Preliminary Investigation on Activin-A as a Candidate Gene Affecting Litter Size in Goats

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Abstract.- The objective of this study was to estimate heritability and genetic correlations of activin-A gene with litter size in Boer and Matou goat, two highly prolific meat goat breeds. The genetic polymorphisms of two exons of the gene were detected and selectively genotyped by both PCR-SSCP-sequencing approach and PCR-RFLP method in the total 380 ewes with litter size records. Our results showed the presence of genetic variations, which consisted of 12 nucleotides changes in the candidate gene. Three of twelve loci, -446C>T, 651A>G and 946A>C were found to be associated with litter size within the second parity in three populations studied. In Boer and Matou, we observed two mutation forms for several pair loci and linkage disequilibrium was found within the gene among two populations. Some genetic implications were gained through polymorphic comparison within the populations. For litter traits, the estimates of genetic and phenotypic correlations were positive and particularly high for genetic correlations. Therefore, we considered activin A as a positional candidate affecting litter size and evaluated their sequence for potential causative genetic variation.

Key words: Activin-A, litter size, Boer and Matou goats, PCR-SSCP, PCR-RFLP, linkage disequilibrium.

INTRODUCTION

Although the mechanisms regulating the activation of primordial follicles and growth of primary and secondary follicles are not completely understood, accumulating evidence indicates that these processes are locally regulated by various paracrine and autocrine factors, among which are activins, growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 (BMP-15) and Kit ligand (KL). For reviews see Findlay et al. (2002), Fortune (2003), and van den Hurk and Zhao (2005).

Activins are heterodimers ($\beta$A$\beta$B, activin AB) or two homodimers ($\beta$A$\beta$A, activin-A; $\beta$B$\beta$B, activin B) of the $\beta$ subunits and so is able to neutralize the activin activity of activin A in the ovary has been most extensively studied. Follistatin is structurally unrelated to the activins, but binds with high affinity to the $\beta$ subunits and so is able to neutralize the activities of activin in a variety of target tissues, including the ovary (Phillips and de Krester, 1998).

Knowledge on genetic parameters and heritability are crucial for the genetic evaluation and for choosing the best selection schemes. Gonads are the main source of activin and related proteins, which contribute to the endocrine regulation of the reproductive system. By inhibiting follicle stimulating hormone (FSH) release without altering leuteinizing hormone (LH) release, activin and inhibin genes may partly be responsible for the differential release of LH and FSH from the pituitary (Stachowiak et al., 2007). Inhibin (INH) related proteins regulate Leydig Cell function (Risbridger et al., 1996), and overexpression of activin gene type II leads to disruption of the normal INH to activin ratio, which leads to reproductive deficiencies. INH and activin-A therefore act to
regulate FSH secretion and are essential for normal gonadal function (Cho et al., 2001).

Activin-A is one of five main groups within the transforming growth factor beta (TGF\(\beta\)) superfamily (Burt and Law, 1994), a group of multifunctional growth and differentiation factors. Recently, studies in mutant sheep and mice have shown that members of the superfamily and their related cell-surface receptors are important intraovarian regulators of ovarian follicular development and/or of ovulation rate (McNatty et al., 2001). Several post-translational processed forms of the alpha-subunit precursor protein could modulate FSH binding to its receptor as well as its biological activity (Schneyer et al., 1991). Activin-A subunit biosynthesis is associated with normal oocyte and follicle maturation, but excessive alpha-activin-A is associated with poor embryo quality in human (Fujiwara et al., 2000). On the other hand, domestic and laboratory animals have been immunized against a variety of activin-A preparations and an increase in ovulation rate has been reported (Medan et al., 2003).

The important role of activin-A gene in reproduction makes it a strong candidate gene for multi-births in mammals; sheep (Hiendleder et al., 1996), human (Montgomery et al., 2000) and pig (Kim et al., 2006). Therefore, we considered activin-A as positional candidate gene and evaluated its sequence for potential causative genetic variation and identified multi-loci within the gene as a putative causative gene for litter size within two unrelated goat populations.

