Association of TG-repeats in the 5'-flanking region of bovine growth hormone receptor (*GHR*) gene with milk production traits and somatic cell count in Holstein cattle

M. Muhaghegh-Dolatabady[*], J. Habibizad and M. R. Bahreini Behzadi

Department of Animal Science, Faculty of Agriculture, Yasouj University, Yasouj, Iran

* Corresponding author, E-mail address: mmuhaghegh@yu.ac.ir

Abstract   The growth hormone receptor (GHR) is a member of cytokine/hematopoietin family that mediates the biological actions of growth hormone (GH) on target tissues. Therefore, the purpose of this study was to examine the association of TG-repeat polymorphisms in the 5'-flanking region of bovine *GHR* gene with milk production traits and somatic cell score (SCS) in Holstein cattle of Iran. The part of 5'-flanking region of *GHR* gene that encompassed TG-repeat was screened by single strand conformation polymorphism (SSCP) method and DNA sequencing. Five hundred eighteen Iranian Holstein cows were genotyped, giving 3 distinct SSCP patterns (A, B, and C). Frequencies of these patterns for the amplified fragment were 0.21, 0.26 and 0.53, respectively. Statistical analysis revealed that TG-repeat had a significant effect on average daily milk production ($P < 0.05$) and tended to associate with fat and protein percentage. No significant difference was observed between TG-genotypes and SCS. The association identified in the TG-repeat of *GHR* gene may have potential to serve as candidate genetic marker for marker assisted selection (MAS) in cattle.

Keywords: *GHR* gene, 5'-flanking region, SSCP, microsatellite, cattle

Introduction

Bovine growth hormone (GH) plays important roles in lactation and growth (Parmentier et al., 1999). An important factor in relation to GH action is growth hormone receptor (GHR) which facilitates growth and development of mammary glands (Feldman et al., 1993). The GHR acts as a biological mediator of GH on target cells by transducing a signal across the cell membrane and inducing transcription of many genes, including insulin like growth factor1 (*IGF-I*) (Argetsinger and Carter-Su, 1996). The gene coding for bovine GHR consists of nine exons (numbered 2 to 10) in the translated part, and a long 5'-noncoding region that includes several alternative untranslated exons, but only exons 1A, 1B, and 1C have been studied in detail (Jiang and Lucy, 2001). Distinct promoters regulate transcription from each of the alternative exons. The P1 promoter, which regulates *GHR* expression in the liver, is associated with exon 1A (Jiang et al., 1999).

DNA polymorphisms, observed in the 5'-flanking region of genes such as promoter, are known to influence transcription rate, thus, expression of protein products. So far, several DNA variants in the 5'-flanking region have been identified in different candi-

date genes for production traits in cattle, sheep and goat. For example, a TG-repeat occurs in or near the P1 promoter of the *GHR* gene in mouse (Menon et al., 1995), human (Pekhletsky et al., 1997), sheep (O'Mahoney et al., 1994), goat (Maj et al., 2007), European bison (Flisikowski et al., 2007) and cattle (Heap et al., 1995). In *Bos taurus* and *Bos indicus*, the TG-repeat is located 90 bp upstream of the initiation codon and 64 bp upstream of the TATA sequence of the *GHR* gene (chromosome 20), and is polymorphic (Lucy et al.,1998). Alleles with 16-20 consecutive TGs commonly occur in *Bos taurus*, whereas the 11-TG-repeat allele is most common in *Bos indicus*.

Although microsatellite markers are usually considered just as neutral DNA markers, several studies indicated that microsatellites located in the promoter regions may affect gene activity (Sandaltzopoulos et al., 1995; Punt et al., 1990; Chen and Roxby, 1997). In many cases, number of repeats in a microsatellite appears to be a key factor for gene expression and expression level (Liu et al., 2000). These findings suggest that variations of TG-repeats in the promoter region of bovine *GHR* gene would be of interest because of GHR

role in mammary development.

Based on above consideration, the aims of this study were to estimate the genotype frequencies of TG-repeats in the 5'-flanking region of bovine *GHR*, and to evaluate their influence on milk production traits and somatic cell count in Holstein cattle of Iran.

Materials and methods

To study the possible association between different genotypes of *GHR* TG-repeats with milk production traits and somatic cell count (SCC), data recorded on 601 Holstein cows at single farm (Qyam) were used. Genomic DNA was extracted from 200 μl peripheral blood samples, collected in tubes in the presence of EDTA, by AccuPrep® Genomic DNA extraction kit.

