pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2015) Vol. 30, No. 2, pp. 91~97
http://dx.doi.org/10.12750/JET.2015.30.2.91

Amphiregulin (*AREG*) Genotypes, Allele Frequencies and the First Parity Litter Size in the Pig

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ABSTRACT

Amphiregulin (AREG), a glycoprotein that is a member of the epidermal growth factor (EGF) family, is expressed by the porcine conceptus and endometrium. *AREG* genotypes were determined based on an SNP in the intron 3 of the gene. Contradictory effects of *AREG* genotypes on reproductive traits in different pig breeds were reported previously. G allele had undesirable effect on reproductive trait in Meishan breed, while it had favorable effects in Polish Landrace and Large White. We determined *AREG* genotypes of 179 pigs including the Duroc, Landrace, Yorkshire, Korean native pig (KNP), and Meishan breeds. Two new SNPs were identified near the previously reported SNP in the intron 3 of *AREG*. Frequencies of *AREG* alleles among the Duroc, Landrace, Yorkshire, and KNP sows were significantly different (p<0.001), indicating association between *AREG* genotypes and pig breeds. The first parity litter size was significantly affected by the breeds (p=0.014), but not by *AREG* genotypes (p=0.148). However, there were breed and *AREG* genotype associated trends in the first parity litter size. The first parity litter size appeared to be higher in Duroc and KNP sows with G allele, while it appeared to be lower in Landrace sows with G allele. Significant variability of *AREG* alleles among pig breeds, for the first time in Duroc and KNP sows, was identified. *AREG* genotypes may influence reproductive traits differentially for each breed and thus, *AREG* genotypes may need to be considered when sows are bred to increase litter size.

(Key words : *amphiregulin*, *AREG*, genotype, allele frequency, litter size)

INTRODUCTION

Genes affecting growth rate of the conceptus and development of endometrium during pregnancy may influence the uterine capacity and litter size in pig. Amphiregulin (AREG) is a glycoprotein that is a member of the epidermal growth factor (EGF) family and it binds to EGF receptor with lower affinity than EGF (Shoyab *et al.,* 1989). The *amphiregulin* (*AREG*) gene maps within the uterine capacity quantitative trait locus (Kim *et al.,* 2002). *AREG* has been implicated in the various aspects of pig reproduction. For oocyte development, follicle stimulating hormone (FSH) stimulated expression of *AREG* in the cultured cumulus-oocyte complexes and the level of *AREG* expression was highest at 2 hr after FSH addition (Prochazka *et al.,* 2011). *AREG* significantly stimulated expression of cumulus expansion-related genes, though to the less extent than FSH. Regulation of cumulus expansion, *AREG*-induced meiotic resumption, and oocyte MI/MII transition is mediated via PI3K/AKT signaling (Prochazka *et al.,* 2012). For embryonic development, AREG promotes the proliferation of trophoblast cells during preimplantation development of porcine embryos *in vitro* (Lee *et al.,* 2009). *AREG* is expressed by the porcine conceptus (Kennedy *et al.,* 1993; Lee *et al.,* 2009).

In addition to its role in oocyte and embryo development, *AREG* may contribute to the fetal development due to its endometrial expression during early pregnancy. Endometrial expression of *AREG* is higher in the White composite gilts than Meishan gilts during early pregnancy and the differential ex-

* This study was supported in part by a grant from the Next-Generation BioGreen 21 Program (No. PJ008047), Rural Development Administration, Republic of Korea.
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pression may contribute to the conceptus growth rate and litter size among different breeds (Kim *et al.,* 2003). Uterine expression of *AREG* in the gilts or sows was the highest on day 20 (Kim *et al.,* 2003) or day 30 (Kim *et al.,* 2009) of pregnancy. Thus, *AREG* may influence the litter size by affecting oocyte, embryonic and fetal development, and with the controlled expression in the uterus.

AREG is consisted of 6 exons and 5 introns (Kim *et al.,* 2003). Two molecular weight forms of mature *amphiregulin*, which differ in glycosylation and in N-terminal peptide core length, have been reported in humans (Johnson *et al.,* 1993). Two mature forms of AREG are consisted of either 78 or 84 amino acids in humans (Johnson *et al.* 1993). The mature form of AREG in pigs is consisted of 84 amino acids (Kim *et al.,* 2003), which is encoded by exons 3 and 4. Mutations in the exons of *AREG* affecting reproductive traits of pig have not been reported. In addition to the different forms of mature AREG reported in humans, two different forms of *AREG* mRNAs were expressed in both Meishan and White composite gilt endometrium on day 30 of pregnancy (Kim *et al.,* 2003). The 112 bp difference between the two different forms of *AREG* mRNAs corresponds to the exon 5 of the *AREG*, which encodes for the cytoplasmic domain (Kim *et al.,* 2003). It is not known whether AREG precursor protein is present in the endometrium without the cytoplasmic domain.