**MATERIALS AND METHODS**

**Samples and breed**

Two highly fertile meat goat breeds, Matou and Boer, were chosen in present study because of a reported kidding rate about 196% (Jiang, 1985), and 192.5% (Tu, 1989), respectively, and a known largest litter size of 5 and 6 (shown in pedigree information of populations sampled) for the former breeds. For two native goats, Boer located in HuBei, and Matou in Hubei and Hunan province (South China), particularly in Shiyan city, Engxi County, Hubei.

Preliminary blood samples with performance records were collected from 209 Boer ewes sharing common rearing conditions sampled from HuBei province 53 Matou and 63 Boer ewes from small flocks with less than 20 in Hubei province. DNA was extracted from leucocytes according to Sambrook et al. (1989) by using phenol/chloroform extraction and ethanol precipitation.

**Polymorphism detection**

Primers were designed in exons 1 and 2 to amplify fragments of the entire activin-A gene based on the combination of cattle and sheep sequence of the ACN gene. The general linear model (GLM) procedure of SAS (Version V 8.1) was used to estimate the effects of each genotyping markers on the studied trait for the first two parties within populations. In addition, the possible linkage disequilibrium and haplotype within the gene were assessed. Single strand conformation polymorphism of polymerase chain reaction products (PCR-SSCP) method was used to screen all potential polymorphisms in exon 1 and exon 2 of activin-A gene. The summary characterizations for positions, optimal PCR conditions and empirical SSCP conditions for each used primer are presented in Table I.

For SSCP analysis, 3 µL of PCR product was mixed with 10 µl formamide dye (95% w/v formamide, 0.025% w/v bromphenol blue, 0.025% w/v xylene cyanol FF, 10 mM EDTA), denatured at 95°C for 10min and immediately chilled on ice. Samples were run at 125 V and 10°C on a nondenaturing polyacrylamide gels (gel size: 20 mm×17 mm×0.1 mm) for 16-30 h depending on the size of the PCR product analyzed and the gel composition used. Bands were visualized by silver staining. Then, all bands showing different patterns on SSCP gels were selected for sequencing directly on both strands.

**Evaluating polymorphism**

In order to balance the desire to maximize the chance of finding the putative mutation with the desire to minimize both cost and effort, polymorphisms were evaluated and prioritized for genotyping on the basis of whether they are likely to affect gene function (Tabor et al., 2002). First, alignment of multiple sequences was performed
ACTIVIN - A GENE AFFECTS LITTER SIZE

Table I. The characterizations and experimental conditions for each primer used in PCR-SSCP analysis

<table>
<thead>
<tr>
<th>name</th>
<th>Sequence(5'-3')</th>
<th>Position in bovine sequence</th>
<th>PCR Mg²⁺, Ta</th>
<th>SSCP A:B:C</th>
</tr>
</thead>
<tbody>
<tr>
<td>P11</td>
<td>F: AGCTGCAATCCCCAAAAATA R: TGACCGAGATGGGACAGGA</td>
<td>promoter (-642…-361)</td>
<td>1.5mM 65°C</td>
<td>29:1 10%</td>
</tr>
<tr>
<td>P12</td>
<td>F: CTCCTGTCCCCATCTGTGTC R: ACCACCGCCCTCTCTAC</td>
<td>promoter (-381…-97)</td>
<td>1.5mM 64°C</td>
<td>10% 8%</td>
</tr>
<tr>
<td>P13</td>
<td>F: GGTTGAGAAGGGGTAGAAG R: CAGCCGAAAAAGGATGG</td>
<td>cds in exon 1 (-128…259)</td>
<td>1.5mM 61°C</td>
<td>29:1 8%</td>
</tr>
<tr>
<td>P21</td>
<td>F: GCCTTGCTCCCTCTGTTCCCT R: GGTTGGCGACCA CTAC</td>
<td>cds in exon 2 (272…604)</td>
<td>1.5mM 64°C</td>
<td>39:1 10%</td>
</tr>
<tr>
<td>P22</td>
<td>F: GGCGGGATGAGCCAGATG R: GCCGGGAGCAAGCAACA G</td>
<td>cds in exon 2 (576…1054)</td>
<td>1.5mM 63°C</td>
<td>29:1 8%</td>
</tr>
<tr>
<td>P23</td>
<td>F: GTGCCACCCTACCCCTTTCC R: TCCTCAGGCTGCTT TTAT</td>
<td>cds in exon 2 (910…1119)</td>
<td>1.5mM 62°C</td>
<td>39:1 10%</td>
</tr>
</tbody>
</table>