For genotyping of the TG-repeat, two new primers (Forward: 5'-gcaatgcgttgtgtgctcta-3' and Reverse: 5'-tggttcctccaggctttatg-3' were designed from the *GHR* sequence (GenBank Accession No. U15731,2), using Primer3 Software (http://frodo.wi.mit.edu/primer3/).

The 324 bp of P1 promoter region (-159 to +165) of the *GHR* gene was amplified using the following polymerase chain reaction (PCR) conditions: one 5 min denaturing cycle at 95 °C, 35 cycles of 95 °C for 45 s, 63 °C for 30 s and 72 °C for 30 s, and one elongation cycle at 72 °C for 7 min. Twenty five ml of PCR mixture was carried out 0.2 ml PCR tubes, using a PCR kit with the lyophilized components. Each tube contained 1.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 20 pmol of each primer and 50 ng genomic DNA.

The SSCP analysis parameters, including the amount of PCR product, acrylamide concentration (8-10%) and the presence or absence of glycerol were optimized for this fragment. For SSCP analysis, 4 μl of each amplification product was added to 8 μl of stop solution (95% formamide, 10mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue). The samples were heat-denatured at 95 °C for 10 min, chilled on ice for 10 min, and the total volume was loaded onto 8% polyacrylamide gel (38:1 acrylamide /bisacrylamide).

Electrophoresis was carried out at room temperature in 0.5X TBE buffer for 20 h. The gels were subsequently fixed in 10% acetic acid, stained with 0.15% $AgNO_3$ and revealed with 1.5% Na_2CO_3.

Statistical analysis

Frequencies of genotypes, alleles and Hardy-Weinberg test were computed using TFPGA version 1.3. The data on milk production traits and somatic cell count were analyzed using the PROC MIXED (SAS Institute Inc. 2001). To test the association between the TG-repeats and the traits, the following model was fitted to the phenotypic data:
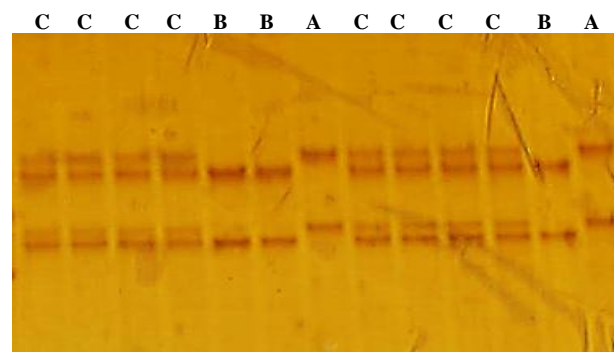
$$Y_{ijklm} = \mu + G_i + S_j + L_k + Si_l + Di_m + e_{ijklm} \qquad (1)$$

where: Y_{ijklm}: Phenotypic value of the milk production traits and somatic cell count of the animal k with a genotype I, μ: overall mean, G_i: fixed effect of the TG genotype, S_j : fixed effect of season, L_k : fixed effect of lactation, Si_l: random effect of sire, Di_m: random effect of dam and e_{ijklm} : random error

Traits analyzed with this model were average of daily milk yield during lactation, fat percentage, protein percentage and SCC. The distribution frequency of SCC values was highly skewed; therefore, the SCC values (expressed in thousands) were transformed to the natural logarithm scale (Shooke, 1993), and converted to somatic cell score (SCS).
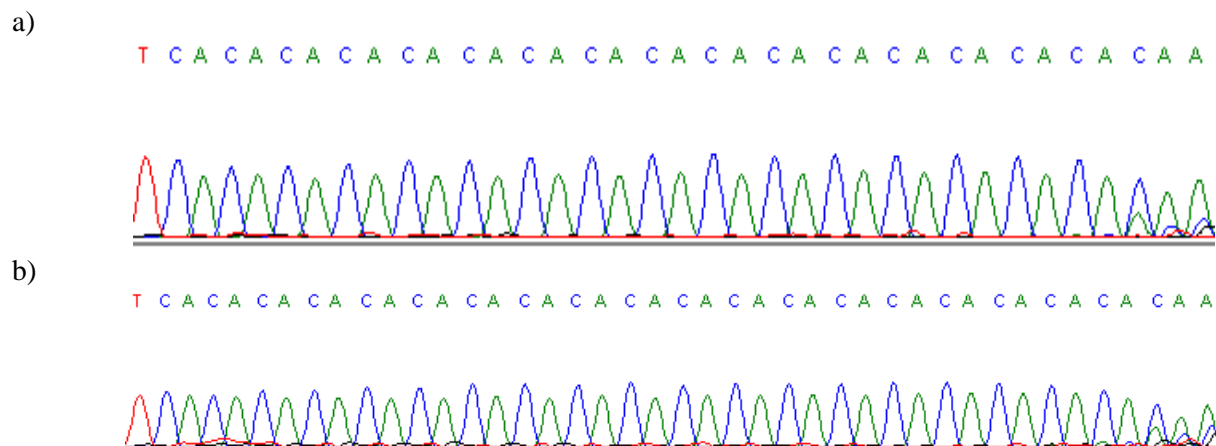
Results

