markers (BIMA 1-BIMA 11) met the criteria.

Cloning and sequencing for identification of AFLP polymorphisms

We converted AFLP markers into more convenient markers for the following reasons. First, the AFLP marker is a dominant marker, which does not discriminate between dominant homozygote and heterozygote genotypes. Second, the AFLP method is not suitable for routine analytical testing of processed meat because of its comparative complexity and damage to the meat DNA. Brugmans et al. (2003) described an efficient protocol for converting AFLP markers using the PCR Walking method (Negi et al. 2000). To identify the mutations that cause AFLP polymorphisms, the sequence information of the genomic DNA flanking the AFLP fragment must be obtained. Our study identified eleven mutations responsible for the original AFLP polymorphisms. Three were insertion/deletion in the AFLP fragment; eight were SNPs at the restriction site (three in *Eco*RI and five in *Taq*I).

All sequences of AFLP fragments were analyzed for homology to the data banks using the website of the NCBI running the Blast programs. One of the AFLP fragments (BIMA 11) was matched with cattle ESTs. These EST sequences were used to reanalyze by BLAST search. It shares 93% similarity with a 3' untranslated region of Homo sapiens microtubule-actin crosslinking factor 1 (Accession No.NM_033044). However, any significant matches were found in other fragments. Next, in order to identify the chromosome location of these markers in cattle, we searched for homology, using the NCBI database with the genome BLAST program (http://www.ncbi.nlm.nih.gov/Genomes/). All of the AFLP fragments, which were extended by the PCR walking method, were significantly

Table 3. The result of cattle genome BLAST on each marker

Marker	Size (bp)	Score	E value	Location	Gene
BIMA 1	782	1,200	0.0	BTA 3	ND*
BIMA 2	625	1,175	0.0	Un	ND
BIMA 3	556	994	0.0	BTA21	ND
BIMA 4	116	219	2E-55	BTA5	Similar to RAS-like, estrogen-regulated, growth inhibitor
BIMA 5	184	350	1E-94	BTA3	ND
BIMA 6	424	787	0.0	BTA11	ND
BIMA 7	433	706	0.0	Un	Similar to PTK2 protein tyrosine kinase 2 isoform a
BIMA 8	625	1,134	0.0	BTA4	ND
BIMA 9	344	660	0.0	Un	ND
BIMA 10	343	346	4E-93	BTA9	Similar to parkin isoform 1
BIMA 11	211	383	2E-104	BTA3	Similar to Microtubule-actin crosslinling factor 1, isoform 4

* No corresponding genes were found in the region of the markers by BLAST progoram.

Table 4. Genotype and allele frequencies obtained from Japanese Black and Holstein cattle

Marker	Japanese Black				Holstein				Probability of identification as F1 (P_i)	Probability of misjudgment (P_m*)
	Genotype frequencies			Allele frequencies	Genotype frequencies			Allele frequencies		
	aa	ab	bb	*a*	aa	ab	bb	*a*		
BIMA 1	0	1	294	0.0017	37	41	22	0.575	0.575	0.0034
BIMA 2	0	25	265	0.0431	19	56	25	0.470	0.470	0.0843
BIMA 3	0	9	91	0.045	14	59	27	0.435	0.435	0.0880
BIMA 4	0	11	89	0.055	30	47	23	0.525	0.525	0.1070
BIMA 5	0	26	264	0.0448	40	44	16	0.620	0.620	0.0876
BIMA 6	0	2	288	0.0034	14	45	41	0.365	0.365	0.0068
BIMA 7	0	0	292	0.0000	13	54	33	0.400	0.400	0.0000
BIMA 8	0	0	297	0.0000	5	45	50	0.275	0.275	0.0000
BIMA 9	0	3	287	0.0052	15	44	41	0.370	0.370	0.0104
BIMA 10	0	9	282	0.0155	15	46	39	0.380	0.380	0.0308
BIMA 11	0	1	310	0.0016	31	48	21	0.550	0.550	0.0032

* P_m was defined as the probability of misjudgment that we incorrectly judge Japanese Black as F1.

Table 5. Probability of identification (P_i) and misjudgment (P_m) by stepwise approach using six markers

Number of marker	Marker	P_i	P_m
2	BIMA 7,8	0.5650	0.0000
3	BIMA 7,8,11	0.8042	0.0032
4	BIMA 7,8,11,1	0.9168	0.0066
5	BIMA7, 8,11,1,6	0.9472	0.0134
6	BIMA 7,8,11,1,6,9	0.9667	0.0236

homologous to cattle genome sequences (Table 3). Eight markers were identified to a cattle chromosome number and the other three were located on an unknown genomic contig. Moreover, three markers (BIMA 4, 7 and 10) were found to be located in the intron region of a known gene.