*a* Numbering, starting at the ATG start site, accords to bovine sequence of the gene without consideration of its intron sequence; *cds*: coding sequence.

*Abb* A:B: acrylamide: bisacrylamide; C: concentration of gel.

using the Clusta IW software (http://www.ebi.ac.uk/clustalw) for comparative information on gene structure, since sequences for a particular sequence-specific function is conserved and hence homologous between species (Ungefroren *et al.*, 1994). Then, we took full advantage of the published characterizations for the gene structure (bovine: Forage *et al.*, 1986; Ungefroren *et al.*, 1994; Robert *et al.*, 2006; human: Debieve and Thomas, 2002) and two predicting tools available on internet, AliBaba2 (Grabe, 2002; http://www.gene-regulation.com/pub/programs.html) and ScanProsite (http://www.expasy.org/tools/scanprosite/), to predict potential functions for all identified polymorphisms except synonymous ones.

Genotyping

We empirically first attempted to genotype all polymorphisms found (if any) for Boer and Matou population samples and then selectively genotyped the other two populations based on the previous assessed priority and the identified minor allele frequency (MAF) in the former populations, which at low risk SNPs with MAF <0.05 were excluded from further analyses, according to Risch (2000).

For relatively lower rate of genotyping error, conventional restriction fragment length polymorphism (RFLP) analysis among the available single nucleotide polymorphism (SNP) genotyping methods was selected to genotype the identified polymorphisms. When a mutation could not create or destroy restriction cutting sequences as all current restriction enzymes suitable for cut are relatively expensive, forced restriction fragment length polymorphism PCR (Forced-RFLP, Wilson *et al.*, 2001) analysis was used. Here we would like to point out, to our experience, that the primer for Forced-RFLP was often unacceptable for PCR so that it often failed to amplify and when the PCR product was not perfect (low and often together with considerable non-specific products), using of nested PCR to take the pool primer pair as nested primer (inner) was very effective but at low fidelity and therefore unfeasible.

After the PCR products were digested by restriction enzymes, the resulting products were size-separated electrophoretically on nondenaturing polyacrylamide gels (8%) and visualized by silver staining.

Statistical analysis

Chi-square test for the homogeneity of genotype distribution was carried out using the same software among populations. The litter size was analyzed using a fixed model,

\[ y_{ijk} = \mu + \alpha_i + \beta_j + \epsilon_{ijk} \]
yijk, the recorded value of traits; μ, the population average; αi, the fixed effect of genotype; βj, the fixed effect of parity; εijk, the random residual effect within populations.

To find significant explanatory genotype effects, preliminary least square analyses (Zhang and Zhang, 1993) were conducted for the trait by GLM procedure followed by Duncan’s test of SAS statistical package (SAS v8.1). When significant effects were found, additive (a) and dominance (d) effects were estimated. Chi-square statistics was conducted for each locus within and between populations respectively for Hardy-Weinberg equilibrium (H-W test) and homogeneity of genotypic frequencies. When a significant chi-square was found, the polymorphism was more likely theoretically to be affected by natural selected for the farmer test, or the distribution for polymorphism analyzed was significantly associated with populations for the latter test.

