Identification of New Microsatellite DNAs in the Chromosomal DNA of the Korean Cattle (Hanwoo)

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ABSTRACT : To isolate the microsatellites from the chromosomal DNA of the Korean cattle (Hanwoo) and to use those for the genetic selection, four bacteriophage genomic libraries containing the chromosomal DNA of six Hanwoo steers showing the differences in meat quality and quantity were used. Screening of the genomic libraries using ^{32}P-radiolabeled 5'-$(CA)_{12}$-3' nucleotide as a probe, resulted in isolation of about 3,000 positive candidate bacteriophage clones that contain $(CA)_n$-type dinucleotide microsatellites. After confirming the presence of microsatellite in each positive candidate clone by Southern blot analysis, the DNA fragments that include microsatellite and flanking sequences possessing less than 2 kb in size, were subcloned into plasmid vector. Results from the analysis of microsatellite length polymorphism, using twenty-two PCR primers designed from flanking region of each microsatellite DNA, demonstrated that 208 and 210 alleles of HW-YU-MS#3 were closely related to the economic traits such as marbling score, daily gain, backfat thickness and *M. longissimus dorsi* area in Hanwoo. Interestingly, HW-YU-MS#3 microsatellite was localized in bovine chromosome 17 on which QTLs related to regulation of the body fat content and muscle hypertrophy locus are previously known to exist. Taken together, the results from the present study suggest the possible use of the two alleles as a DNA marker related to economic trait to select the Hanwoo in the future. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 10 : 1329-1333)***

Key Words : Bacteriophage Genomic Library, Microsatellite, Chromosomal DNA, Korean Cattle

INTRODUCTION

The Korean Cattle (Hanwoo), with their genetic sources, have been well-preserved for several thousand years because of the unique geographical situation in Korean peninsula. The Hanwoo had played an important role in agricultural sector as a labor source until their beef production became favorable to Korean consumers in the 1970s. Recently, Korean people begin to give more attention for the necessity to sustain and preserve Hanwoo, because of consumers' higher preference on the Hanwoo meat (Cho and Ko, 1998). Equivalent importance of the Hanwoo production has been emphasized because of their unique genetic resources maintained during the history of Korea and their economical value in the open era of meat market under World Trade Organization (WTO) treaty.

The major goal of genomic research in livestock has been focused on developing genetic map and identifying genes associated with important economic traits. Recent advent of the so-called "biotechnology" has quickly changed the methodology for the detection of DNA polymorphism and successfully resulted in gene identification, breeding and improvement of domestic animals. Since there are several millions of microsatellites pertaining to bovine genome and they are highly polymorphic, via many of genomic researchs such as EST (Ruyter-Spira et al., 1996; Grosse et al., 2000), somatic cell panels (Fridolfsson et al., 1997; Lahbib-Mansais et al., 1999), radiation hybrid map (Yang et al., 1998), and single nucleotide polymorphism (Yang et al., 1998; Heaton et al., 1999), it is now well-recognized that microsatellites are useful tools to develop the linkage map and to identify the functional candidate genes associated with quantitative traits. Therefore, the study reported herein was conducted to identify the microsatellite DNAs from the Hanwoo chromosome and use those for the genetic selection of the Hanwoo.

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MATERIALS AND METHODS

Bacteriophage genomic library construction

Blood samples were collected from the Hanwoo steers at slaughter house after completion of their evaluations of meat quality and weight. Pure chromosomal DNAs were isolated from the blood samples using the Genomic DNA isolation kit (Genotein Corporation, Korea). Partially digested chromosomal DNAs with *BamH*I enzyme were utilized to construct the bacteriophage library by use of Lamda Fix/*Xho*I partial fill-in vector kit and Giga Gold Packaging kit (Stratagene, La Jolla, CA, USA). After packaging of bacteriophages, the genomic libaries were titered, amplified and stored at -80°C.

Library screening

The Hanwoo bacteriophage genomic library was used to screen the microsatellite DNA in Hanwoo chromosome with slight modifications of the standard protocol

Table 1. Construction of Hanwoo genomic libraries

Bacteriophage genomic DNA library	BGL I*	BGL II	BGL III	BGL IV
Meat quantity (grade)	Mixed	A	B	A
Meat quality (grade)	Mixed	1+	3	1+
Number of independent clones	520,000	3,590,000	1,206,000	2,010,000

* Library composed of ungraded mixture of chromosomal DNAs from three different steers of Hanwoo.
Meat quantity grades: A; highest, B; medium, C; lowest. Meat quality: 1+ ; highest, 3; lowest.

