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# Forensic Characterization of Four New Bovine Tri-nucleotide Microsatellite Markers in Korean Cattle (Hanwoo)

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## ABSTRACT

We identified four new bovine tri-nucleotide microsatellite loci and analyzed their sequence structures and genetic parameters in 105 randomly selected Korean cattle (Hanwoo). Allele numbers of the loci B17S0808, B15S6253, B8S7996, and B17S4998 were 10, 11, 12, and 29, respectively. These alleles contained a simple or compound repeat sequences with some variations. Allele distributions of all these loci were in Hardy-Weinberg equilibrium ($P > 0.05$). Observed heterozygosity and expected heterozygosity ranged from 0.54 (B15S6253) to 0.92 (B17S4998) and from 0.599 (B15S6253) to 0.968 (B17S4998), respectively, and two measures of heterozygosity at each locus were highly correlated. Polymorphism information content (PIC) for these 4 loci ranged from 0.551 (B15S6253) to 0.932 (B17S4998), which means that all these loci are highly informative (PIC > 0.5). Other genetic parameters, power of discrimination (PD) and probability of exclusion (PE) ranged from 0.783 (B15S6253) to 0.984 (B17S4998) and from 0.210 (B15S6253) to 0.782 (B17S4998), respectively. Their combined PD and PE values were 0.9999968 and 0.98005176, respectively. Capillary electrophoresis revealed that average peak height ratio for a stutter was 13.89% at B17S0808, 26.67% at B15S6253, 9.09% at B8S7996, and 43.75% at B17S4998. Although the degree of genetic variability of the locus B15S6253 was relatively low among these four microsatellite markers, their favorable parameters and low peak height ratios for stutters indicate that these four new tri-nucleotide microsatellite loci could be useful multiplex PCR markers for the forensic and population genetic studies in cattle including Korean native breed.

(**Key words :** Tri-nucleotide repeats, Bovine, Microsatellite, Genotyping)

## INTRODUCTION

Korean cattle (Hanwoo) are major livestock species in Korea. They are now being raised primarily for beef production. Their increasing economic value brought up the forensic issues such as individual identification and parentage verification. These forensic issues can be resolved by microsatellite genotyping.

Microsatellite markers are DNA stretches containing tandem repeats of 1 to 6 nucleotide unit and have been increasingly used as genetic markers for forensic and population genetic studies mainly due to their high allelic polymorphism and wide distribution throughout the eukaryotic genomes (Tautz and Renz, 1984; Vaiman et al., 1992). Since the first description of bovine microsatellite loci in the early 1990s, many bovine microsatellite loci, mostly di-nucleotide repeats, have been identified (Fries et al., 1990; Vaiman et al., 1992; Ma et al., 1996; Reed et al., 2001). With advent and improvement of multiplex PCR methods, the microsatellite markers were extensively used for evaluating differences of cattle breeds and determining population substructures by many laboratories (MacHugh et al., 1998; Beja-Pereira et al., 2003; Freeman et al., 2004; Cymbron et al., 2005). However, it has proven difficult to combine the microsatellite data sets and compare the population genetic parameters from different studies mainly due to different sets of markers genotyped in those studies (Freeman et al., 2006). International Society for Animal Genetics (ISAG) recommended 12 microsatellite markers for routine use in bovine identity and parentage analysis with numerous inter-laboratory comparison tests (http://www.isag.org.uk/ISAG/all/ISAG2008_CattleParentage.pdf). Recently, van de Goor et al. (2009, 2011) proposed a repeat-based nomenclature of 16 microsatellite markers including the 12 ISAG-recommended markers and reported a large-scale population data in a successive process of standardization for bovine genotyping, similarly to the standardization procedures in human (Butler, 2005).