Considering a high possibility of introducing bias into least square means within parity result from a larger fluctuation between contiguous parities within Haimen (a well known phenomenon for quantitative trait where mean and variance are linked, according to Sancristobal-Gaudy et al. (2001), linkage disequilibrium test between the observed genotypes and putative genotypes was taken as an additional approach of evaluating association using Haploview (V 3.32) program (Barrett et al., 2005). For the putative genotypes, ewes were assigned respectively the FecF/Fec+ genotype if they have at least one litter size record of three but no records of more than three but were assigned the FecF/FecF genotype if they have at least one litter size record of four or more, as described by Davis et al. (2002). If linkage disequilibrium was shown, the polymorphism (marker) was most likely associated with the trait. This inferring approach may be fallible and biased for mutations with little but causal effect on trait because bias and misclassification are more likely to obscure small-to-moderate relative risks than larger relative risks (Tabor et al., 2002).

Additionally, the same program was used to assess the population-based linkage disequilibrium for pairs of polymorphisms with MAF 5% (Du et al., 2007) within the gene for optimizing polymorphism selection and information about possible haplotype for analysis (Lewontin, 1988). Combination of genotypes for loci with MAF 5% (Risch, 2000) was treated as a alternative “genotype” to single locus for association analysis (5% is set as minimum population frequency for analysis by rule of thumb) (Tabor et al., 2002), we used the numerals 1- 3 for simplicity, to represent genotypes for each locus where 1 is homozygote for “wild-type” allele (predominant allele in Boer goat population sample), 2 is homozygote for “mutate-type” allele and 3 is heterozygote.

RESULTS

PCR-SSCP and sequencing

PCR-SSCP results from 6 pairs of primers showed different patterns on SSCP gels respectively. Sequencing of PCR products with different patterns from Boer and Matou revealed six polymorphisms within exon 1 and six in exon 2 (Table II). Any one of those polymorphisms was confirmed within the other two populations after it was detected in the subsequent selective genotyping analysis.

Assessing polymorphism

The majority of two exons sequences (flanking primers excluded) of goat activin-A gene were obtained and submitted to GenBank. Based on the similarity with bovine sequence, the locus and type of variant for each detected polymorphism has been described in Table II. By sequence alignment analysis, some implications from several interesting sequence structure features were gained: First, Alignment of a 630- bp goat 5’noncoding sequence obtained with that of bovine (full -622bp) showed a strong conservation (95% homology). A striking difference between them is a 7 bp-insert at -354 (TGCTGGG) as an extended imperfect 10 bp-repeat at -344 (TGCTGGGTTG) containing another (TGGG)n repeat (belong to a known repeat family) in goat compared to bovine. Additionally, a potential (GGCT)3 repeat domain present in this location. Second, comparison of the same sequence with human promoter revealed two relative high conservative sequences at -630 to -361 (identities=81%) and -305 to -1 (identities=89%)
little similarity mainly due to an extended alternating TG sequence repeat in human (Debieve and Thomas, 2002). Also, the sequence including -446C>T transition, -453GGCTGGCCGGG444, is identical to two dispersed 10 bp-repeats in two non-corresponding regions of human, from -479 to -468 and –570 to –557. Third, 184Ala and 299Val deduced from two associated polymorphic sites are identical to the homologues of ovine and bovine, but reverse, Val and Ala respectively, to those of pig, human and rattus. Fourth, two non-synonymous substitutions, 299Val>Ala and 311Thr>Pro, within TGFβ domain (by similarity) of the gene which is high conservative among several mammal species are semi-conserved on the base of analysis by ClustalW software. Nonetheless, both “mutate-type” alleles for these two polymorphisms corresponded respectively to homologues of other mammal species even those in the same family (goat 299 Val>Ala vs. mouse 310Val and rattus 310Ala; goat 311Thr>Pro vs. sheep 311Pro), which appear to suggest that these two variants are unlikely to affect this gene function.

To our knowledge, none of these changes located in any published potential or identified functional site of the gene in mammals. The polymorphism at -65 closes to the possible AP-2-binding site at -66 based on the required consensus 5’-GSCCCDSS-3’ (Thompson et al., 1994) and a potential transcriptional start site at -64 by similarity with cattle sequence (Ungefroren et al., 1994). The predicting results showed several potential motives covering polymorphic sites for non-coding polymorphisms under the condition of default value for all parameters but nothing for non-synonymous ones (Table II). Then, all variants found here were assessed roughly for their risks in Table II.