Three different SSCP patterns, A, B and C (Figure 1), were observed for the amplified fragment of 5'-flanking region of bovine *GHR* gene in 518 animals. The samples revealing other SSCP patterns were excluded from further analysis. Sequence analysis of different SSCP patterns revealed that the TG-repeats contained only two alleles differing by 4 repeat units (Figure 2). According to sequence analysis, the SSCP patterns of A, B and C equaled to homozygous for 20-TG-repeats ($TG_{20/20}$), homozygous for 17-TG-repeats ($TG_{17/17}$) and heterozygous ($TG_{17/20}$), respectively.

C C C C B B A C C C C B A

Figure 1. Different SSCP patterns of amplified fragment at the 5'-flanking region of *GHR* gene (A = $TG_{20/20}$, B = $TG_{17/17}$, C = $TG_{17/20}$).

Genotypes of TG-repeats in the 5'-flanking region of *GHR* had a significant effect on the average daily milk yield ($P < 0.05$) and tended to associate with fat ($P = 0.058$) and protein percentage ($P = 0.089$). No significant association was found between these geno-

a)

b)

Figure 2. Sequence chromatographs of TG-repeats (CA-repeat) in the 5'-flanking region of *GHR* gene obtained by reverse primer. a) Short allele with 17-TG- repeats, b) Long allele with 20-TG-repeats

types and the SCC ($P = 0.245$). The $TG_{17/17}$ genotype had significantly lower average daily milk yield compared to $TG_{17/20}$ and $TG_{20/20}$ genotypes. The short allele (TG_{17}) had negative effect on milk yield and positive effect on milk fat and protein percentage (Table 1). The expected genotypic frequencies were similar to the observed ones, suggesting that genotypic distributions were at Hardy-Weinberg equilibrium (Table 2).

Discussion

Many studies have already reported on the association between length variation in TG-microsatellite of *GHR* gene and performance traits in livestock. For example, the short allele (TG_{11}), which was identified in *Bos taurus* and *Bos indicus* cattle, was associated with growth traits in Angus steers (Hale et al., 2000). Angus steers having the short length of TG-repeats had lower weaning weight, carcass weight, and marbling scores in comparison with steers with the longer allele. Similar results were observed in *Bos indicus*-influenced composite cattle (Curi et al., 2005) and in Japanese Black cattle (Ohkubo et al., 2006). In, addition, the TG-repeat polymorphism in the 5'-noncoding region of the bovine gene *GHR* was significantly associated with mean body live weight, daily live weight gain, cold carcass weight and weight of lean and fat in valuable cuts in Polish Black and White cattle. The longest allele with 21 TG repeats was superior for most carcass traits, but when growth rate was considered, the allele homozygote for 21-TG-repeats was inferior to other genotypes of TG-repeat (Maj et al., 2004). On the other hand, no associations were found between TG-repeat variants and milk production traits in Polish goat breeds (Maj et al., 2007). However the results of our study revealed that the TG-repeat polymorphisms were significantly associated with average daily milk

yield and tended to associate with fat and protein percentage.

Table 1. Genotypic frequencies, allelic frequencies and *P* value of Hardy-Weinberg equilibrium (HWE) for the different genotype of TG- repeats.

