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A specific marker gene analysis for expression of black-dotted nose in Hanwoo

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Abstract

The RAPD-PCR method was used to screen the specific DNA markers for the black-dotted nose in Hanwoo. This study attempted to provide a tool to control the expression of the black-dotted nose in Hanwoo population in Korea. A total of 5 URP markers were used and analyzed the DNA variants among the 5 URP markers. A specific DNA marker was found in the URP 5 marker and named R5SNP. From the sequence analysis of R5SNP, 852 base sequences were detected. After compared with the registered DNA sequences in the data base, UMD 3.1 reference method used to check the homology of the base sequences between them and 99% of the sequence homology was detected. Also, a total of 11 SNPs variants found which were associated with the expression of the black-dotted nose. In our study, a specific segment of DNAs was found that was only in the black-dotted nose. This segment of DNAs was not associated with the normal yellow-coloured nose of Hanwoo. The R5SNP marker was found in this study could be used as an indicator for distinguishing Hanwoo and other foreign breeds and also for the early detection of the Black-dotted nose in Hanwoo.

Keywords: RAPD markers; Hanwoo; Cattle; Black-Dotted Nose

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Introduction

For breed classification and registration in cattle, the phenotypic colour patterns are very crucial. In Korea, Hanwoo, the Korea native cattle, can be registered as a purebred only for the solid colour of yellow or brownish-yellow on a whole body and no black-dots on nose. In 1974, the report of the National Agricultural Cooperative Foundation showed that 91% was brownish-yellow, 3.0% partially other colours, and 0.3% for the totally different colour patterned. Nevertheless, it was not inevitable to discard the non-standard coloured Hanwoo due to unexpected expression in progeny from the phenotypically normal coloured parents. In mammals, the two colour genes of phaeomelanin (red/yellow) and eumelanin (brown/black) play a major role in determination of coat colour on body. The two loci of Extention (E) and Agouti (A) determine the contents and patterns of colours based on their allele combinations (Robbins et al., 1993; Jackson, 1993). In reality, the decision for the purebred Hanwoo cannot be made sorely by the MC1R analysis. To overcome such limitations, the breed formation, genetic characteristics, genetic diversity and association with other breeds have widely been analyzed by using genetic markers of blood protein, milk protein, microsatellites, mitochondrial DNA, Y chromosomes and etc. (Edwards et al., 2000; Troy et al., 2001; Hanotte et al., 2000, 2002). However, selection of Hanwoo by such genetic markers is yet too difficult to be practically applied. Genetic differences between cattle within a breed by the analysis of DNA sequence variation (Smith et al., 1996.) in specific regions on DNA sequences have been actively studied. The genetic diversity of Cuttle Fish species (Yoon et al., 2010.) has been proven by using methods such as RAPD (Random Amplified Polymorphic DNA.) and the genetic differences between species were identified

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(Yoon, 2006). Studies of genetic variation for the receptor known as gene marker of Melanocyte-stimulating hormone (αMSH) suggested that it determines the hair colour of Hanwoo. Slominski et al. (2004), Selz et al. (2007), Lee et al. (2002) reported that E+ allele of MC1R genotypes is more associated with the colour patterns on nose of cattle. Lee et al. (2002) found that the colour pattern in Chick-So (Other Korea native cattle with dark stripped colour patterns on body) is highly associated with pigmentation of nose. However, the studies on melanogenesis in pigmentation of nose have not been much made. Therefore in our study, we attempted to find the potential marker by using RAPD technique (Michael et al., 1990; Williams et al., 1990; Meunier et al., 1993) as an indicator to genetically and phenotypically differentiate the black-dotted nose of Hanwoo, the normal yellow-coloured nose of Hanwoo, and other foreign breeds such as Hereford and Holostein.

Material and Methods

Sample selection and genomic DNA extraction

The blood samples were taken from 19 black-dotted nose of Hanwoo, 18 normal yellow-coloured nose of Hanwoo, and 14 Holstein cows from the farms in Ansung, Korea and then, DNAs were extracted by using DNA Extraction Kit (Toyobo, Japan), which then was dissolved by using magnetic beads in the sterilized water.