Although many bovine microsatellite markers including

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ISAG-recommended ones were commercialized (Finnzymes Diagnostics, Finland) and have been commonly used for cattle genotyping, there is evidence that some of the microsatellite loci display low polymorphism and/or produce non-amplifying null alleles (Dakin and Avise, 2004; van de Goor et al., 2011) and thus they are insufficient for a reliable genotyping, especially in closely related animals or those from herds subjected to rigorous selection (Radko et al., 2010). In addition, it is well known that many microsatellite markers often show one or more stutter bands due to a slipped-strand mis-pairing during amplification, making allele designation difficult, especially in case that di-nucleotide microsatellite markers are employed for genotyping (Guichoux et al., 2011). Unfortunately, all the bovine microsatellite markers employed currently are di-nucleotide repeats, unlikely to the human genotyping markers which are tetra-nucleotide microsatellites (Butler, 2007). Tri-, tetra-, or penta-nucleotide repeats appear to be significantly less prone to slippage than di-nucleotide repeats (Edwards et al., 1991).

Therefore, it is still important to identify more microsatellite loci that would reliably amplify and show high degree of polymorphism in a given species. In this study, we report four new bovine tri-nucleotide microsatellite markers and examined their genetic parameters in a population of Korean cattle (Hanwoo), which will give us a better chance to study population genetics and forensic science of cattle including Korean native breed.

## MATERIALS AND METHODS

### 1. DNA Extraction

DNA was isolated from hair root samples using guanidinium thiocyanate (GuSCN) glass fiber filter (Ivanova et al., 2006). Approximately ten hair roots from each animal were put into each well of 96-well plate and incubated for 2 hrs at 55℃ in 80 μl lysis buffer (50 mM NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 0.2% SDS) containing 0.5 μl proteinase *K* (20 mg/ml). Then, 100 μl binding solution (6M GuSCN, 10 mM Tris-HCl pH 6.4, 20 mM EDTA pH 8.0, 1% Triton X-100) was added in each well. The lysates were transferred to 96-well glass fiber filter plates (MultiScreen® HTS, Millipore, USA), which had been assembled in vacuum manifold (MultiScreen® HTS vacuum manifold, Millipore), and applied to vacuum pressure (−25 inches-Hg, for 1 minute). Each well of the filter plates was washed twice with 150 μl of 80% ethanol and dried under the vacuum. DNA samples from the filter plates were eluted into 96-well V-bottom collection plate (Corning, USA) by adding 50 μl TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 8.0) in each well under the vacuum. The isolated DNA samples were stored at −20℃ for PCR.

### 2. PCR Amplification and Genotyping

PCR amplification was carried out in 20 μl reaction mixture containing 10-30 ng of template DNA, 1 unit hot-start *Taq* polymerase (Genetbio, Korea), 2 μl of 10X reaction buffer, 2 mM $MgCl_2$, 200 μM of dNTPs, and 5 pmol of each primer (TAMRA labeled). Thermal cycling was conducted using the GeneAmp PCR® System 9700 (Applied Biosystems, USA) under the following conditions: 95℃ for 10 min, 30 cycles of 95℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 30 sec, and a final extension at 72℃ for 30 min. The PCR products were subjected to capillary electrophoresis using Applied Biosystems 3130xl Genetic Analyzers. After denaturation with GeneScanROX™500 (Applied Biosystems, USA) and Hi-Di™ formamide (Applied Biosystems, USA), the products were separated in 36 cm capillary filled with POP-7™ polymer (Applied Biosystems, USA) under the condition of 15 sec injection time, 2 kV injection voltage, 15 kV run voltage and 60℃ run temperature. The results were analyzed using GeneMapper® IDv3.1 software (Applied Biosystems, USA) with respect to internal size standard GeneScanROX™500 (Applied Biosystems, USA).

### 3. Allele Sequencing and Nomenclature

PCR products showing clear bands on a 7M urea PAGE gel were applied to Solg™ PCR Purification Kit (SolGent, Korea) for DNA purification. Purified DNA samples were cloned into TA plasmid vector using TOP cloner™ TA kit (Enzynomics, Korea). Sequencing reactions were performed for M13 primer using BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The reaction products were purified using MagneSil® Green (Promega, USA) and separated in a 36 cm capillary filled with POP-7™ polymer in Applied Biosystems 3130xl Genetic Analyzers. Nomenclature for new bovine tri-nucleotide microsatellites proposed in this study is based on the number of repeat units and recommendations for human microsatellite

nomenclature (Gill et al., 1997).

