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Effects of Mitochondrial DNA Polymorphism on Growth Traits of Hanwoo

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mt DNA 다형이 한우 성장에 미치는 영향

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SUMMARY

한우의 mt DNA cytochrome oxidase subunit I, II, 및 III complex지역의 유전적 다형현상을 제한효소를 이용하여 검출하였다. PCR primer 6 종에 대하여 20가지 제한효소를 처리하였으며, Pst I, Pvu II, Rsa I, Eco RI, Bgl II, and Msp I 제한효소를 사용하여 유전적 변이를 검출하였다. 검출된 변이체와 한우의 성장과의 관련성을 조사한 결과 cytochrome oxidase subunit III complex 지역의 유전염기서열을 근거로 제작한 primer Mt9 좌위에서 제한효소 PvuII를 이용한 절단형과 체중형질 인 WT15($P<0.05$) 및 WT18($P<0.01$)에서 고도의 유의성이 관찰되었다. 아울러, Mt9-Pvu II($P=0.07$), Mt6-Bgl II($P=0.05$), and Mt8-Rsa I($P=0.05$) 좌위 또한 WT9, WT15, and WT15에서 각각 통계적 유의성이 관찰되었다. 따라서 본 결과는 cytochrome oxidase subunit III complex segments가 candidate gene으로서 기초적 유전정보 제공은 물론 유전적 개량을 위해 사용될 수 있을 것으로 사료된다.

(Key words : mitochondrial DNA polymorphism)

INTRODUCTION

Mitochondrial(mt) DNA, which are generally maternally inherited and non-recombining patterns in the nature of animals, is closed circular double helix DNA and the length of the sequence is approximately 16,500 bp(Brown, 1980). Mt DNA encodes for 13 hydrophobic polypeptides, 22 tRNAs and 2 rRNAs, which are related to respiratory-chain and oxidative phosphorylation systems(Anderson et al., 1981). The respiratory chain is a series of 5 multi subunit enzyme complexes located on the inner mitochondrial membrane. Most of the mitochondrial protein-coding genes, particularly cytochromesubunits, as well as rRNAs, have been used in phylogenetic or population genetic studies.

Interesting findings were focused onthe genetic or non-genetic differences on the cytochromesubunits in mt DNA because these subunits may be related to aging and muscle development process. Harman (1972) and Fleming (1985) were the first to postulate that mitochondria may play a role in the aging process. The aging hypothesis of mito-

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chondria proposes that aging results from the accumulation of detrimental mitochondrial DNA mutations during life (Linnane et al., 1989). In human skeletal muscle, muscle fibers that lack cytochrome c oxidase activity also appear and accumulate in an age related manner (Muller et al., 1990; and Brieiley et al., 1996). Several reports have described a decrease in mitochondrial respiratory enzyme activities in human skeletal muscle (Trounce et al., 1989; Cooper et al., 1992, Boffoli et al., 1994; Boffoli et al., 1996) and liver (Yen et al., 1989) with aging. Therefore, genetic variants in the region of cytochrome oxidase subunits may explain different levels of cytochrome subunit activities, which may effect on early muscle development as well as aging. Marin et al., (1998) also reported that bovine cardiac mitochondrial function significantly increased in mitochondrial oxidative phosphorylation and mitochondrial gene expression during the early stages of growth and development. Mitochondrial respiratoryfunction also decreased during aging (Marin et al., 1994). Therefore, genetic di fferences on the region of cytochrome oxidase subunit in mt DNA may explain some variation of growth and aging process.

To further understand the regulation of mitochondrial function and biogenesis from early to late muscle development and aging, point mutations in the cytochrome oxidase subunit region of mt DNA were aimed to analyze genetic effects on animal growth as weights.

MATERIALS AND METHODS

1. Animals

Two hundred thirty one Korean native steers, which were part of the 33th progeny test in 2002 and 143 bulls, were used from Daekwanryung branch of the National Livestock Research Institute (NLRI). The cattle were fed a postweaningcorn and soybean meal diet, which was formulated to meet NRC (1984) requirements for growing beef cattle.

2. Sample Preparation

Total DNA was prepared from EDTA-blood samples of 231 steers and 143 bulls from Daekwanryng branch of National Livestock Research Institute. The high salt procedure including proteinase K and SDS lysis steps was adapted for the DNA extraction. DNA was ethanol precipitated and resuspended in distilled water.