Genotype	Genotype frequency (%)	Number of Geno-types	Allele frequency (%)	*P* value of HWE
$TG_{20/20}$	0.21	126	0.475	0.61
$TG_{17/20}$	0.53	319		
$TG_{17/17}$	0.26	156	0.525	

Such associations would suggest the existence of a direct effect of the TG- microsatellite on traits by changing the expression of *GHR* gene. In general, microsatellites located in intragenic regions (promoters, 5'- and 3'-untranslated regions, and introns) can be important regulators of gene expression by influencing the transcription rate, RNA stability, splicing efficiency, and RNA-protein interactions. Several lines of evidence indicated that microsatellite length polymorphisms were involved in transcriptional regulation and, therefore, they are an important source of variation in quantitative traits (Kashi et al., 1997; King et al., 1997; Li et al., 2002). For example, variation in the number of CA-repeats causes differing levels of epidermal growth factor receptor gene expression and concomitant protein production (shorter alleles produced greater protein), which may have potential as a predictor of clinical outcome in human breast cancer (Brandt et al., 2006). In addition, variable expression of NadA, an outer membrane protein and adhesion of the pathogen *Neisseria meningitidis*, is mediated by changes in the number of TAAA repeats located upstream of the core promoter of *nadA* gene (Martin et al., 2005). Furthermore, GT- repeat polymorphisms in the promoter region of Tilapia prolactin 1 (*prl 1*) are associated with

Table 2. Least squares means (LSM) and standard errors (SE) for average daily milk yield, fat and protein percentage, and somatic cell score (SCS) referred to each SSCP pattern.

TG-genotype	Milk (kg)		Fat (%)		Protein (%)		SCS	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
TG$_{20/20}$	34.91[ab]	1.043	3.01[ab]	0.093	2.78[ab]	0.075	175.39[a]	65.287
TG$_{17/17}$	33.86[b]	1.008	3.12[a]	0.090	2.83[a]	0.073	242.19[a]	63.434
TG$_{17/20}$	35.35[a]	0.955	2.99[b]	0.087	2.73[b]	0.069	181.57[a]	58.089

differences in *prl* 1 gene expression and the growth response of salt-challenged fishes (Streelman and Kocher, 2002). Also, transcription rates were incrementally decreased by stepwise increases in the repeat number from 16 to 20 for a CA-microsatellite located near an enhancer element in intron 1 at the human epidermal growth factor receptor gene (Gebhardt et al., 1999). It has been reported that stepwise increases in the repeat number from 0 to 21 for a CA-microsatellite, located in the promoter region of the human matrix metalloproteinase-9 gene, produced incremental increases in transcription rates (Shimajiri et al., 1999).

The effect of variable number of TG-microsatellite in the 5'-flanking region of bovine *GHR* gene was studied using reporter gene *luc* constructs. No effect of TG-variants was shown, various numbers of TG-repeats had similar transcriptional activity (Zhou and Jiang, 2006). Maj and Zwierzchowski (2008) also confirmed these findings by Real-time PCR analysis on liver mRNA samples of Polish Black-and-White bulls with different number of TG-repeats.

Alternatively, the association of TG- genotypes in the 5'-flanking region of *GHR* gene could be explained by linkage disequilibrium with nearly located alleles that are directly responsible for milk production traits. Several quantitative trait loci (QTL) affecting milk production traits were reported on chromosome 20 (Arranz et al., 1998; Coppieters et al., 1998; Khatkar et al., 2004). An effort to fine map QTL on BTA20 in Holstein–Friesian cattle resulted in a relatively narrow region including the growth hormone receptor and prolactin receptor (*PRLR*) genes (Blott et al., 2003; Viitala et al., 2006). In these studies, *GHR* Phe279Tyr had the largest influence on milk yield, protein and fat percentages, while the effect of PRLR Ser18Tyr was markedly significant on protein and fat yields. These findings suggested the existence of an interaction between the two substitutions in explaining the QTL effects on milk production traits in BTA20.

Conclusions

The current results showed that the *GHR* TG-variants can be selected for genetic improvement of milk production traits in Holstein cattle. Although, variations of *GHR* TG-repeats are indicated to have similar transcriptional activity (Zhou and Jiang, 2005; Maj and Zwierzchowski, 2008), the present study provides evidence that in Holstein cattle QTL(s) segregate on the chromosomal region including *GHR* affect milk production traits on chromosome 20.

Acknowledgements

References

Argetsinger, L.S., Carter-Su, C., 1996. Growth hormone signaling mechanisms: involvement of the tyrosine kinase JAK2. *Hormone Research* 45, 22-24.

Arranz, J.J., Coppieters, W., Berzi, P., Cambisano, N., Grisart, B., Karim, L., Marcq, F., Moreau, L., Mezer, C., Riquet, J., Simon, P., Vanmanshoven, P., Wagenaar D., Georges M., 1998. A QTL affecting milk yield and composition maps to bovine chromosome 20: a confirmation. *Animal Genetics* 29, 107-115.