MC1R gene analysis for the black dotted nose and the normal yellow-coloured nose of Hanwoo

For the PCR-RFLP of MC1R, the construction of MC1R primer was designed based on the information from NCBI's (http://www.ncbi.nlm.nih.gov) nucleotide sequence (Gene accession no. AF445642) as described in some reports (Klungland et al., 1995; Chung et al., 2000) (Forward primer: 5' *CAG TGC CTG GAG GTG TCC AT* 3', Revers primer 5' *GGC CAG CAT GTG GAC GTA GA* 3'). The PCR method by using the nano-spectrometer selected DNA(O.D:1.8-1.9), each group of the DNA concentration was quantified and standardized of about 200ng/ul±30 and also, 10 pmol forward/reverse primer, 2.5mM DNA 1ul, 10XPCR buffer 2.5ul, and distilled water of 2 unit Taq polymerase (Toyobo, JPN) were added to perform the PCR. The reaction condition with pre-denaturation at 95°C for 10 min, 30 sec at 95°C denaturation, 30 sec annealing at 60°C, 72°C of extension for 1 min and 35 cycles were performed. Lastly, at 72°C for 5 min from the final-extension, PCR was completed. In order to analyze the aspect of MC1R gene variants, the PCR product of 10ul with MspI(Toyobo, JPN) 2 units of 10×M buffer 2ul was mixed at 37°C, which reacted for 4 hrs. After enzyme treatment and 2% agarose gel electrophoresis, the pattern of DNA band was classified according to the method as described in the papers of Klungland et al. (1995) and Park et al.(2012).

RAPD-PCR analysis

The choice of primer for PCR was made by 5 sets of URP markers (JK, Anseong, KOR) which contained GC content over 60% of the total sequence by RAPD. For PCR amplification with a total of 25ul PCR preparation solution, 100ng/ul DNA was mixed with 2ul of 10x reaction buffer, 2.5mM of dNTPs, 10pmol of URP primer, 2unit Taq DNA polymerase and finally, distilled water was added. After denaturation for 5 min at 95°C following with denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C, which was repeated 40 times, the final extension was conducted for 8 min at 72°C and then finally, stopped at 4°C. To check the consistence of the experiment, the whole experiment was conducted 3 times and was proven consistent. To see the polymorphic patterns of the gene, the electrophoresis was made for 2 hrs at 100 V on 3% agarose gel.

Cloning of specific DNA marker

After analyzing the genetic diversity for all the individuals, the specific marker gene identified was extracted from the black-dotted nose of Hanwoo using Gel Extraction kit (TOYOBO, Osaka, JAP). The amplified single marker band was re-checked on 3% agarose gel. The 4ul of the extracted marker was mixed with 2x ligase buffer 10ul, T4 DNA ligase 4ul, and T-easy vector 2ul and then ligation was induced for 3 hrs at room temp. Finally, 10ul of the recombinant plasmid vector mixed with 50ul of component cell (DH5a, TOYOBO, Osaka, JAP) was stabilized for 20 min at 4°C and then, the vector was induced for 1.5 min at 42°C and mixed with 500ul of SOC for 1.5 hr at 37°C. It was then inoculated onto X-gal LB plate for 18 hrs at 37°C and then, the white colony, which was proven the completion of insertion, was extracted and inoculated again into LB-Broth Ampicillin for 24 hrs at 37°C.

Sequence analysis of DNA markers

Transgenic plasmid DNA from the cloned *E. coli* was extracted using the PLASMID Kit (TOYOBO, Osaka, JAP). After 10ul of the extracted plasmid product was treated with 10U/ul of EcoR I for 3 hrs at 37°C, target gene was identified and then the remaining plasmid DNA was added with 5xBuffer and BIG-Dye (3.1V). The T7 Primer was added and followed by denaturation for 10 sec at 96°C, annealing for 5 sec at 50°C, extension for 4 min at 60°C, and 35 cycles of amplification. After, in order to refine the sequence, big-dye product was mixed with isopropanol to stand at room temp for about 15 min and then, removing the supernatant of 70% EtOH precipitation, then by adding 100% formamide for about 3 min of denaturation at 96°C. The purified product analyzed by the Capillary method of 3730XL Sequencer (ABI, NY, USA) was able to be sequenced with using the Sequmen II Program (DNAster, Madison, USA).