AREG genotypes of A1A1, A1A2 and A2A2 were determined based on a C/T substitution in the intron 3 of the gene (Jiang *et al.,* 2002). The *AREG* polymorphism was close to fixation for alternative alleles in the Meishan with A1 allele and Large White (also described as Yorkshire) with A2 allele in their founders (King *et al.,* 2003). In contrast, the highest frequency was found for the A1A1 genotypes (0.72 and 0.63) and the lowest for the A2A2 genotypes (0.02 and 0.02) of the *AREG* genes in Polish Large White and Polish Landrace pigs, respectively (Mucha *et al.,* 2013). A Meishan allele (A1) of the *AREG* was associated with an undesirable effect of the number born in the first parity in comparison to a Large White allele (A2), having 1.65 (A1A1) and 2.06 (A1A2) piglets fewer born than A2A2 homozygotes in an F2 population of Meishan × Large White pigs (Jiang *et al.,* 2002). In contrast to the study of Jiang *et al.* (2002), the *AREG* genotype had a significant effect on both litter weight at piglet age of 21 days and litter weight at weaning among the pig breeds in Poland including Landrace, Large White, and Line 990, where the A1A1 genotype of *AREG* was the favorite one in comparison to A1A2 (Katska-Ksiazkiewicz *et al.,* 2006). In a recent study, the number of piglets born alive were significantly higher ($p<0.05$) with A1A1 genotype (12.76) than A1A2 genotype (12.46) in parities 2^{nd} to 4^{th} of Polish Large White and Polish Landrace pigs (Mucha *et al.,* 2013). In the first parity and in parities 5^{th} to 8^{th}, despite the lack of significant differences, sows of the A1A1 genotype had higher number of piglets born alive and higher number of piglets alive on 21^{st} day values compared to sows of the A1A2 and A2A2 genotypes (Mucha *et al.,* 2013). The data suggest that the *AREG* genotype may affect the reproductive traits of number born and litter weight differentially among different pig breeds. The objective of this study was to determine the *AREG* genotypes, allele frequencies and its association with the reproductive traits among the current populations of commercial pig breeds, including Duroc and Korean native pig (KNP), in Korea. *AREG* genotypes and allele frequencies of Duroc and KNP have not been reported previously.

MATERIALS AND METHODS

1. Experimental Animals and Primer Design

The experimental protocol and standard operating procedures on experimental animals were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science, RDA (Jeonju, Republic of Korea), in compliance with standard international regulations. Blood samples were collected and DNA was isolated from 179 pigs of 5 different breeds, including Duroc (n=40 sows), Landrace (n=45, including 40 sows and 5 boars), Yorkshire (n=46, including 43 sows and 3 boars), KNP (n=38, including 33 sows and 5 boars), and Meishan (n=10) at the National Institute of Animal Science (NIAS), Rural Development Administration (RDA), Republic of Korea. Two sets of primers were designed to amplify across the intron 3 of *AREG* based on the porcine *AREG* cDNA (GenBank accession No. NM_214376) (Kim *et al.,* 2003) and the *AREG* gene sequence in the Genome Browser for pig (http://genome.ucsc.edu/). PCR primer pairs were designed using either Primer3 online-tool or Vector NTI version 11.5 (Invitrogen, Carlsbad, CA). The first set of primers [the forward primer, pAmphi-intron3-F2 (659F): (5'-AGCACCTGGAAGCAGTAACC-3') and the reverse primer, pAmphi-intron3-R2 (723R): 5'-CTTTCCCCACATCGTTCAC-3')] were derived from exons 3 and 4 of the *AREG* gene, respectively (Kim *et al.,* 2003). The

expected 782 bp fragment was smaller than the previously investigated 899 bp fragment, amplified by a different set of primers (Jiang *et al.,* 2002), but it would provide similar allele pattern after the restriction enzyme *Sty*I digestion. The second set of primers, with the same forward primer, pAmphi-intron3-F2 (659F), located on exon 3, and a different reverse primer, pAmphi-intron3-RR1 (5'-GACCTAGAATATATTTGAGTCAAG-G-3'), located on intron 3, were to amplify a smaller 602 bp fragment, while providing more straightforward allele pattern after the restriction enzyme *Sty*I digestion than the previous report (Jiang *et al.,* 2002). The first set of primers was used at the beginning of the study and the amplification results were confirmed by using the second set of primers. Then the second set of primers was used for most of the studies.