(Sambrook and Russell, 2001). In order to easily separate each bacteriophage clone that contains microsatellite DNA, the optimal number of bacteriophage clone were determined by titration. A chemically synthesized oligonucleotide, 5`-$(CA)_{12}$-3', was radiolabeled with ^{32}P-γ-ATP (Amersham Bioscience, USA) by use of T4 polynucleotide kinase, and purified by Quick Spin column (Roche Applied Science, Germany) before use for hybridization. Nitrocellulose membranes spotted with transferred bacteriophage DNAs were incubated at 55°C in a prehybridization buffer (6X SSC, 1X Denhardt, 0.1% SDS) for two hours and hybridized at the same temperature in hybridization buffer (6X SSC, 1X Denhardt, 0.5% SDS, 5mM EDTA) with radiolabeled probe (5×10^5 cpm/ml hybridization buffer) for 12 h. After the hybridization the solution was decanted and washed three times with 2×SSC/0.1% SDS at 55°C for 15 minutes, and twice in 2×SSC/0.1% SDS at 55°C for 15 minutes. The membranes were air-dried, covered with plastic wrap, and subjected to X-ray film exposure at -80°C for 3-5 days.

Southern blot analysis and DNA sequencing

After library screening, the candidate bacteriophage clones that contain microsatellite DNA fragments were isolated and subjected to DNA purification. Purified bacteriophage DNA was digested with several restriction digestion enzymes and separated in a 0.6% agarose gel. Southern blot analysis with the same radiolabeled probe that was used for library screening was carried out as described previously (Yeo et al., 2002). The small DNA fragments hybridized with radiolabeled probe were ligated into plasmid vectors and subjected to DNA sequencing using the automated DNA sequencer (ABI 3100, the Institute of Biotechnology at Yeungnam University).

Genomic DNA isolation and microsatellite analysis

Three hundred thirty one heads of bulls registered in the Hanwoo Improvement Center (National Agricultural Cooperative Federation) were selected based upon their phenotypic standards and recorded pedigree. The grades of marbling score, backfat thickness and *M. longissimus dorsi* area were measured at market days (720 days) according to the standards of the Korean Animal Products Grading Service. Chromosomal DNA of each bull was isolated from the blood cells by phenol-chloroform extraction method (Sambrook and Russell, 2001). Polymerase chain reaction (PCR) for microsatellite was performed using 20 ng of Hanwoo genomic DNA as template with 1×PCR buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs, 15 pM of each primer, and 1 unit of *Taq* polymerase (Takara Co., Japan) in 30 μl reaction volume. The PCR was performed for 5 min at 94°C, and followed by 35 cycles of 30 sec at 94°C, 30 sec at 50-60°C, 1 min at 72°C, and extension for 10 min at 72°C at the end of cycles. The PCR products were separated in a 6% polyacrylamide gel electrophoresis. The precise size of microsatellite allele was determined by comparison with 10 and 100 bp standard markers. Duncan's multiple range test was performed by use of SPSS statistical program.

PCR primer design and chromosome localization

PCR primers used for microsatellite DNA analysis were designed using the Primer 3 (Whitehead Institute for Biomedical Research, http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The locations of microsatellites were analyzed using a new version of COMPASS software (with the kind help of Dr. Harris A. Lewin, University of Illinois at Urbana-Champaing, IL, USA) to predict the position of each microsatellite on Hanwoo chromosomes (Larkin et al., 2003).

RESULTS AND DISCUSSION

Bacteriophage genomic library construction

Four bacteriophage genomic libraries of the Hanwoo were generated using the chromosomal DNA obtained from six Hanwoo steers showing differences in their meat quality and quantity (Table 1). Results from titering of each library, except BGL I library, revealed that libraries contain 1 to 3.5 millions of independent clones. One hundred and thirty bacteriophage clones were randomly selected and their DNAs were digested with *Not* I restriction enzyme after purification, and subjected to pulse field gel eletrophoresis (PFGE) to test the quality of each library (data not shown). Among the one hundred and thirty clones used for the analysis, other than 5 clones, all the bacteriophage clones contained insert with average size of about 20 kilo base pairs (kb).