Blott, S., Kim, J.J., Moisio, S., Schmidt-Kuntzel, A., Cornet, A., Berzi, P., Cambisano, N., Ford, C., Grisart, B., Johnson, D., Karim, L., Simon, P., Snell, R., Spelman, R., Wong, J., Vilkki, J., Georges, M., Farnir, F., Coppieters, W., 2003. Molecular dissection of a quantitative trait locus: a phenylalanine-to-tyrosine substitution in the transmembrane domain of the bovine growth hormone receptor is associated with a major effect on milk yield and composition. *Genetics* 163, 253-266.

Brandt, B., Meyer-Staeckling, S., Schmidt, H., Agelopoulos, K., Buerger, H., 2006. Mechanisms of EGFR gene transcription modulation: relationship to cancer risk and therapy response. *Clinical Cancer Research* 12, 7252-7260.

Chen, Y., Roxby, R., 1997. Identification of a functional CT-element in the *Phytophthora infestans* piypt1 gene promoter. *Gene* 198, 159-164.

Coppieters, W., J. Riquet, J.J. Arranz, P. Berzi, N. Cambisa-

no, B. Grisart, L. Karim, F. Marcq, L. Moreau, C. Nezer, P. Simon, P. Vanmanshoven, D. Wagenaar, M. Georges., 1998. A QTL with major effect on milk yield and composition maps to bovine chromosome 14. *Mammalian Genome* 9, 540–544.

Curi, R.A., De Oliveria, H.N., Silveria, A.C., Lopes, C.R., 2005. Effects of polymorphic microsatellites in the regulatory region of IGF1 and *GHR* on growth and carcass traits in beef cattle. *Animal Genetics* 36, 58-62.

Feldman, M., Ruan, W., Cunningham, B.C., Well, J.A., Kleinberg, D.L., 1993. Evidence that growth hormone receptor mediates differentiation and development of mammary gland. *Endocrinology* 133, 1602-1608.

Flisikowski, K., Krasińska, M., Maj, A., Siadkowska, E., Szreder, T., Zwierzchowski, L., Żurkowski, M., 2007. Genetic polymorphism in selected gene *loci* in a sample of Białowieża population of European bison (*Bison bonasus*). *Animal Science Papers and Reports* 25, 221-230.

Gebhardt, F., Zanker, K. S., Brandt, B., 1999. Modulation of epidermal growth factor gene transcription by a polymorphic dinucleotide repeat in intron 1. *Journal of Biological Chemistry* 274, 13176-13180.

Hale, C.S., Herring, W.O., Shibuya, H., Lucy, M.C., Lubahn, D.B., Keisler, D.H., Johnson, G.S., 2000. Decreased growth in Angus steers with a short TG-microsatellite allele in the P1 promoter of the growth hormone receptor gene. *Journal of Animal Science* 78, 2099-2104.

Heap, D., Lucy, M.C., Collier, R.J., Boyd, C.K., Warren, W.C., 1995. Rapid communication: Nucleotide sequence of the promoter and first exon of the somatotropin receptor gene in cattle. *Journal of Animal Science* 73, 1529.

Jiang, H.L., Lucy, M.C., 2001, Variants of the 5'-untranslated region of the bovine growth hormone receptor mRNA: isolation, expression and effects on translational efficiency. *Gene* 265, 45–53.

Jiang, H., Okamura, C.S., Lucy, M.C., 1999. Isolation and characterization of a novel promoter for the bovine growth hormone receptor gene. *Journal of Biological Chemistry* 274, 7893-7900.

Kashi, Y., King, D.G., Soller, M., 1997. Simple sequence repeats as a source of quantitative genetic variation. *Trends in Genetics* 13, 74-78.

Khatkar, M.S., Thomson, P.C., Tammen, I., Raadsma, H.W., 2004. Quantitative trait loci mapping in dairy cattle: review and meta-analysis. *Genetics Selection Evolution* 36, 163-190.

King, D.G., Soller, M., Kashi, Y., 1997. Evolutionary tuning knobs. *Endeavour* (Camb.) 21, 36-40.

Li, Y. C., Korol, A.B., Fahima, T., Beiles, A., Nevo, E., 2002. Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Molecular Ecology* 11, 2453-2465.

Liu, L., Dybvig, K., Panangala, V.S., Van Santen, V.L., French, C.T., 2000. GAA trinucleotide repeat region regulates M9/pMGA gene expression in *Mycoplasma gallisepticum. Infection and Immunity* 68, 871-876.