2. PCR Amplification, SNP Identification and Genotyping

PCR reactions were carried out with each primer set in a 20 μl volume containing 50 ng genomic DNA, 1.5 mM $MgCl_2$, 10 pmol of each primer, 200 mM dNTP and 0.8 U *Taq* polymerase U Top-*Taq*™ DNA polymerase (Qiagen, Germany). Amplification was performed under the following PCR conditions: 10 min at 95℃; followed by 35 cycles of denaturation for 30 sec at 95℃, annealing for 30 sec at 60℃ and extension for 1 min at 72℃; and a final extension of 5 min at 72℃. By using the first set of primers, the 782 bp fragment was expected to contain a part of exon 3, intron 3, and a part of exon 4 of *AREG*. With the second set of primers, the 602 bp fragment was expected to contain a part of exon 3 and a part of the intron 3 of *AREG*. Both amplicons contained a *Sty*I restriction site that was present in all genotypes, which served as an internal control, and an additional *Sty*I restriction site that was specific to *AREG* genotypes. A number of pigs were sequenced to confirm the *AREG* genotypes of each breed. The genotypes of the *AREG* gene among the Duroc, Landrace, Yorkshire, KNP and Meishan pigs (n=179) were determined.

3. Data Analysis

The *AREG* sequence variability in the pig breeds and the association of the *AREG* alleles with the first litter size were investigated. The Chi-square analysis was conducted for the *AREG* sequence variability with the records from the Duroc, Landrace, Yorkshire, and KNP sows (n=156) using the MINITAB program (MINITAB Inc., USA). The records of first parity litter sizes of the Duroc, Landrace, Yorkshire, and KNP sows (n=113) were analyzed for estimating the effect of the *AREG* genotypes by a general linear model using the MINITAB program (MINITAB Inc, U.S.A). The model for analysis was as follows.

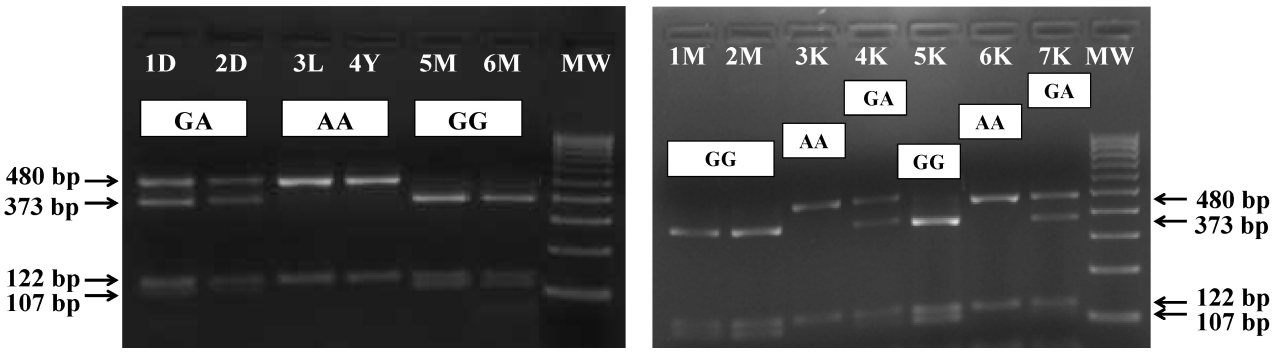
$$Y_{ijklm} = \mu + BREED_i + GENO(BREED)_{j(i)} + MONTH_k + YEAR_l + e_{iklm,}$$

where, Y_{ijklm} is the first parity litter size in the sows, μ is the overall mean for the trait, *BREEDi* is the effect of the *i*-th breed, *GENO(BREED)j(i)* is the effect of the *j*-th SNP genotype within the *i*-th breed, $MONTH_k$ is the effect of the *k*-th month of birth, $YEAR_l$ is the effect of the *l*-th year of birth, and *eiklm,* is the residual effect.