Screening and sequencing of microsatellite DNAs

Since the dinucleotide microsatellites, such as $(CA)_n$ or $(TG)_n$, are known to be the most abundant and highly polymorphic, radiolabeled 5`-$(CA)_{12}$-3' probe was utilized

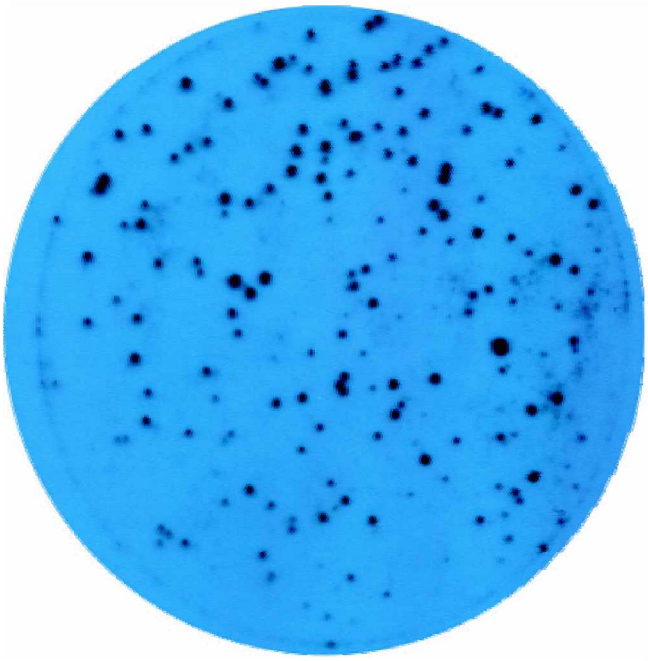

Figure 1. Screening of Hanwoo bacteriophage genomic library. About 500-1,000 bacteriophage clones were plated onto 150mm petri dish containing LB-agar. ^{32}P-Radiolabeled 5'-$(CA)_{12}$-3' probe was used to screen microsatellite DNAs.

Table 2. PCR Primers used for microsatellite DNA analysis

Primer name	Repeat length	Predicted chromosome	Predicted marker
HW-YU-MS#1	$(CA)_{10}$	16	EST1013
HW-YU-MS#2	$(CA)_{12}$	12	COL4A1
HW-YU-MS#3	$(CA)_{18}$	17	EST1050
HW-YU-MS#4	$(CA)_{17}$	3	GBP1
HW-YU-MS#5	$(CA)_{10}$	14	D10884
HW-YU-MS#6	$(CA)_{17}$	19	TP53
HW-YU-MS#7	$(CA)_{13}$	4	IGFBP3
HW-YU-MS#8	$(CA)_{12}$	13	CHGB
HW-YU-MS#9	$(CA)_{10}$	NA	EST1592
HW-YU-MS#10	$(CA)_{14}$	24	BCL2
HW-YU-MS#11	$(CA)_{5}$	NA	PAM
HW-YU-MS#12	$(CA)_{9}$	3	JUN
HW-YU-MS#13	$(CA)_{15}$	14	ANGPT1
HW-YU-MS#14	$(CA)_{14}$	NA	SVIL
HW-YU-MS#15	$(CA)_{6}$	24	ME2
HW-YU-MS#16	$(CA)_{9}$	13	B4GALT5
HW-YU-MS#17	$(CA)_{4}$	NA	GC20
HW-YU-MS#18	$(CA)_{10}$	NA	RCN1
HW-YU-MS#19	$(CA)_{12}$	NA	SC4MOL
HW-YU-MS#20	$(CA)_{19}$	1	IL12A
HW-YU-MS#21	$(CA)_{4}$	NA	COL10A1

The predicted locations of the PCR primers used for the microsatellite DNA analysis and DNA markers closely located to the microsatellite DNA fragments was analyzed by use of COMPASS program (see Materials and Methods).

to screen the bacteriophage clones that contain the microsatellite fragment. About 500 to 1,000 bacteriophage clones were plated onto the 150 mm Petri dish in diameter.