Comparative analysis of nucleotide sequences for different breeds

NCBI 4.6.1 reference and UMD 3.1 reference were used for the cross analysis of the nucleotide sequence homology between Hanwoo with the black-dotted nose and Hereford.

Dendogram analysis

After analyzing the homology of the base sequences of the gene for the black-dotted nose in Hanwoo and Herford, different SNPs for different breeds were used for phylogenic analysis. They were analyzed by MEGA5.05 following the method of Tamura et al. (2004, 2011). The dendrogram was drawn by the un weighted pair group method with arithmetic averages (UPGMA). The significant results from the analysis was obtained by the method of Nei and Li (1979); $d_{xy}=1-(2n_{xy}/(n_x+n_y)$, where n_x and n_y= no. of SNPs; and, $2n_{xy}$= no. of common SNPs between the individuals. The larger score for the genotypes indicates the distant similarity and the smaller score indicates the close similarity.

Genomic DNA PCR

Each group of about 300 of the genomic DNA concentrations was standardized. And from the specific DNA sequences analyzed from the black-dotted nose, forward primer 5' *GGT TAA TTG CGC GCT TGG CG* 3'and revers primer 5' *AGA AAG CGC CAC GCT TCC CG* 3' was designed. Hence, a total of 10pmol primers were added with 2.5mM dNTP 4ul, 10XPCR buffer 5ul, distilled water of 16.5ul and 2 unit Taq polymerase, making a total of 25ul for the PCR. The pre-denaturation reacted at 95°C for 10 min, 95°C at 30 sec denaturation, 64°C for 3 sec annealing, and 72°C for 1 min extension, 35 times of repeated cycles, then 72°C for 5 min final-extension of PCR. The electrophoresis of 2% agarose gel confirmed the pattern of DNA bands.

Results

MC1R genetic variation of the black- dotted nose and the normal yellow-coloured nose in Hanwoo

The extracted DNAs from the nose of Hanwoo and analyzing the variations of MC1R for its variants in the black-dotted nose of Hanwoo showed the genotypes of ED/ED and E+/e, whereas Hanwoo with normal yellow-coloured nose showed the genotype of E+/E+ (Fig. 2) as generally detected in Hanwoo.

General analysis of specific gene patterns of the black dotted nose in Hanwoo

The analyzed specific and non-specific sections of DNA fragment, which was used by the method of RAPD-PCR, were the same as shown in Fig. 3. The expression band of homogeneity from the black-dotted nose resulted in the case of URPO1 to URPO4 of 500bp; 700bp, 800bp,

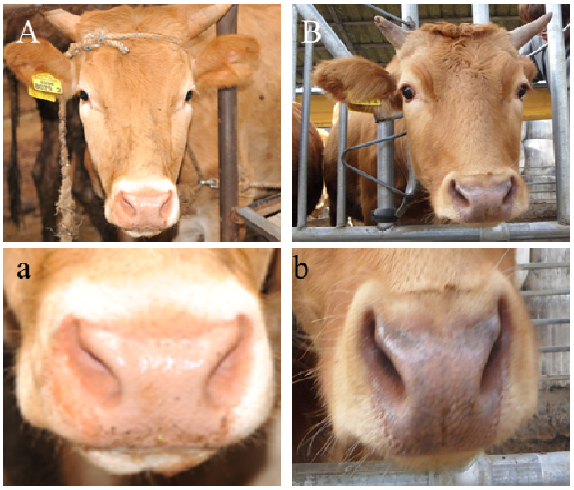

Fig. 1: Criteria for the classification of the nose and coat colour of Hanwoo. A-B: Yellow coat colour, a: Yellow-coloured nose, b: Black-dotted nose.