Genotyping
We initially genotyped all except -522C>G polymorphisms found here for Boer and Matou populations and then selected five interesting of the 11 loci to determine the other two populations (15% MAF within one of populations; spanning exon 1 and exon 2, non-coding region and coding region, Table III). All the five selected polymorphisms found in the former two populations are found in the latter two populations. Eight of the eleven loci were genotyped by PCR-RFLP analysis and the other 3, -155C>T, 129G>A and 792T>C by Forced-RFLP analysis for which the restriction enzymes were used in Table III, respectively. The primers with corresponding PCR conditions used for the latter analysis, respectively, are

5’-TCAGGGGCAACTCGACcG-3’ and 5’-CCCAAGGCAATCCAGGACAAG-3’ with 1.5mM, 57°C for -155C>T,
5’-GGGTAGAGGAGGCTAGAAG-3’ and 5’-ACCTTCCCAGTCGCGG-3’ with 1.5mM, 58°C for 129G>A, and
5’-TGCGTTGCTCCTCTGTTCCT-3’ and 5’-CCAGCCCAGCtCTGGA-3’

Table II.- The assessments for each identified polymorphism.

<table>
<thead>
<tr>
<th>Polymorphism*</th>
<th>Type of variant</th>
<th>Predicted functional effect</th>
<th>Risk of phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>-522C&gt;G</td>
<td>Regulatory region</td>
<td>SP 1, WT 1, ETF</td>
<td>Low</td>
</tr>
<tr>
<td>-506&gt;G</td>
<td>Regulatory region; insertion</td>
<td>SP 1, AP-2, E 1, MyoD, MZF b</td>
<td>Low</td>
</tr>
<tr>
<td>-446C&gt;T</td>
<td>Regulatory region</td>
<td>SP 1, NF-1</td>
<td>Low</td>
</tr>
<tr>
<td>-155C&gt;T</td>
<td>Regulatory region</td>
<td>AP-2, SP 1</td>
<td>Low</td>
</tr>
<tr>
<td>-65G&gt;C</td>
<td>5’UTR</td>
<td>SP 1, NF-1</td>
<td>Low</td>
</tr>
<tr>
<td>129G&gt;A</td>
<td>Synonymous</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>567G&gt;A</td>
<td>Synonymous</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>651A&gt;G</td>
<td>Synonymous</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>792T&gt;C</td>
<td>Synonymous</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>906C&gt;T</td>
<td>Synonymous</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>911T&gt;C</td>
<td>Non-synonymous (semi-conserved)</td>
<td>299Val&gt;Ala</td>
<td>High</td>
</tr>
<tr>
<td>946A&gt;C</td>
<td>Non-synonymous (semi-conserved)</td>
<td>311Thr&gt;Pro</td>
<td>High</td>
</tr>
</tbody>
</table>

*aNumbering for non-coding polymorphisms bases on the goat sequence obtained (GenBank: bankii907831) while for coding ones corresponding to the bovine full coding sequence (GenBank: M13273), both starting at the ATG start site.

bThe consensus sequence for MZF 1, NGNGGGGA, was taken from Debieve & Thomas (2002).
with 1.5 mM, 62°C for 792 T>C (the deliberately introduced point mutations are shown in lower-case letters). Observations of genotypes for each genotyping marker within four goat populations were given in Table III.

Statistics analysis

The allele and genotypic frequency and chi-square values are not shown here, because it is easy to calculate them by simple formula based on the available observational genotypes (Table III). Chi-square statistics were not significant (P>0.05 for H-W test) for all but four genotyping loci (Table III) within populations and significant (P<0.05 for distribution for polymorphism) within all loci except -506>G, -155>C>T and 651A>G among populations analyzed (the loci with zeros in the observational cells of the contingency table was excluded from this analysis). The significant differences were found between LSMs for litter size of two of genotypes within only three of all loci genotyped within populations (P<0.05, P=0.01) (Table IV, data for other loci not shown).