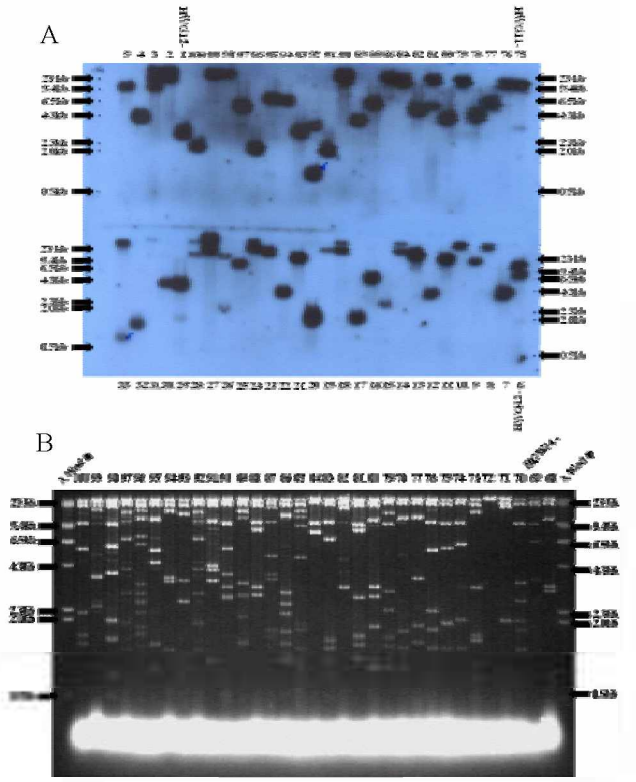

Figure 2. Southern blot analysis of microsatellite DNAs. A typical result of restriction enzyme digestion (*EcoRI* in the case of Figure 2) of bacteriophage DNAs that obtained from the library screening (A) and a typical result of Southern blot analyis (B) are shown. The numbers on the left and right sides indicate the sizes (base pairs) of the DNA standards. The numbers of at the top (A and B) or bottom of each figure are clone numbers.

because it was turned out to be the optimal number of clones to easily isolate the individual clone after screening. Using this approach three thousand of bacteriophage clones hybridized with the probe were individually isolated and kept in buffer for subsequent analysis. The results from the several rounds of library screening showed that approximately 20% of the Hanwoo bacteriophage clones were hybridized with the probe (Figure 1). Considering average size of phage clone generated in the present study is 20 kb, the results indicate that $(CA)_n$-type microsatellites exist at a frequency of once per every 100 kb in Hanwoo chromosome and agree with other (Zhu et al., 1999).

In order to confirm the presence of microsatellite DNA fragment and facilitate subcloning into plasmid vector for the subsequent DNA sequencing, the bacteriophage DNAs of the positive candidate clones subjected to obtained from library screening were purified. And the purified DNAs were employed for Southern blot analysis with the same probe used for the library screening after digestion of DNAs with restriction enzymes such as *EcoR*I, *BamH*I and *Hind*III (Figure 2). After Southern blot analysis DNA fragments with less than 2 kb in size and hybridized with the probe

Table 3. Performances of marbling, daily gain, backfat thickness and *M. longissimus dorsi* area related with microsatellite DNA marker in Hanwoo steers

Microsatellite allele			Performances (±standard error)			
Locus (bp)	Existence	No. of heads	Marbling degree	Daily gain (g)	Backfat thickness (mm)	*M. longissimus dorsi* area (cm^2)
208	Yes	158	7.60±0.36	683±6	7.12±0.24	74.64±0.68
	No	173	7.20±0.31	680±6	6.78±0.21	73.61±0.63
210	Yes	186	7.63±0.31	694±5^{a}	7.34±0.23^{A}	74.94±0.61^{A}
	No	145	7.08±0.36	665±6^{b}	6.34±0.21^{B}	73.03±0.71^{B}
208/210	Yes	59	8.54±0.60^{a}	705±8^{A}	8.27±0.41^{A}	76.22±1.10^{A}
	No	272	7.14±0.25^{b}	676±5^{B}	6.65±0.17^{B}	73.64±0.51^{B}
Total/mean		331	7.39±0.24	681±4	6.94±0.16	74.1±0.46

$^{a, b}$ means significant difference within column at α<0.05 and $^{A, B}$ means significant difference within column at α<0.01.

Figure 3. Microsatellite DNA markers related with high and low groups in marbling score using Hanwoo microsatellite primer (HW-YU-MS#3).

were gel purified for ligation into the plasmid vector for subsequent DNA sequencing. To evaluate the microsatellite length polymorphism and eventually identify possible DNA markers that are highly associated with genetic traits determining the economic ally important value, unique PCR primers were designed from flanking region of each $(CA)_n$-repeated sequences. A total of twenty-two PCR primers were designed and utilized for this study. The positions of microsatellites from which PCR primers were designed and the previously characterized DNA markers that are closely linked to each microsatellite were determined by the COMPASS computer program (Table 2).

Application of Hanwoo microsatellite primers

To test and identify the valuable microsatellite primers, among the PCR primers obtained in this study, thirty of Hanwoo bulls belonging to 5% of each tails in normal distribution curve for each economic trait were selected. The designed primers were employed to find specific allele associated with either high or low performance in several economic traits. Among 22 pairs of PCR primers evaluated in this study the HW-YU-MS#3 primer revealed evident allelic difference at PCR-amplified products of 208 and 210 bp between high and low groups (Figure 3). During prescreening, 10 out of 15 animals in the high marbling score group showed 210 bp and 12 of them showed 208 bp. A marked contrast to this was seen among the 15 animals in the low marbling score group, where only one showed 210 bp and none had the 208 bp allele. And then these alleles were further tested to screen 331 heads of Hanwoo for distribution of these alleles and performances.