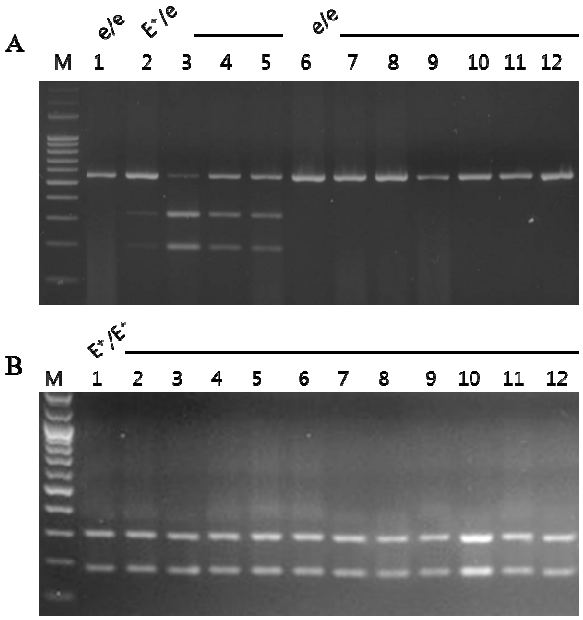

Fig. 2: Genotype mutation of the *MC1R* gene in yellow-coloured nose of Hanwoo and black-dotted nose in Hanwoo. PCR-RFLP analysis for detection of the MC1R genotype mutation in cattle. A: Black-dotted nose in Hanwoo, B: Yellow-coloured nose of Hanwoo.

and 2000bp, correspondingly distributed in various ways. For the case of URPO5, the band can only be distributed below 800bp, and showed low diversity compared to other primers. Especially in the case of the 2000bp of URPO4 and the formation of 800bp of URPO5, only the black dotted nose showed the specific gene expression (Fig. 3).

Selection of specific gene and base sequence analysis

Based on the analysis for the genetic diversity of specific DNA fragment (R5SNP: Random 5 specific

Table 1: Comparison of nucleotide sequences of specific fragment sequence of searched gene from UMD 3.1 reference (HanWoo) and NCBI 4.6.1 reference (Hereford)