### 4. Statistics

Allele variability was tested using the following software packages. FSTAT 2.9.3 program was employed in calculating allelic frequencies and the mean number of alleles (Goudet, 2001). Possible deviation from Hardy-Weinberg equilibrium was examined by the probability test (Guo and Thompson, 1992) using GENEPOP v3.4 (Raymond and Rousset, 1995). Other statistical parameters, such as power of discrimination (PD), polymorphism information content (PIC), and power of exclusion (PE) were calculated using PowerState v1.2 program (Tereba, 1999).

## RESULTS AND DISCUSSION

We identified four new bovine tri-nucleotide microsatellite loci (B17S0808, B15S6253, B8S7996, and B17S4998) from *Bos taurus* whole genome sequence data (NCBI GenBank) by using a Tandem Repeats Finder software (Benson, 1999) and assessed their genetic variability in a population of 105 randomly selected Korean cattle (Hanwoo). Table 1 shows locus name, chromosomal location, primer sequences for amplification, and the amplicon length of each locus. The number of alleles detected at loci B17S0808, B15S6253, B8S7996, and B17S4998 was 10, 11, 12, and 29, respectively (Table 2). The alleles were designated on the basis of the number of repeat units and the recommendations for the nomenclature of human microsatellites (Gill et al., 1997). The probability tests for Hardy-Weinberg equilibrium are shown in Table 3. *P*-values at each locus were 0.673 (B17S0808), 0.153 (B15S6253), 0.104 (B8S7996), and 0.405 (B17S4998), all of which were in Hardy-Weinberg equilibrium ($P > 0.05$). Many other bovine microsatellite markers including ISAG-recommended markers showed significant deviations from Hardy-Weinberg equilibrium in southwestern European breeds (Beja-Pereira et al., 2003), Brazilian Nellore cattle (Cerviniet al., 2006), and many Asian cattle breeds including Korean native breed (Chung et al., 2006; Sun et al., 2008). Deviations from Hardy-Weinberg equilibrium could change allele frequencies of the loci from generation to generation. Therefore, the loci showing allele distribution deviated from Hardy-Weinberg equilibrium should be carefully used for paternity tests of motherless or fatherless case (Selkoe and Toonen, 2006). Over the past several years, di-nucleotide microsatellite markers (BM1824, BM2113, ETH10, ETH255, ETH3, INRA23, SPS115, TGLA122, TGLA126, TGLA227, TGLA53, and amelogenin X/Y) have been also employed for individual identification and paternity test in the Beef Traceability System of Korea. The forensic parameters about Hardy-Weinberg distribution and stutter peaks obtained from this system would be helpful to evaluate the usefulness of these di-nucleotide microsatellites for the paternity test, especially in fatherless or motherless cases.

For these new tri-nucleotide microsatellite loci, observed heterozygosity and expected heterozygosity ranged from 0.54 (B15S6253) to 0.92 (B17S4998) and from 0.599 (B15S6253) to 0.968 (B17S4998), respectively and two measures of heterozygosity at each locus were highly correlated. Takezaki and Nei (1996) suggested that useful markers for evaluating genetic variation should have an average heterozygosity higher than 0.3 in the population. Thus, all these markers are appropriate for the forensic study in Korean cattle (Hanwoo). Other statistical parameters for these loci such as

Table 1. Four new bovine tri-nucleotide microsatellite markers

| Locus | Primer sequences (5'-3') | allele size (bp) | Chromosomal region |
|---|---|---|---|
| B17S0808 | F: TAMRA-GAACTCCCCTGGGAATATAAA<br>R: CGGACACGACTGAAGTGAC | 102~134 | Ch.17<br>30480786-30480900 |
| B15S6253 | F: TAMRA-TAGTTGAACGCATGGCCCTA<br>R: GGGCGTTACCAAGAACCTGA | 145~182 | Ch.15<br>47186201-47186363 |
| B8S7996 | F: TAMRA-GCTCCTGATCTTTAAAACTGGATT<br>R: CTGGGACGTGAGTGGACAGA | 206~267 | Ch.8<br>9667915-9668161 |
| B17S4998 | F: TAMRA-GCTGACCTTAGGAACAAGGTAT<br>R: AAATAGAATGTTGCCTGTGG | 341~418 | Ch.17<br>64994862-64995230 |