Genotyping for genotype frequency

PCR-RFLP was conducted to investigate genotype frequencies in both breeds. Table 4 presents the genotyping results, genotype frequencies and allele frequencies obtained from Japanese Black and Holstein. In this study, we investigated about 300 animals in Japanese Black and 100 animals in Holstein. Allele *a* indicates the Holstein-specific allele detected by AFLP. The allele frequencies ranged from 0.000 to 0.055 (Japanese Black) and 0.275 to 0.620 (Holstein).

Hardy-Weinberg equilibrium (HWE) was tested for each locus using genotypic results of the Holstein population. None of the loci showed significant departure from HWE at $p<0.05$ for the probability test in the population. Linkage disequilibrium between a pair of loci was subsequently tested using a likelihood ratio test (Slatkin and Excoffier 1996). No locus pairs showed significant disequilibrium at $p<0.05$. Therefore, calculations of identification and misjudgment probability described below were based on assumption of no linkage among eleven markers.

Calculation of Identification probability

The efficiency of the eleven markers was evaluated for the ability to distinguish between Japanese Black and F1. We estimated two measures, the probability of identification as F1 (P_i) and probability of misjudgment (P_m) that we incorrectly judge Japanese Black as F1. These probabilities were defined based on the estimated allelic frequencies of each marker in the present study (Table 4). Table 5 shows the two probabilities provided by the combination of these

markers. These methods are described in detail as follows.

At first, we applied BIMA 7 and BIMA 8, which were efficient markers since both were Holstein-specific alleles that were not detected in Japanese Black. The individual identifying probabilities, which were consistent with the allelic frequency in Holstein, were P_{i7} = 0.400 (BIMA 7) and P_{i8} = 0.275 (BIMA 8). The two markers combined provided more efficient probability $P_{i7,8} = 1-(1-P_{i7})(1-P_{i8}) = P_{i7}+P_{i8}-P_{i7}P_{i8}$ = 0.565, while the misjudgment probability using the two markers, $P_{m7,8}$, was estimated at 0.00.

Taking allele frequencies into account, the markers that were high frequencies in Japanese Black could not contribute to this procedure because it increases P_m in the breed identification test. Therefore five markers (BIMA 2, 3, 4, 5 and 10), which have more than P_m:0.01, were excluded from this system.

In the other four markers, we took a stepwise approach and calculated the P_i and P_m using P_i and P_m of each markers, which was calculated as follows: $P_\Sigma = 1-(1- P_a)(1-P_b)(1-P_c)\ldots$, where P_a and P_b were the probabilities of the markers BIMA a and BIMA b, respectively. For example, P_i using four markers were: $P_{i7,8,11,1} = 1-(1-P_{i7})(1-P_{i8})(1-P_{i11})(1-P_{i1})$ = 0.9168. The calculation of whole efficiencies for discrimination depend on the number of markers. We concluded that the most efficient combination for a breed discrimination test was using four markers (BIMA 7, 8, 11 and 1), which provided more efficient probability (P_i = 0.9168; P_m = 0.0066) than that of six markers developed in our previous study. BIMA 6 and 9 were useful as additional markers when more detection efficiency (P_i = 0.9667) was needed. Compared with the previous markers, these markers developed in this study were more efficient in both probabilities. Especially, the probability of misjudgment was improved (P_m = 0.0198 to 0.0066) to provide a reliable result in breed discrimination test.

These markers could be useful for discriminating not only between Japanese Black and F1, but also between Japanese Black and Holstein. In Japanese Black, frequencies of Holstein specific alleles (allele a, Table 4) were low, so the P_i and P_m were calculated by using the expected genotypic frequencies given by the estimated frequencies of allele a. The results showed more effective probability with P_i = 0.81 and P_m = 0.00 using two markers (BIMA 7 and 8) and P_i = 0.993 and P_m = 0.007 using four markers (BIMA 1, 7, 8 and 11).

This study highlights the potential of AFLP procedure as a useful tool for developing DNA markers, which could discriminate between Japanese Black and F1, and contribute to the reduction of incorrect or falsified labeling of food. It would be expected that this work could similarly be applied to other food products.

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