A common perfect linkage disequilibrium between 567G>A and 911T>C was seen within and among two breed population samples. Quantifying for linkage disequilibrium between polymorphisms revealed that polymorphism -506>G was in linkage disequilibrium with -65G>C within Boer (r²=0.70) and Matou (r²=0.54). On the other hand, with the restriction of either MAF 10% or frequency of genotype “2” 5 %, only -506>G, -446C>T, -65G>C, 567G>A, 651A>G and 911T>C genetic variations within Boer and all but -155C>T and 792T>C genotyping loci within Matou were further characterized for distribution of possible hypotypos. A common poverty (less than total three times each) even absence (none unexpectedly) of 3 certain 2-marker combination genotypes, 22, 23 and 32, for many (40-50 %) of the previous pairs of loci was found within Boer and Matou samples. Such poverty suggested that the haplotype frameworks with co-occurrence of two “mutate-type” alleles are lack for those pairs in whose populations.

No significant difference (P>0.05) for the analysis trait was found between the five 4-marker combinations (with population frequency 5%) of genotype for -506>G, -446C>T, 567G>A and 651A>G polymorphisms within Boer, 1111 (20%), 1111 (9%), 1333 (6%), 2111 (7%) and 3111 (15%), by least square analysis. However, a correlation analysis of the genotype with percentages of ewes with at least one litter record > 2 indicated 1113 (44%) and 3111 (38%) differ (Fisher’s Exact Test, P<0.05) from 1111 (11%), respectively. The same analyses were not done within the other three populations because of its limited sample size.

DISCUSSION

Association study

Activin gene is a gonadal glycoprotein that binds to the activin I and activin II subunits to form heterodimer complexes known as Activin I (activin-A) or activin II (activin- B) respectively; it also regulates the secretion of pituitary FSH. Many studies have evaluated genetic parameters for growth traits in sheep (Tosh and Kemp, 1994, Notter, 1998, Mousa et al., 1999; Neser et al., 2001), but there are few studies describing genetic correlation between growth and reproduction traits for goat breeds.

There are many publications describing fecundity genes in some sheep flocks (McNatty et al., 2001; Montgomery et al., 2001; George, 2005). However, fewer studies for the same goal in goat have been available in literature although high reproductive goat breeds, empirically, as prolific sheep breeds are substantially larger than many general ones in mean litter size. The reason may be owing to the goat are now less economically important than cattle, sheep, and pig (Luikart et al., 2001). Without any previous reports for QTL, we studied goat fecundity trait through candidate gene approach. This approach has been argued for: first, non-replication of results (summarized by both Tabor et al., 2002; Leopoldo, 2005); second, limitations of the ability to include all possible causative genes and polymorphisms (summarized by Tabor et al., 2002); third, insufficient current knowledge (summarized by Tabor et al., 2002) and polygenic nature of most traits in animals (possible epistasis).

Nevertheless, Leopoldo (2005) concluded that meta-analysis could be considered as helpful.
analytical tool to synthesize and discuss livestock.

Table III.- Distribution of genotype for each genotyping marker within two goat populations.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Population</th>
<th>Boer (N=209)</th>
<th>Matou (N=53)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RE</td>
<td>WW</td>
<td>WM</td>
</tr>
<tr>
<td>-506&gt;G</td>
<td>Eco88</td>
<td>129</td>
<td>66</td>
</tr>
<tr>
<td>-446C&gt;T</td>
<td>Hae</td>
<td>128</td>
<td>60</td>
</tr>
<tr>
<td>-155C&gt;T</td>
<td>Sfr303</td>
<td>196</td>
<td>12</td>
</tr>
<tr>
<td>-65G&gt;C</td>
<td>BseL</td>
<td>140</td>
<td>62</td>
</tr>
<tr>
<td>129G&gt;A</td>
<td>MroN</td>
<td>179</td>
<td>26</td>
</tr>
<tr>
<td>567G&gt;A</td>
<td>Bsp143</td>
<td>157</td>
<td>46</td>
</tr>
<tr>
<td>651A&gt;G</td>
<td>Van91</td>
<td>116</td>
<td>57</td>
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<tr>
<td>792T&gt;C</td>
<td>Xag</td>
<td>190</td>
<td>13</td>
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<tr>
<td>906C&gt;T</td>
<td>Eco88</td>
<td>207</td>
<td>2</td>
</tr>
<tr>
<td>911T&gt;C</td>
<td>Bsp143</td>
<td>157</td>
<td>46</td>
</tr>
<tr>
<td>946A&gt;C</td>
<td>Ban</td>
<td>203</td>
<td>6</td>
</tr>
</tbody>
</table>