Table 2. Allele frequencies of four new tri-nucleotide microsatellite loci in Korean cattle (Hanwoo)

| B17S0808 | | B15S6253 | | B8S7996 | | B17S4998 | |
|---|---|---|---|---|---|---|---|
| Allele | Frequency | Allele | Frequency | Allele | Frequency | Allele | Frequency |
| 19 | 0.005 | 11* | 0.621 | 10* | 0.117 | 19 | 0.010 |
| 20 | 0.267 | 12 | 0.015 | 19.2* | 0.233 | 20* | 0.044 |
| 21 | 0.296 | 14 | 0.068 | 20.2* | 0.053 | 21 | 0.010 |
| 22* | 0.160 | 15 | 0.053 | 21.2 | 0.005 | 21.2 | 0.005 |
| 23 | 0.092 | 16 | 0.010 | 25 | 0.083 | 22.2 | 0.034 |
| 24 | 0.083 | 17* | 0.160 | 25.2* | 0.126 | 23.2* | 0.150 |
| 25 | 0.034 | 18* | 0.039 | 26.2* | 0.073 | 24.2 | 0.005 |
| 26 | 0.034 | 19 | 0.019 | 27.2 | 0.034 | 25 | 0.019 |
| 29 | 0.010 | 20 | 0.005 | 28 | 0.005 | 25.2 | 0.034 |
| 30 | 0.019 | 22 | 0.005 | 28.2 | 0.223 | 26.2 | 0.053 |
| | | 23 | 0.005 | 29.2* | 0.029 | 27.2 | 0.058 |
| | | | | 30.2 | 0.019 | 28.2 | 0.063 |
| | | | | | | 29.2 | 0.010 |
| | | | | | | 30 | 0.039 |
| | | | | | | 31 | 0.029 |
| | | | | | | 33 | 0.024 |
| | | | | | | 34* | 0.092 |
| | | | | | | 35* | 0.097 |
| | | | | | | 36 | 0.029 |
| | | | | | | 36.2* | 0.019 |
| | | | | | | 37.2 | 0.034 |
| | | | | | | 38.2 | 0.015 |
| | | | | | | 39.2 | 0.034 |
| | | | | | | 40.2 | 0.044 |
| | | | | | | 41.2 | 0.019 |
| | | | | | | 42.2 | 0.005 |
| | | | | | | 43.2 | 0.010 |
| | | | | | | 44.2* | 0.010 |
| | | | | | | 45.2 | 0.005 |

* Sequenced 16 alleles. GenBank accession numbers of allele 22 at locus B17S0808, alleles 11, 17, and 18 at locus B15S6253, alleles 10, 19.2, 20.2, 25.2, 26.2, and 29.2 at locus B8S7996, and alleles 20, 23.2, 34, 35, 36.2, and 44.2 at locus B17S4998 are serial numbers from JQ954758 to JQ954773.

polymorphism information content (PIC), power of discrimination (PD), and power of exclusion (PE) were estimated (Table 3). PIC values for these 4 loci ranged from 0.551 (B15S6253) to 0.932 (B17S4998). PIC is an indicator of the degree of informativeness of a marker. According to the criteria of Botstein et al. (1980), all these loci are highly informative (PIC > 0.5). PD values of the loci B17S0808 (0.923), B8S7996 (0.952), and B17S4998 (0.984) were very high over 0.9, but that of the locus B15S6253 (0.783) was relatively low. PE values of B17S0808, B8S7996, and B17S4998 were as high as 0.610, 0.703, and 0.782, respectively but that of B15S6253 was relatively low (0.210). Combined PD and PE values across the four microsatellite loci were 0.9999968 and 0.980051757, respectively. Although the degree of genetic variability of the locus B15S6253 was relatively low among these four new microsatellite loci, the favorable parameters