3. Design of Primers

In Table 1, the primers for the cytochrome oxidase subunit I (COX I), II (COX II), and (COX III), region in the mt DNA were designed based on the mt DNA sequence (GenBank accession number, J01394). For the optimal size of the PCR fragments in the analysis procedures of single strand conformation polymorphisms (SSCP) or restriction fragment length polymorphism (RFLP), length of the PCR products were mediated to be expected around 1,000bp 1,200bp. All the Mt primer sets showed restriction enzyme sites, and therefore, SSCP analysis, which is a powerful tool to detect any single mutation site, was not performed.

4. Weight Traits

Weights were recorded at birth (BW), 3 (WT3), 6 (WT6), 12 (WT12), 15 (WT15) and 18 month (WT18) of age.

5. Polymerase Chain Reaction

PCR was conducted with a final volume of 20 ul, including 2 ul of 10 X reaction buffer (10 mM Tris, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$), 10 uM dNTP, 10 pM of each primer, 50 ng of genomic DNA, and two units of Taq DNA polymerase. After denaturation for 2 min at 95℃, PCR cycles for the mt primers were adapted

Table 1. Primer sequences for the regions of the cytochrome oxidase subunit (COX) I, II, and III of the mitochondrial DNA

Primer	Sequence	Position	Region
Mt5F1	ATAGCCCCATTTCACTTC	4587~4604	COX I
Mt5R2	CGTTGTAGATTTGGTCGTC	5834~5852	
Mt6F	TTGGGCCGGTATAGTAGGAACAGC	5781~5758	COX II
Mt6R	TCGTCGAGGCATGCCAGATAGTC	6981~7003	
Mt7F	CGACGATACTCCGACTAC	6998~7015	COX II
Mt7R	TTTTATAATATTGACGCAGAT	8039~8059	
Mt8F	GTCCAGGCTTATATTACGGTCAA	7936~7958	COX III
Mt8R	TAGGCCAATTATTAGCAGGGTCAT	9087~9110	
Mt9F	CTAATCGGAGGAGCTACACTTG	8806~8827	COX III

Mt9RATTTATTATTCTTTTTCGGACTA9806-98281:mitochondrial DNA cytochrome oxidase subunit priming region forward primer.
2: mitochondrial DNA cytochrome oxidase subunit priming region reverse primer.

to 94℃ for 45 sec for denaturation, 57℃ for 1 min for annealing, and 72℃ for 1.5 min for polymerization (MJ research P-200).

6. Restriction Enzyme Digestion

Six microliter aliquot from each PCR reaction mixture was used for restriction endonuclease digestion using 2 units of enzyme under the conditions suggested by the manufacturers. The enzyme used in this experiment were: Alu I, Hae III, Rsa I. Sca I, Msp I, Bgl I, Bgl II, Hinf I, Bam HI, Hind III, Pst I, Pvu II, Hae II, Xho I, Hph I, Kpn I, Apa I, Eco RI, Sma I and Taq I (Promega). These restriction enzymes showed sufficient activity to be used directly in the PCR mixture. Six microlitttres of the reaction solution for each digestion was loaded on 2% LE agarose gels. Restriction fragments were separatedby electrophoresis at 4V/cm for 1h. The gels were stained with ethidium bromide and photographed under UV light.

7. Statistical Analysis

Least squares means and standard errors were determined for all measurements with a model including fixed effects of the cytochrome oxidase subunit genotypes, parity, and castration, and a covariate for age of animal. Analysis of variance was conducted using Statistical Analysis System (SAS) general linear models (GLM) procedures, and least squares means were compared using Fisher's least significant difference test (SAS, 1985) with a comparison error rate of 0.05.