Samples	5'	Sequence	3'
BDnHW	1	ATT GGG AAT TCC TGC - AG CCC GGG GGA TCC ACT AGT TCT AGA GCG GCC G-C CAC CCC GGT GGA GCT CCA GCT TTT GTT	78
NnHW		. . . . . AC.	
HF		c. g . ct Gc. GG . . . TG . . . T. C T. T . . T . . C TCC AA G T A T A T . G TG AC. . CA . . G . . C A G AAG GGG GCG	
	79	CCC TTT AGT G-A GGG TTA ATT GCG CGC TTG GCG TAA TC- - AT GGT CAT AGC TGT TTC CTG TGT GAA ATT GTT ATC CGC	156
		. . . . .	
		. G nCn G G . G A A ACC . CC . nA . CA CCn . A C. G G G G C. C . . C . . C . . . A C AG . A G C . . . . GC . CA . A . . .	
	157	TCA CAA TTC CAC ACA ACA TAC GAG CCG GAA GCA TAA AGT GTA AAG CCT GGG GTG CCT AAT GAG TGA GCT AAC TCA CAT	234
		. . . . .	
		C. . . . AA . . . . . G . . . . . C. . . C . C. . . . . G . . . . G . . C . . A . . . C. . . n . . . A . . . A	
	235	TAA TTG CGT TGC GCT CAC TGC CCG CTT TCC AGT CGG GAA ACC TGT CGT GCC AGC TGC ATT AAT GAA TCG GCC AAC GCG	312
		. . . . .	
		C. . A . . . C A . . . C . . . G . . . . . CC A . . . G . . . . . A G . . C . . . . . A . . AA . . A . . . A	
	313	CGG GGA GAG GCG GTT TGC GTA TTG GGC GCT CTT CCG CTT CCT CGC TCA CTG ACT CGC TGC GCT CGG TCG TTC GGC TGC	390
		. . . . .	
		. . . . . CC C. . A AA . . . . . A . nA . . . . AC . . G . . . A . . A . . . A . . . A . . . A . nA . . . C.	
	391	GGC GAG CGG TAT CAG CTC ACT CAA AGG CGG TAA TAC GGT TAT CCA CAG AAT CAG GGG ATA ACG CAG GAA AGA ACA TGT	468
		. . . . .	
		. . . . . G A . . . . C. . . C . . . . . G . n. . . . C C. n . . . . . C C. . . . . A . . . . . A A	
	469	GAG CAA AAG GCC AGC AAA AGG CCA GGA ACC GTA AAA AGG CCG CGT TGC TGG CGT TTT TCC ATA GGC TCC GCC CCC CTG	546
		. . . . .	
		. . . . . A . . . . . --- --- --- --- --- --- --- --- --- --- --- ---	
	547	ACG AGC ATC ACA AAA ATC GAC GCT CAA GTC AGA GGT GGC GAA ACC CGA CAG GAC TAT AAA GAT ACC AGG CGT TTC CCC	624
		. . . . .	
		--- ---	
	625	CTG GAA GCT CCC TCG TGC GCT CTC CTG TTC CGA CCC TGC CGC TTA CCG GAT ACC TGT CCG CCT TTC TCC CTT CGG GAA	702
		. . . . .	
		--- ---	
	703	GCG TGG CGC TTT CTC ATA GCT CAC GCT GTA GGT ATC TCA GTT CGG TGT AGG TCG TTC GCT CCA AGC TGG GCT GTG TGC	780
		. . . . .	
		--- ---	
	781	ACG AAC CCC CCG TTC AGC CCG ACC GCT GCG CCT TAT CCG GTA ACT ATC GTC TTG AGT C	852
		. . A . . . . . G . . . . . C . . . . . C . . . . . G . . C	
		--- -	

nuclear pair), the gene sequence from the black-dotted nose was cross-analyzed by NCBI 4.6.1 reference and UMD 3.1 reference yielded the same result shown in Table 1. In the result from URP-marker 5 around 800bp, which was analyzed from the black-dotted nose, 852 base sequences were detected. By analyzing the homology for the sequence of *Bos Taurus* NCBI 4.6.1 reference, 1 bp to 511bp of R5SNP showed the homology with 511bp to 1014bp of sequence ID: ref|NW_001501683.1 by 80%. However, UMD 3.1 reference was not mapped, which indicated the base sequence was assumed to be only specific for the black-dotted nose of Hanwoo. This result was compared with Hanwoo specific base sequences obtained from Insilicon Co. ltd in Suwon. It was found 11 SNPs (single nucleotide polymorphism) were detected, showing 99% of homology among them.

Dendrogram analysis

To estimate the genetic distance of SNPs in specific gene sequences between the black-dotted nose and the normal yellow-coloured nose in Hanwoo, unweighted pair-group method of analysis (UPGMA) by MEGA software was used and the result was shown in Fig. 4.

The genetic distances were 0.012 between the black-dotted nose and the normal yellow-coloured nose, 1.093 between the black-dotted nose and the Hereford. From these results, the specific base sequences in the black-dotted nose in Hanwoo could be used as the specific gene marker for Hanwoo differentiated with other foreign breeds.