As summarized in Table 3 marbling score, daily gain, backfat thickness and *M. longissimus dorsi* area were significantly increased in the presence of 210 bp. The marbling degree, considered as the most important economic trait in Hanwoo, was significantly higher in individual animal possessing both 208 and 210 bp (44.1% judged either 1+ or 1 in marbling score) than average (32%). Analysis of microsatellite length polymorphism revealed that groups having 210 bp gain about thirty grams more body weight per day compared to the animal without 210 bp (Table 3). In fact, the Hanwoo bulls gained 22 kg of body weight at 720 days of feeding. It is worth mentioning that the HW-YU-MS#3 microsatellite reside on chromosome 17 on which QTL related to body fat and muscle growth were previously assigned (Konfortov and Miller, 1998; Casas et al., 1999). It will be of interest to test the HW-YU-MS#3 microsatellite with increased number of Hanwoo heads in the near future and use as a profitable tools for MAS (marker- assisted selection) in Hanwoo.

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REFERENCES

Casas, E., S. D. Shackelford, J. W. Keele, R. T. Stone, S. M. Kappes and M. Koohmaraie. 1999. Evidence of quantitative traits loci affecting growth and carcass composition traits in cattle segregating the muscle hypertrophy locus. J. Anim. Sci. 77(Supp. 1):126-127.

Cho, B. D. and Y. D. Ko. 1998. Hanwoo meat. In: Domestic animal industry in Korea (Ed. J. K. Jung). World Association of Animal Science in Korea. pp. 3-25.

Fridolfsson, A. K., T. Hori and A. K. Wintero. 1997. Expansion of the pig comparative map by express sequence tag (EST) mapping. Mamm. Genome 8:907-912.

Grosse, W. M., S. M. Kappes and R. A. McGraw. 2000. Linkage mapping and comparative analysis of bovine sequence tags (ESTs). Anim. Genet. 31:171-177.

Heaton, M. P., W. W. Laegreid, C. W. Beattie, T. P. L. Smith and S. M. Kappes. 1999. Identification and genetic mapping of bovine chemokine genes expressed in epitherial cells. Mamm. Genome 10:128-133.

Konfortov, B. A. and J. R. Miller. 1998. Carboxypeptidase E gene in bovine is located on chromosome 17. 26th International conference on Anim. Genet. pp. 56-56.

Lahbib-Mansais, Y., G. Dalias and D. Milan. 1999. A successful strategy for comparative mapping with human ESTs: 65 new assignments in the pig. Mamm. Genome 10:145-153.

Larkin, D. M., A. E. D. Wind, M. Rebeiz, P. A. Schweutzer, S. Bachman, C. Green, C. L. Wright, E. J. Campos, L. D. Benson, J. Edwards, L. Liu, K. Osoegawa, J. E. Womack, P. J. de Jong and H. A. Lewin. 2003. A cattle-human comparative map built with cattle BAC-ends and human genome sequence. Genome Research 13:1966-1972.

Ruyter-Spira, C. P., R. P. Crooijmans and R. P. Dijkhof. 1996. Development and mapping of polymorphic microsatellite markers derived from chicken brain cDNA library. Anim. Genet. 27:229-234.

Sambrook, J. and D. W. Russell. 2001. Molecular cloning. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. NY. pp. 6:28-30.

Yang, Y. P., C. E. Rexroad, J. Schlapfer and J. E. Wormack. 1998. An integrated radiation hybrid map of bovine chromosome 19 and ordered comparative mapping with human chromosome 17. Genomics 48:93-99.

Yeo, J. S., J. W. Kim, T. K. Chang, D. H. Nam, J. Y. Han and C. B. Choi. 2002. Detection of DNA fragment to differentiate Korean Cattle. Asian-Aust. J. Anim. Sci. 15(8):1071-1075.

Zhu, B., J. A. Smith, S. M. Tracey, B. A. Konfortov, K. Welzel, L. C. Schalkwyk, H. Lehrach, S. Kollers, J. Masabanda, J. Buitkamp, R. Fries, J. L. Williams and J. R. Miller. 1999. A 5×genome coverage bovine BAC library: production, characterization, and distribution. Mamm. Genome 10:706-709.