RESULTS AND DISCUSSION

1. Genetic Variants

Genetic variants of mt DNA were revealed by restriction endonuclease analysis in this experiment. The nuclotide sequence data of polymorphic sites in mt DNA have been widely used for studies of molecular evoluation, the genetic structure of populations and the estimation of genetic relationships between and within in species. Genetic variants using restriction enzymes were reported for cattle by Laipis et al., 1982, Watanabe et al., 1989, Bhat et al., 1990, and Amano et al., 1994. Laipis

Table 2. Number of restriction fragments with each enzyme digestion of polymerase chain reaction amplicons for the region of cytochrome oxidase subunit in Hanwoo mt DNA

Enzyme	Primer sets					Reaction temperature
	Mt5[1]	Mt6	Mt7	Mt8	Mt9	
Pst I	-	-	-	2	-	37℃
Hae III	-	-	2	2	4	37℃
Rsa I	3	3	-	2	3	37℃
Alu I	3	2	-	2	2	37℃
Bgl II	-	2	-	-	-	37℃
Pvu II	-	-	-	-	2	37℃
Eco R I	-	-	2	-	-	37℃
Sca I	2	-	3	-	2	37℃
Msp I	2	-	-	-	-	37℃
Hinf I	5	3	4	2	2	37℃
Hph I	2	-	2	3	3	37℃
Taq I	-	2	2	-	3	65℃

- : not determined.

[1] : mitochondrial DNA priming region for cytochrome oxidase subunit I (Mt5), II (Mt6 and Mt7), and III (Mt8 and Mt9).

et al. (1982) reported the first mt DNA polymorphisms between and within a single lineage of Holstein cows. A total of 20 restriction enzymes, which were Alu I, Hae III, Rsa I. Sca I, Msp I, Bgl I, Bgl II, Hinf I, Bam HI, Hind III, Pst I, Pvu II, Hae II, Xho I, Hph I, Kpn I, Apa I, Eco RI, Sma I and Taq I (Promega), were used to detect genetic variants in Hanwoo mt DNA. As previous reports for mt DNA polymorphisms by several studies, several restriction enzymes, which were Pst I, Pvu II, Rsa I, Eco RI, Bgl II, and Msp I, showed genetic differences in the region of cytochrome oxidase subunit I, II, and III within Hanwoo breed. Watanabe group (1985) have used 17 restrictin enzymes to find genetic variants in mt DNA, and they observed genetic differences using Hind III, Taq I and Msp I restriction enzymes. Later, the same group reported more genetic variants in mt DNA using Bam HI, Bgl II, Eco RV, Hind III, Pst I, and Sca I in Philippine cattle. Bhat et al. (1990) also reported genetic variants in mt DNA using Bam HI, Bgl II, Hind III, Hpa I, Pst I, and Ava II in Holstein and Indian water buffalo. Chung et al. (1995) also found polymorphism using Pst I, Sca I, and Hpa I. Our finding of the genetic variants might not be same restriction sites by many previous studies because previous reports did not make any location of the restriction cleavage sites on the mt DNA. However, this experiment was aimed to find gentic variants in the specified regions, which were the cytochrome oxidase subunitI, II, and III of the mitochondrial DNA in Hanwoo.

Cleavage sites were generated for mt DNA sequences based on *Bos taurus* with GenBank accession number (J01394) using a computer

Table 3. Allele frequencies of mt DNA restricted by Rsa I, Msp I, Bgl II, Eco RI, Pst I, and Pvu II restriction enzymes in the region of cytochrome oxidase subunit in Hanwoo mt DNA

Segment	Allele	Frequency	Segment	Allele	Frequency
Mt5-Rsa I	A	0.6629	Mt7-Eco R I	A	0.2857
	B	0.3371		B	0.7143
Mt6-Rsa I	A	0.9534	Mt8-Rsa I	A	0.9523
	B	0.0466		B	0.0477
Mt6-Msp I	A	0.8936	Mt8-Pst I	A	0.2988
	B	0.1064		B	0.7012
Mt6-Bgl II	A	0.3763	Mt9-Pvu II	A	0.4385
	B	0.6237		B	0.5614

program (DNAstar v.1.0). In this analysis, 41 different types of restriction enzymes, which showed 214 cleavage sites, were found. All the restriction cleavage sites detected in this experiment were belonged to the 214 cleavage sites, which were generated by computer programs except for Msp I restriction site. The mt DNA sequence reported in GenBank did not have Msp I restriction sites on the region of cytochrome oxidase complex I, II, and III. It may be explained by a hypothesis that Hanwoo has different genetic composition comparing with Bos Tauruscattle, even though Hanwoo is in the line of Bos Taurus for the evolutional relationship.