Comparison of specific gene expressions

After we constructed the specific primer for the black-dotted nose in Hanwoo using R5SNP base sequences, we

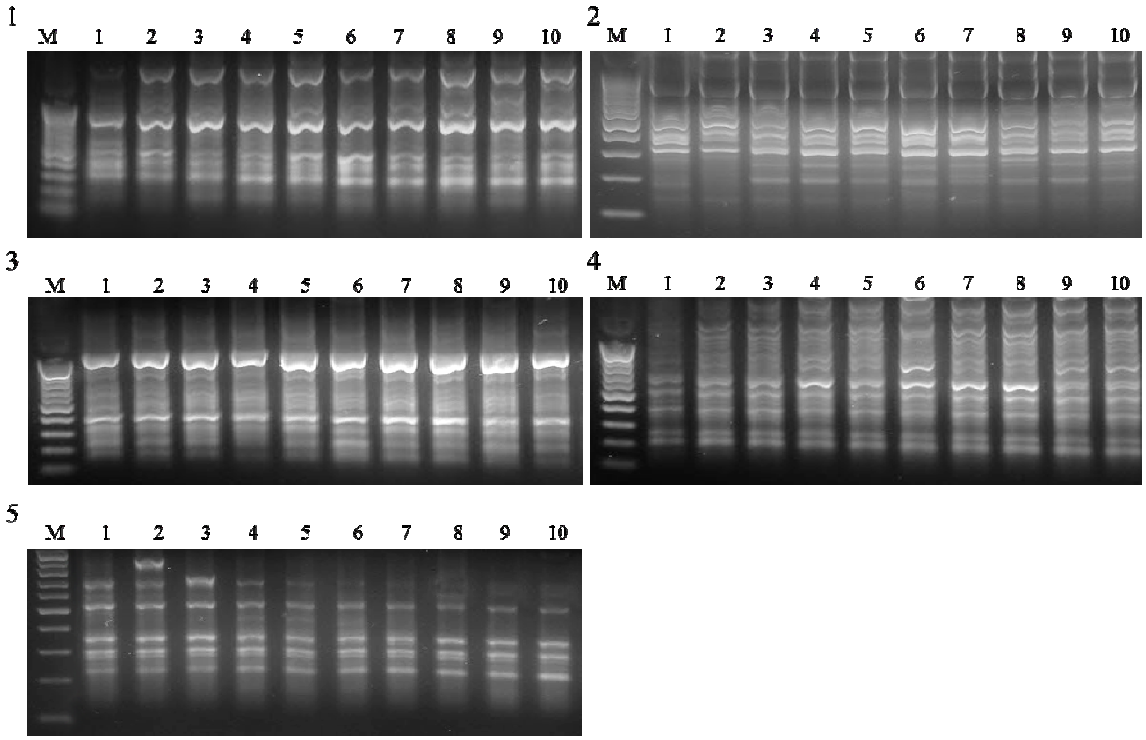

Fig. 3: RAPD profiles using URP primer in the yellow-coloured nose of Hanwoo and black-dotted nose in Hanwoo. The number corresponds to the serial of the genotypes. M : 100bp DNA ladder, lane 1-5 : black-dotted nose, lane 6-10 : yellow-coloured nose.

A

Tag No.	BDnHW	HW	HF
1	***		
2	0.012	***	
3	1.093	1.079	***

B

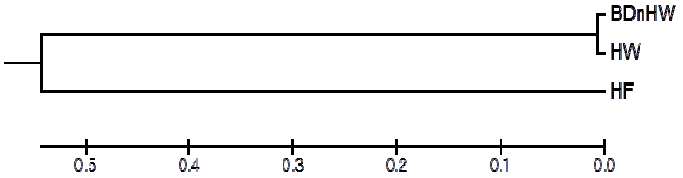

Fig. 4: Evolutionary relationships of taxa. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 1.09229610 is shown. The analysis involved 3 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 808 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. A : Pairwise genetic distance , B : Dendrogram , BDnHW : Black-Dotted Nose in Hanwoo , HW : yellow-coloured nose of Hanwoo, HF : Hereford.

analyzed the expression patterns for the black-dotted nose, the normal yellow coloured nose, and Holstein (Fig. 5). The 19 Hanwoo with black-dotted nose showed the single band around 720 bp in R5SNP but for Hanwoo with normal yellow coloured nose and Holstein, it also showed the single band but showed polymorphic bands in other regions. Therefore, all the black-dotted nose in Hanwoo showed a single band for R5SNP base sequences but Hanwoo with normal yellow coloured nose and Holstein showed different polymorphic band patterns.