*aRE, restriction enzyme; W, wild-type allele; M, mutant-type allele;  
bObservational cells for “MM” with superscripts * and ** means significant at P<0.05 and P<0.01, respectively for H-W test for the locus (line) within the population (row).

Table IV.- Least square means (LSM), standard error (SE) and allele substitution effects for the litter size of the second parity at preliminary useful loci.

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>locus</th>
<th>WW LSM±SE</th>
<th>WM LSM±SE</th>
<th>MM LSM±SE</th>
<th>Additive effect</th>
<th>Dominant effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>WW</td>
<td>WM</td>
<td>MM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boer</td>
<td>209</td>
<td>651A&lt;G</td>
<td>1.80±0.07a</td>
<td>2.02±0.09</td>
<td>1.75±0.13b</td>
<td>-0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>Matou</td>
<td>53</td>
<td>946A&gt;C</td>
<td>1.54±0.14b</td>
<td>2.20±0.15b</td>
<td>2.00±0.47</td>
<td>0.23</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*aMeans in the same line with different superscripts in capital and small letters significantly differ at P<0.05 and P<0.01, respectively.

For evaluation of non-coding polymorphism in present study, we attempt to take advantage of motif-discovery tool available on the internet for possible regulatory element including the polymorphic site. An example for supporting our method of non-coding polymorphism-prioritizing (see method part) is that the promoter activity and expression of human MSH6 gene is altered by polymorphisms identified in binding site for transcription factor SP1 (Gazzoli and Kolodner 2003). Another two cases, however, indicated that it is fallible: despite each of polymorphism determined functionally inactivated a different SP1 site, no evidence for an association of the LEP promoter genotype with the analyzed fatness traits (Stachowiak et al., 2007); and despite association of activin and inhibin promoter polymorphisms at -16 with premature ovarian failure, no association was found in the difference of promoter activity of a luciferase reporter and the phenotype (Harris et al., 2005). Furthermore, Tompa et al. (2005) assessed 13 tools for prediction of regulatory elements and found that the absolute measures of correctness of these programs are low.

We suggested that 651A<G, -446C>T, both -446C<T and 946A>C within activin-A gene preliminarily associated with the little size in the second parity among the Boer and Matou populations, respectively. It appeared that there was a synergistic effect on increasing the largest litter size of ewe between heterozygote for -446C<T and heterozygote for 946A>C, because majority of individuals with both these heterozygote mutations...
have litter size records of four. It is thereby likely that polymorphism at -446 is a regulatory one in goat.

We also conducted combination genotypes test for association with the study trait. A binding studies of Burkart et al. (2005) might be account for the significance of combination for genotypes or haplotypes within regulatory region in association study, while mutation of the C/EBPβ binding site increased the basal activity of the activin promoter two fold and the incorporation of a second mutation at the CRE element at -117 (which dramatically reduced promoter activity) abolished this increasing in the basal promoter activity. We showed that combination of genotype and homozygotes for wide-types for -506<-G, -446C<T and 567G<A respectively and heterozygote for 651A<G (i.e., 1113) significantly associated with the proportion of individuals with at least one litter size record 3 in Boer population sample. This result was in coincidence with that from the least square analysis for only 651A<G.

We think that one polymorphism which was predicted to locate in (create or destroy) any transcription factor binding site, and therefore represent a putatively functional regulatory polymorphism, should have higher priority than predicted nonbinding polymorphisms in non-coding region. Importantly, our results suggested that association study through a candidate gene approach should be conducted for multi-loci within multi-populations.