The restriction enzymes, which were Hae III, Alu I, Sca I, Hinf I, Hph I, andTaq I, had restrcition cleavage sites, but no genetic variants were detected. No restriction cleavages sites were found by Bgl I, Bam HI, Hind III, Hae II, Xho I, Kpn I, Apa I, and Sma I enzymes (Table 2). For the mt DNA polymorphism patterns, two different allele types were detected in all restriction cleavage experiemets, designated A or B. Allele frequencies were estimated for all the loci. Different allele frequencies were calculated by each enzyme reaction for the Mt6 and Mt8 loci. Mt6 and Mt8 loci with Rsa I restriction enzyme showed similar allele frequencies (Table 3). These allele frequencies were not compared with previous reports because these allele frequencies may be not the same enzymatic cleavage sites. These allele cleavage sites were focused on the region of cytochrom oxidase complex in Hanwoo mt DNA.

2. Effects of Genotypes

Mitochondria have been suggested as being responsible for genetic variation in cytogenetic effects on traits of economic impotance because they possess DNA and cytogenetic inheritance. Therefore, it is possible that genetic variants of the mt DNA may be an useful molecular marker for genetic improvement of beef cattle. Faust et al. (1989) suggested that cytogenetic effects of mt DNA can affect animal growth as well asreproduction. Therefore, finding genetic differences within breed should be done in mt DNA as well as genomic DNA. Escepially, the region of cytochrome oxidase subunit may be a candidate segment for muscle development and aging. The mitochondria also, which are the main source of energy in the cell, aging hypothesis proposes that aging results from the accumulation of detrimental

Table 4. Least squares means and standard errors for birth weight, weight on 6 and 9 month by genotypes for the region of cytochrome oxidase subunit in Hanwoo mt DNA

Segment	Allele	Frequency	Segment	Allele	Frequency
Mt5-Rsa Ⅰ	A	0.6629	Mt7-Eco R Ⅰ	A	0.2857
	B	0.3371		B	0.7143
Mt6-Rsa Ⅰ	A	0.9534	Mt8-Rsa Ⅰ	A	0.9523
	B	0.0466		B	0.0477
Mt6-Msp Ⅰ	A	0.8936	Mt8-Pst Ⅰ	A	0.2988
	B	0.1064		B	0.7012
Mt6-Bgl Ⅱ	A	0.3763	Mt9-Pvu Ⅱ	A	0.4385
	B	0.6237		B	0.5614

[1] : mitochondrial DNA region of cytochrome oxidase subunit I restricted by Rsa I restriction endonuclease.

mitochondrial DNA mutations during life (Linnane et al. 1989). If the detrimental mitochondrial DNA mutations during life in the region of cytochrom oxidase subunit are inferfered to animal production at certain stages of aging and growth, they would cause cellular and tissue dysfunction.

Significant effects for the weight traits were detected by several restriction enzymes in the region of cytochrome oxidase subunit in Hanwoo mt DNA. Genetic variants in Mt9 loci, which was the region of COX III, restricted by PvuII enzyme showed significant differences among genotypes for WT15 ($P<0.05$) and WT18 ($P<0.01$) in Table 5. Mt9-Pvu II ($P=0.07$), Mt6-Bgl II ($P=0.05$), and Mt8-Rsa I ($P=0.05$) loci also explained some variation in WT9, WT15, and WT15, respectively in Table 4. There was no significance detected at early age. If mt DNA polymorphism is highly related to aging process of animal, then genetic variants can affect in late of growing stages rather than early growing stages. Therefore, we may expect that significant genetic effects on growth traits couldbe observed in the late of age stages. In this study, genotypic effects on weight were found in the late of age stages, which are WT15 and WT18. However, it is unclear that these results are from either hypothesis of mt DNA mutation correlated to animal aging and development process or statistical differences of different rate of gene expression for individuals in the late growing stages. Growing stages from birth to 18 month in animal are varied, and high individual variation would be expected in beef cattle.