Discussion

The emergence of non-standard coloured cattle was not inevitable to discard the non-standard coloured Hanwoo due to unexpected expression in progeny from the phenotypically normal coloured parents. It is an economically costly process in terms of time, effort and money to the Korea livestock industry. However, the studies on non-standard coloured Hanwoo of nose has not been much made. The only distinction way of non-standard colour cattle, it is just a MC1R genetic variation or evaluation of appearance in the Hanwoo (Lee et al., 2002). The most of coat and nose colours in cattle are determined by the relative expression of phaeomelanin and eumelanin (Selz et al., 2007). The variation in MC1R is known to have much influence melanogenesis produced by α-MSH (Slominski et al., 2004). The genotype of E^+/E^+ was proven for the normal yellow coloured nose. However in our studies, for the black-dotted nose, the

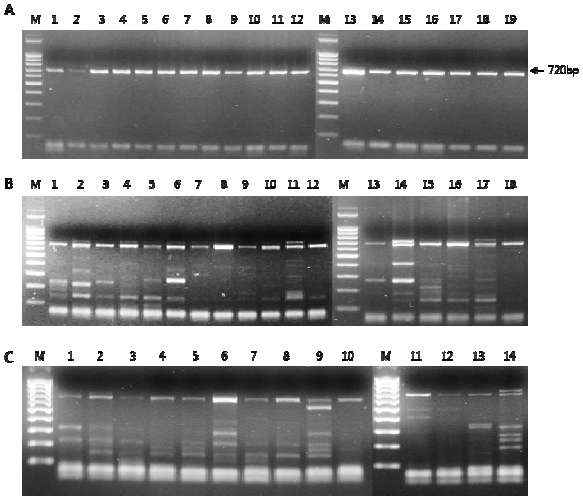

Fig. 5: PCR analysis of specific DNA fragment gene (R5SNP) from the yellow-coloured nose of Hanwoo and black-dotted nose in Hanwoo and Holstein. 720bp is a target gene fragment. A: black-dotted nose in Hanwoo group, B: yellow-coloured nose of Hanwoo group, C: Holstein group.

genotype of E^D/E^D was mostly detected. These results were able to suggest a possibility about the genetic polymorphism in non-standard cattle. Another results of many reports to genetic polymorphism in cattle species, were able the differences of the species in the colour and external section can be measured through genetic markers (Kim et al., 2011; Khatun et al., 2012). These have been used to determine evolutionary relationships within and between species, genera or higher taxonomic categories (Cornuet et al., 1999). This procedure detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of an arbitrary nucleotide sequence. Our study demonstrated that the short random oligonucleotides of arbitrary sequences can be used to differentiate non-standerd hanwoo to variation of RAPD fingerprints in breed-specific DNA. From the analysis of random PCR, around 800 bp was found a specific DNA marker only in the black-dotted nose of Hanwoo and throughout the analysis, 852 sequences of R5SNPs were analyzed. The similarity analysis for the base sequences among Hanwoo using UMD 3.1 reference showed 99% of homology. However, in PCR analysis, the expression of R5SNP was detected by a single band in the black-dotted nose but the polymorphic bands were detected in the normal yellow-coloured nose in Hanwoo. The specific DNA sequence can be used to differentiate non-standard Hanwoo species like a Kim et al (2011) reports. Hence, the specific base sequences for the black-dotted nose was very different from those for the normal yellow-coloured nose and could be used an indicator for detection of the black-dotted nose in Hanwoo. We could define the genetic association and specific base sequences for the potential expression of the black-dotted nose in future progeny of Hanwoo. By use of R5SNP base sequences, we could test the expression of the black-dotted nose and breed specification in early stages. We would do further researches to attempt the functional analysis for R5SNP base sequences and see whether it affects melanogenesis and consequently utilize the resulting information to discard the non-standard colour patterns in Hanwoo.

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