Genetic variation analysis

In addition to genetic improvement for the interesting economic trait, breeding programs aimed to preserve biodiversity. We therefore further characterized the polymorphisms among populations for any evolutionary implication. The possible haplotypes and linkage disequilibriums within the candidate gene were evaluated considering that knowledge of the extent and range of LD in animal populations is extremely valuable in studying population history and characterizing/managing genetic resources and diversity (Du et al., 2007).

Not only the common features among populations were the common polymorphisms of which no one had been determined to be population-specific in present study, but also the common strong linkages disequilibrium within exon 1 and exon 2 of activin-A, respectively. This observation was supported by the findings of Luikart et al. (2001) who showed that phylogeographic structure was surprisingly weak among domestic goat populations. In addition, this result further was supported by the recent findings of Chen et al. (2006) who concluded that Matou was clustered closely with Boer into the same branch, based on cytochrome b gene sequence information.

Moreover, Boer and Matou shared the common poverty of haplotypes with co-occurrence of two “mutant” forms for some polymorphism pairs within and across two exons of activin gene. One possible explanation is that the selection operated on multiple loci in epistasis within the gene in those populations. Interestingly, these findings helped to determine the phasing which allele falls on heterozygous chromosome for those pair of polymorphisms and whether genotyping for them is right. On the other hand, some absent genotype combinations within Boer population were found within Matou population with sufficiently high frequencies. This finding indicated that direction and intensity of selection were different for the loci between the populations.

According to our results and study of Delegeane et al. (1987) and by sequence alignment analysis, some implications from several interesting sequence structure features were gained: First, Alignment of a 630-bp goat 5‘noncoding sequence obtained with that of bovine (full -622bp) showed a strong conservation (95% homology), which suggests that regulation of the promoter for express of the gene was homologous between goat and bovine. A striking difference between them is a 7 bp-insert at -354 (TGCTGGG) as an extended imperfect 10 bp-repeat at -344 (TGCTGGGTTG), containing another (TGGG)_n repeat (belong to a known repeat family) in goat compared to bovine; and such feature has a functional consequence in some instances. Furthermore, the ACN311Pro is non-polar as well as high conservative across other several animals including sheep, bovine, pig, human, mouse and ratus, while 311Thr is polar, which may affect the secondary structure of the
expression product in that region due to unique structure of proline which occurs in proteins frequently in turns or bends.

Another interesting feature was the conspicuous differences in MAF as well as in homogeneity of genotypic frequencies within loci across populations. Also, the sequence including -446C>T transition, -453GGCTGGCCGG-444, is identical to two dispersed 10 bp-repeats in two non-corresponding regions of human, from -479 to -468 and –570 to -557. This feature may be has an implication on the base of homologous between species (see evaluating polymorphisms).

The polymorphism 946A>C/311Thr>Pro within the activin gene TGFβ domain had sufficient and close MAF among Matou populations (20.8%), whereas was observed only six times as heterozygote (1.4 %) in 209 Boer goats. Nevertheless, there was a common significant poor homozygote for mutate form for this locus within the studied population (Table III, P<0.01 for H-W test). It is therefore high likely that this mutation has functional consequences considering its location and has been under directional selection with a disfavor for “mutate-type” homozygote in goat, and then has been homogenizing in Boer population.

Another minor allele, -446T, in Boer (24%), and Matou (48%) was in population sample. However, we could not simply concluded this association with these breeds due to either the different directions of selection or mutation for this locus among breeds because of a relatively high minor allele frequency and a limited population samples within populations, namely, some of this difference may stem from unrepresentative sampling for Nubian.

**CONCLUSIONS**

This study elucidated significant association of activin-A gene in two highly prolific meat goat breeds, Boer and Matou. These polymorphisms are marker for potentially linked polymorphisms with impact on protein structure and/or expression level. Polymorphisms of this candidate gene can be used as marker for improving goat breeding. However, further validation for the usefulness of these markers is necessary.

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**Conflict of interest**

The authors declare that there is no conflict of interest.

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