RESULTS

1. *AREG* Genotypes and Sequence Variation

AREG genotypes were described as A1A1, A1A2 and A2A2 according to the references (Jiang *et al.,* 2002; Katska-Ksiazkiewicz *et al.,* 2006; Mucha *et al.,* 2013) and in the introduction of the manuscript. They are now described as GG, GA and AA genotypes based on the SNP sequence. *AREG* alleles were described as A1 or A2 according to the references (Jiang *et al.,* 2002; King *et al.,* 2003) and in the introduction of the manuscript. They are now described as G or A alleles based on the SNP sequence. After *Sty*I digestion of PCR amplicons, GG, GA and AA genotypes of the *AREG* were identified among different breeds (Fig. 1A and 1B). This is the first report of *AREG* genotypes of Duroc and KNP breeds. Sequencing results revealed that the *AREG* polymorphism was rather a G/A substitution (Fig. 2). This substitution is at the nucleotide 525 of the 717 bp intron 3 of *AREG*, which is rs338575180 [A/G] (Chromosome 8, position 74573709). In addition to the previously reported SNP (Jiang *et al.,* 2002), two additional SNPs were identified among the different breeds (Fig. 2, bottom panel). The sequences, including the SNPs, from five breeds were deposited at the GenBank (GenBank accession no. KF541319～KF541326). The two newly characterized G/C and C/G SNPs, and the previously reported C/T and now clarified as G/A SNP are dispersed within the 29 bp span as noted in GA genotype (Fig. 2, bottom panel). The 3 SNPs are rs343652032, rs325615549, and rs338575180 in the dbSNP of NCBI, and they were submitted by two separate investigators before and after the deposition of sequences from



(A) (B)

Fig. 1. PCR-RFLP analysis of the *AREG* genotypes. (A) *AREG* genotypes of the two Duroc (D) sows in lanes 1 and 2, Landrace (L) and Yorkshire (Y) boars in lanes 3 and 4, and a Meishan (M) sow and a boar in lanes 5 and 6, are shown with the molecular weight (MW) marker in lane 8. Fragments of 602 bp amplified by PCR with pAmphi-intron3-F2 (659F) and pAmphi-intron3-RR1 primers were digested with the restriction enzyme *Sty*I digestion. GG genotype yielded 373 bp and 122 bp, 107 bp bands, whereas AA genotype yielded 480 bp and 122 bp bands. GA genotype yielded 480 bp, 373 bp, 122 bp and 107 bp bands. (B). PCR-RFLP analysis of the *AREG* genotypes of the two Meishan (M) sows in lanes 1 and 2, and 5 Korean native pig (K) sows in lanes 3 to 7 are shown with the MW marker in lane 8. Whereas the Meishan sows were fixed with GG genotype, the Korean native pig sows had GG, GA or AA genotypes.