Table 3. Forensic relevance parameters for four tri-nucleotide microsatellites in Korean cattle (Hanwoo)

| | B17S0808 | B15S6253 | B8S7996 | B17S4998 |
|---|---|---|---|---|
| $He_{obs}$ | 0.830 | 0.540 | 0.880 | 0.920 |
| $He_{exp}$ | 0.825 | 0.599 | 0.879 | 0.968 |
| n*A* | 10 | 11 | 12 | 29 |
| HWE(*P*) | 0.673 | 0.153 | 0.104 | 0.405 |
| PD | 0.923 | 0.783 | 0.952 | 0.984 |
| PIC | 0.770 | 0.551 | 0.832 | 0.932 |
| PE | 0.610 | 0.210 | 0.703 | 0.782 |

$He_{obs}$, observed heterozygosity; $He_{exp}$, expected heterozygosity; n*A*, number of alleles observed; HWE(*P*), probability test for Hardy-Weinberg equilibrium; PD, power of discrimination; PIC, polymorphism information content; PE, power of exclusion.

shown in Table 3 indicate that these four new tri-nucleotide microsatellite markers could be useful multiplex PCR markers for the forensic and population genetic studies.

We also analyzed stuttering for these tri-nucleotide microsatellite markers. Average peak height ratio for stutters with loss of one repeat unit (-3bp) was 13.89% (B17S0808), 26.67% (B15S6253), 9.09% (B8S7996), and 43.75% (B17S4998). Other stutters with loss of two or more repeat units were observed only in locus B17S4998. These peak height ratios were much lower than those for stutters from the ISAG-recommended di-nucleotide microsatellite loci (Shackell et al., 2005). These results well agree with the fact that tri-, tetra-, or penta-nucleotide repeats are significantly less prone to slippage than di-nucleotide markers (Edwards et al., 1991). High stutter percentage makes microsatellite genotyping difficult to discriminate stutter peaks and minor allelic peaks (Shackell et al., 2005). Thus, these tri-nucleotide microsatellite loci with low peak height ratios for stutters could be useful markers for multiplex genotyping.

We also analyzed the sequence structure of these four tri-nucleotide microsatellite markers by sequencing several alleles for each locus, which were indicated in Table 2 with their GenBank accession number. Allele 22 at locus B17S0808 was a compound repeat structure with $(ACT)_{15}(GCT)_7$ repeats. Three alleles (11, 17, and 18) at locus B15S6253 were all simple repeat structures with $(TGC)_{11-18}$ repeats. There were two different types of repeat structure in the six sequenced alleles at locus B8S7996. One allele (10) was a simple repeat structure with $(AGC)_{10}$ repeats. The other five alleles (19.2, 20.2, 25.2, 26.2, and 29.2) were compound repeat structures with $(AGC)_{8-9}A(AGC)_2A(ACG)_{9-19}$ repeats. Alleles at locus B17S4998 were also classified into two groups with different types of repeat structure. Three (20, 34, and 35) of the six sequenced alleles were simple repeat structure with $(AGC)_{20-35}$ repeats. The other sequenced alleles (23.2, 36.2, and 44.2) were compound repeat structures, which were interrupted by a internal consensus sequence; $(AGC)_{5-6}(AGA)_{0-1}CACGACTGAAGCGACGC(AGC)_{12-33}(ACC)_{0-1}$. In this study, only several alleles were sequenced, which means that it cannot rule out the possibility that there are different repeat structures in other alleles at the same locus and even in the same alleles of different individuals because sequence polymorphism may appear in the alleles with same length (Spolnicka et al., 2008).

In this study, we identified and characterized four new bovine tri-nucleotide microsatellite markers in a small population of Korean cattle (Hanwoo). Next, we will design a new multiplex PCR system including our new tri-nucleotide microsatellite markers and apply it for the forensic and population genetic studies in cattle of different populations or breeds, which could confirm a general usefulness of these tri-nucleotide microsatellite markers.

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