Mt DNA is more vulnerable to damage than nuclear DNA, and therefore, mutation rate comparing with nuclear DNA is 10 times higher. Because mt DNA lacks protective histones, and has few and inefficient repair mechanisms as well as a high rate of turnover. Therefore, numerous mitochondrial DNA mutations, which are nuclotide deletions in general, have been demonstrated to appear and accu-mulate with age in a variety of animal tissues. Also, mitochondrial DNA shows increased damage with age. Muller et al. (1990) and Brierley et al. (1996) reported that muscle fibres that lack cytochrome c oxidase activity also appear and accumulate in an age related manner in human skeletal muscle. If mt DNA polymorphisms in the region of cytochrome oxidase are highly related to aging and growth, the genetic variants can be

Table 5. Least squares means and standard errors for weight on 12, 15 and 18 month by genotypes for the region of cytochrome oxidase subunit in Hanwoo mt DNA

Segment	Allele	BW, kg	WT6, kg	WT9, kg
		p=0.4984	p=0.7455	p=0.6880
Mt5-Rsa Ⅰ[1]	A	25.02±0.41	166.35± 5.68	234.25± 6.17
	B	25.52±0.62	163.30± 7.68	230.09± 8.50
		P=0.2740	P=0.3386	P=0.5101
Mt6-Rsa Ⅰ	A	24.93±0.60	164.82± 7.24	237.99± 7.60
	B	22.37±2.25	138.25±26.22	219.43±26.33
		P=0.1073	P=0.7947	P=0.4088
Mt6-Msp Ⅰ	A	24.44±0.41	162.44± 5.10	226.05± 5.22
	B	26.73±1.39	166.82±16.55	238.18±15.65
		P=0.3596	P=0.4780	P=0.1514
Mt6-Bgl Ⅱ	A	24.22±0.64	160.83± 8.06	222.00± 8.33
	B	24.93±0.47	167.77± 6.15	236.04± 5.97
		P=0.9216	P=0.9755	P=0.5373
Mt7-Eco R Ⅰ	A	24.92±1.39	164.55±11.44	231.15±16.47
	B	24.76±0.76	164.16± 6.77	241.69± 8.38
		P=0.3769	P=0.4224	P=0.7506
Mt8-Rsa Ⅰ	A	25.23±0.60	159.40± 5.48	231.27± 6.36
	B	28.39±3.49	135.27±29.47	221.20±31.37
		P=0.3191	P=0.8139	P=0.6722
Mt8-Pst Ⅰ	A	24.90±0.62	159.97± 8.48	230.96± 8.411
	B	25.65±0.43	162.33± 5.93	235.22± 6.33
		P=0.3764	P=0.3100	P=0.0722
Mt9-Pvu Ⅱ	A	24.74±0.50	156.19± 5.84	224.23± 6.28
	B	25.36±0.51	164.25± 5.55	239.36± 6.02

[1] : mitochondrial DNA region of cytochrome oxidase subunit I restricted by Rsa I restriction endonuclease.

directly used in the beef breeding area because unlikely genomic DNA that there is a single cause to animal growth and aging. However, we believe that the root of the growth and aging process are almost certainly multifactorial events. Even though our research purpose was aimed to detect genetic differences in mt DNA that may effect on growth and aging, major problems are still remained unclearly as followed.

The cytochrome oxidase subunit is too great to be explained from the very low levels of mitochondrial DNA mutations. In general many studies

reported that high frequency of mutation sites is around D-loop region. In addition, mutant mtDNA is extremely recessive. Studies on cells and single muscle fibres have demonstrated that mitochondrial DNA is extremely recessive and high levels of mutated mtDNA must be present before there is either a biochemical or clinical abnormality (Elizabeth et al., 1997). Our results may be reached to the same aspect for the recessive gene action, but not severe recessive patterns were not observed in this experiment. Therefore, our results suggested that mt DNA mutation in the region of cytochrome oxidase subunit III might be auseful genetic marker to aid genetic improvement of growth in Hanwoo.

CONCLUSION

Genetic variants of the mt DNA for the cytochromeoxidase subunit III significantly influenced weight on 15 and 18 months, but genotypes from cytochrome oxidase subunit I and II did not explain significant variation in weight traits. Our findings indicate that mt DNA segments may be used in marker assisted selection programs to improve weight traits in the late of growing stages for beef cattle. Consequently, results of the present study, and future genotypic data from these animals, based on variation in the mt DNA loci, will provide critical information of genetic improvement as a source of candidate genes.

IMPLICATION

There were several reports that mt DNA polymorphisms have been linked to differences in performance availabilityof certain traits in beef cattle (Mannen et al., 1998). Even though some preliminary results for mt DNA effects on important traits, extensive researches were not taken because of shortage for the structured data set, which is materially related reference Hanwoo population in several generations. Therefore, to better understand the effects of mt DNA genotypes on growing stages, it may be necessary further investigation with well unequivocally assigned Hanwoo population.

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