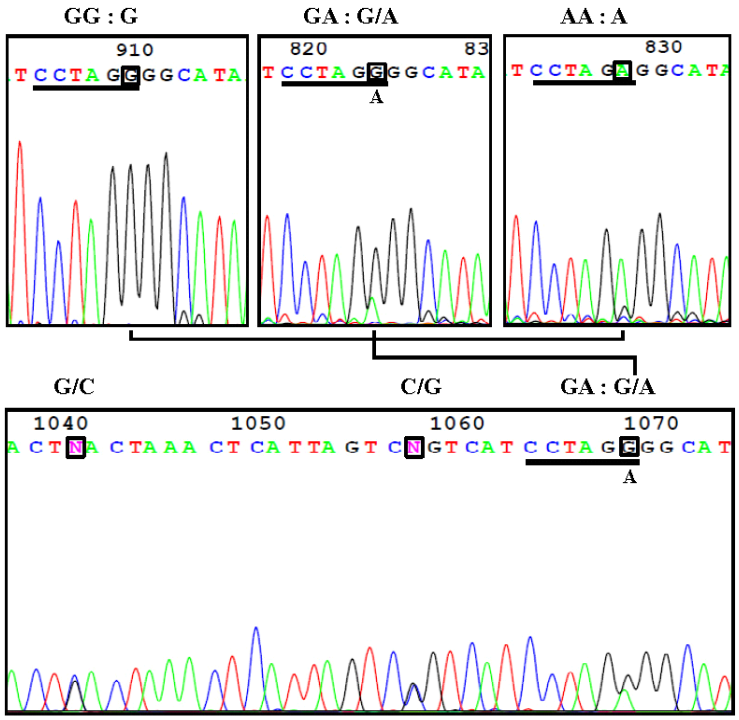


Fig. 2. Sequence analysis of *AREG* genotypes. (Top panel). Sequence analysis confirmed the *AREG* genotypes of GG, GA, and AA from left to right. The *Sty*I restriction site of CCWWGG is underlined and the nucleotides for substitution are noted within the rectangle. GG genotype had G, while GA genotype had G/A and AA genotype had A, respectively. (Bottom panel). There were additional G/C and C/G substitutions in front of the designated G/A SNP for *AREG* GA genotype within the 29 bp span and they are rs343652032, rs325615549, and rs338575180 in the dbSNP of NCBI. Out of the G/C, C/G and G/A substitutions, only the G/A substitution is recognized by *Sty*I. The *Sty*I restriction site of CCWWGG is underlined and the nucleotides for substitutions are noted within the rectangles.

this study to the GenBank, respectively. Out of the 3 SNPs, the last G/A is the site for *Sty*I restriction digestion (Jiang *et al.,* 2002). The positions of the newly identified G/C and C/G SNPs were preserved in their respective *AREG* genotypes among the breeds investigated.

2. *AREG* Allele Frequencies, Genotypes and First Parity Litter Size of Among Pig Breeds

Frequencies of G (previously described as A1) allele of *AREG* among the Duroc, Landrace, Yorkshire, KNP, and Meishan pigs (n=179) were 0.30, 0.11, 0.01, 0.38 and 1.00, respectively (data not shown). The polymorphism was almost fixed for alternative alleles in the Meishan (G) and Yorkshire (A) breeds. The allele frequencies of *AREG* among the Duroc, Landrace, Yorkshire, and KNP sows (n=156) were determined. Frequency of G allele of *AREG* was the highest in the KNP sows, followed by Duroc, Landrace and Yorkshire sows with their allele frequencies of 0.38, 0.30, 0.10 and 0.01, respectively (Table 1). The allele frequencies of *AREG* among the Duroc, Landrace, Yorkshire, and KNP sows were significantly different ($p<0.001$, Table 1), suggesting association between sequence variability of *AREG* and pig breeds. The frequencies of G allele in the KNP or Duroc sows were almost four or three times higher than that of Landrace, respectively (Table 1). First parity litter size was significantly different among the Duroc, Landrace, Yorkshire, and KNP sows ($p=0.014$, Table 2), whereas the first parity litter size was not significantly affected by the *AREG* genotypes ($p=0.148$, Table 2). However, there was a tendency of variation in the first parity litter size among the different breeds when the data were analyzed by *AREG* genotypes.

Table 1. Frequency of G and A alleles of *AREG* among the Duroc, Landrace, Yorkshire, KNP sows

	G	A	Total
Duroc	24 (0.30)	56 (0.70)	80
Landrace	8 (0.10)	72 (0.90)	80
Yorkshire	1 (0.01)	85 (0.99)	86
KNP	25 (0.38)	41 (0.62)	66
Total	58	254	312

AREG allele frequencies of each breed are shown with percentage in ( ). *AREG* allele frequencies were statistically different ($p<0.001$) among the breeds (Chi-Sq = 44.267, DF = 3).

DISCUSSION

The main findings of this study was that allele frequencies of *AREG* among the Duroc, Landrace, Yorkshire and KNP sows were significantly different ($p<0.001$, Table 1), suggesting association between sequence variability of *AREG* and pig breeds. The frequency of G allele of *AREG* was the highest in the KNP sows, followed by Duroc and Landrace sows with their allele frequencies of 0.38, 0.30 and 0.10, respectively. Thus, if traits were associated with higher allele frequencies in one breed, for example reproductive traits, their reproductive traits may be improved more rapidly than traits with lower allele frequencies through mating. However, the allele frequencies of *AREG* were almost fixed in the Meishan (G) and Yorkshire (A) breeds, similar to the previous report by King *et al.* (2003). Therefore, if traits associated with low allele frequencies or allele frequencies were fixed in one breed, the allele frequencies of their offspring may not be altered significantly by breeding. A recent study showed the high G allele frequency of 0.81 in Polish Landrace pigs (Mucha *et al.,* 2013) and it

Table 2. Least squares means and standard errors for the first parity litter size by *AREG* genotypes*