Lucy, M.C., Johnson, G.S., Shibuya, H., Boyd, C.K., Herring, W.O., 1998. Rapid communication: Polymorphic (GT)n microsatellite in the bovine somatotropin receptor gene promoter. *Journal of Animal Science* 76, 2209-2210.

Maj, A., Zwierzchowski, L., 2008. Nucleotide sequence polymorphisms in the promoter region of bovine growth hormone receptor gene (*GHR*) have no effect on its expression level in liver. *Animal Science Papers and Reports* 26, 5-15

Maj, A., Korczak, M., Bagnicka, E., Zwierzchowski, L., Pierzchala, M., 2007. A TG-repeat polymorphism in the 5-noncoding region of the goat growth hormone receptor gene and search for its association with milk production traits. *Small Ruminant Research* 67, 279-284.

Maj, A., Korczak, M., Oprządek, J., Zwierzchowski, L., Dymnicki, E., 2004. TG-repeat length polymorphism in the 5'-noncoding region of the growth hormone receptor gene in cattle and its association with meat production traits. *Animal Science Papers and Reports* 22, 297-305

Martin, P., Makepeace, K., Hill, S.A., Hood, D.W., Moxon, E. R., 2005. Microsatellite instability regulates transcription factor binding and gene expression. *Proceedings of the National Academy of Sciences* 102, 3800-3804.

Menon, R.K., Stephan, D.A., Manbir, S., Morris, S. M., Zou, L., 1995. Cloning the promoter-regulatory region on the murine growth hormone receptor. *Journal of Biological Chemistry* 270, 8851-8859.

O'Mahoney, V.O., Brandon, M.R., Adams, T.E., 1994. Identification of a liver-specific promoter for the ovine growth hormone receptor. *Molecular and Cellular Endocrinology* 101, 129−139.

Ohkubo, T., Yano, H., Takahashi, S., Kimura, N., Tanaka, M., 2006. Bos indicus type of growth hormone receptor gene is retained in Japanese Black cattle. *Journal of Animal Breeding and Genetics* 123, 410–430.

Parmentier, I., Portetelle, D., Gengler, N., Prandi, A., Bertozzi, C., Vleurick, L., Gilson, R., Renaville, R., 1999. Candidate gene markers associated with somatotropic axis and milk selection. *Domestic Animal Endocrinology* 17, 139-148.

Pekhletsky, R.I., Chernov, B.K., Rubtsov, P.M., 1997. Variants of the 5′-untranslated sequence of human growth hormone receptor mRNA. *Molecular and Cellular Endocrinology* 90, 103-109.

Punt, P.J., Dingemanse, M.A., Kuyvenhoven, A., Soede, R.D.M., Pouwels P.H., Van den Hondel, C.A.M.J.J., 1990.

Functional elements in the promoter region of the *Aspergillus nidulans* gpdA gene coding glyceradehyde-3-phosphate dehydrogenase. *Gene* 93, 101-109.

Sandaltzopoulos, R., Mitchelmore, C., Bonte, E., Wall, G., Becker, P.B., 1995. Dual regulation of the *Drosophila hsp26* promoter *in vitro*. *Nucleic Acids Research* 23, 2479-2487.

Shimajiri, S., Nobuyuki, N., Tanimoto, A., Murata, Y., Hamada, T., Wang, K-Y., Sasaguri, Y., 1999. Shortened microsatellite d(CA)21 sequence down-regulates promoter activity of matrix metaloproteinase 9 gene. *Federation of the European Biochemical Societies (FEBS) Letters* 455, 70-74.

Shook, G. E., 1993. Genetic improvement of mastitis through selection on somatic cell count. *Veterinary Clinics of North America: Food Animal Practice* 9, 563–581.

Streelman, J.T., Kocher, T.D., 2002. Microsatellite variation associated with prolactin expression and growth of salt challenged tilapia. *Physiological Genomics* 9, 1–4.

Viitala, S., Szyda, J., Blott, S., Schulman, N., Lidauer, M., Mäki –Tanila, A., Georges, M., Vilkki, J., 2006. The role of the bovine growth hormone receptor and prolactin receptor genes in milk, fat and protein production in Finnish Ayrshire dairy cattle. *Genetics* 173, 2151-2164.

Zhou, Y., Jiang, H., 2006. A milk trait-associated polymorphism in bovine growth hormone receptor gene does not affect receptor signaling. *Journal of Dairy Science* 89, 1761-1764.