	GG	GA	AA	Total
Duroc	11.08 ± 3.44 (n=1)	9.92 ± 0.93 (n=22)	9.15 ± 0.92 (n=17)	10.05 ± 1.41[a] (n=40)
Landrace	5.42 ± 2.76 (n=1)	9.13 ± 1.35 (n=5)	10.73 ± 0.84 (n=23)	8.43 ± 1.19[a] (n=29)
Yorkshire	-	-	10.54 ± 0.87 (n=26)	10.54 ± 0.87[ab] (n=26)
KNP	7.89 ± 2.04 (n=2)	7.63 ± 1.20 (n=11)	4.76 ± 1.46 (n=5)	6.76 ± 1.11[ac] (n=18)

* First parity litter size was significantly different among the Duroc, Landrace, Yorkshire, and KNP sows ($p=0.014$). Tukey's simultaneous test was carried out to distinguish litter size between breeds. Different letters within same column differ significantly ($p<0.05$). However, the first parity litter size was not significantly affected by the *AREG* genotypes ($p=0.148$).

is different from either our result showing the G allele frequency of 0.10 in Landrace. The study also showed the high G allele frequency of 0.85 in Polish Large White and it is different from the previous result showing the G allele frequency of 0.04 in Large White pigs (King *et al.* 2003) or 0.01 in Yorkshire from our results. The data suggest that the *AREG* allele frequency may vary among different breeds and even among certain lines within the breeds, and *AREG* genotypes may affect the reproductive traits differentially in them.

This is the first report of *AREG* genotypes of KNP breeds. It was unexpected that KNP has GG, GA and AA genotypes (Fig. 1B), and the allele frequency of *AREG* in KNP was not fixed like either Meishan (G) or Yorkshire (A). KNP is generally believed to have come from northern China to Korean peninsula approximately 2000 years ago (Kim & Choi 2002). KNP was restored from the brink of extinction in 1980s by bringing the remaining stocks together in Korea and it was registered as a breed in 2006 (Kim *et al.,* 2005; Kim 2008; Kwon 2006). KNP pigs have been bred with Landrace or Yorkshire pigs to produce specialty meat, while investigating the growth and meat quality traits (Kim *et al.,* 2007; Kim *et al.,* 2011; Niu *et al.,* 2013). It has not been reported whether the *AREG* genotypes affect the reproductive and other economically important traits in the KNP crossbred pigs.

In the Duroc and KNP sows, the first parity litter size appeared to be higher with GG or GA genotype than AA genotype (Table 2). On the contrary, the first parity litter size appeared to be lower with GG or GA genotype than AA genotype in the Landrace sows. Trend of the higher first parity litter size in the Duroc and KNP sows with GG or GA genotype than AA genotype in this study is comparable to the previous report showing that GG genotype was favorite rather than GA genotype (Katska-Ksiazkiewicz *et al.,* 2006), but the breeds investigated were Landrace, Large White, and Line 990 sows in Poland. The result of this study in Duroc and KNP sows are also comparable to a recent study showing that the number of piglets born alive in parities 2^{nd} to 4^{th} of Polish Large White and Polish Landrace pigs were significantly higher ($p<0.05$) in GG genotype (12.76) than GA genotype (12.46) (Mucha *et al.,* 2013). However, the results in the Duroc and KNP sows were contradictory to the report in an F2 population of Meishan × Large White pigs, where the Meishan allele (G) of the *AREG* was associated with an undesirable effect of the number born in the first parity (Jiang *et al.,* 2002).

Trend of the lower first parity litter size in Landrace sows with GG or GA genotype than AA genotype in this study (Table 2) was in agreement with the report of Jiang *et al.* (2002), though the breed was Meishan. The result of this study was contradictory to the report (Katska-Ksiazkiewicz *et al.,* 2006), where GG genotype of Landrace sows was the favorite one in both litter weight at piglet age of 21 days and litter weight at weaning in comparison to GA genotype. However, *AREG* allele frequencies of Landrace, Large White, and Line 990 sows were not included in the report. The variability of *AREG* alleles among different breeds and trends in litter size associated with *AREG* genotypes and breeds suggest that *AREG* genotypes may affect reproductive traits differentially in each breed or possibly in certain lines of the breed. The results may be relevant to breeding and crossbreeding sows for increased litter size (Petry & Johnson 2004). Further studies using other independent lines of these breeds and populations with the increased sample size are needed to validate the current findings.

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Received May 2, 2015, Revised June 10, 2015, Accepted June 14, 2015