



Genetic polymorphism of retinol binding protein 4 locus and its association with litter traits in pigs

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ABSTRACT

Retinol binding protein 4 (RBP4) gene was investigated to find its association with litter traits using PCR-RFLP method in RBP4 locus. The *MspI* polymorphism was observed in the third intron region of RBP4 gene. Genotypes, AA, AB and BB were observed with frequency of 0.48, 0.28 and 0.24 respectively. Homozygotes were predominant in LWY and crossbred whereas, heterozygote was predominant in Duroc. The frequencies of RBP4 allele A were 0.61 in the LWY, 0.48 in Duroc and 0.71 in the LWY × Ankamali pigs. RBP4/*MspI* genotype had no significant association with litter traits. Sows carrying RBP4 - AA genotype had higher litter size and weight at birth, but not significant. Five single nucleotide polymorphisms were identified in RBP4 565bp fragment nucleotide sequences, 4 were found in intronic regions and 1 silent substitution in exon region.

Key words: Litter traits, Pig, Polymorphism, Retinol binding protein 4 (RBP4) gene

Reproductive traits play an important role in determining the efficiency of production in swine. The gene assisted selection or marker-assisted selection in conjunction with traditional selection methods seems as the most effective mean to improve the reproduction performance. Retinol binding protein 4 has a role in providing the conceptus with appropriate amounts of retinoic acid in the early critical phase of pregnancy around day 12 and in buffering retinoic acid oversupply. Retinoic acid (vitamin A) is derived from endogenous retinol and delivered to target cells by retinol binding protein. Retinoic acid is implicated in the regulation of gene transcription and the availability of retinol to the conceptus is transported from maternal capillaries across epithelium to the uterine lumen. At the time of implantation and trophoblastic elongation, RBP4 is sequestered in the uterine lumen (Rothschild *et al.* 2000). The most advanced conceptuses secrete the RBP, necessary for their development. The transport and buffering ability of RBP at this critical time in pregnancy makes RBP4 a strong candidate gene for litter traits in swine. The present study was conducted to find out the association between RBP4 gene with litter traits in pigs.

MATERIAL AND METHODS

Study included 180 sows (98 Large White Yorkshire (LWY), 25 Duroc and 57 crossbreds of Large White Yorkshire × Ankamali) from Center for Pig Production and Research, Mannuthy. Animals are fed with concentrate feed and weaning of piglets was done at 45 days after birth. Blood (5ml) was collected from each animal in a tubes containing EDTA as anticoagulant and DNA was isolated by standard phenol chlorophorm isolation procedure. PCR amplification (25µl, final volume) was performed in thermal cycler (BOIRAD, USA) using 50ng of genomic porcine DNA, 1×PCR buffer, 1.8mM MgCl₂, 0.1mM each dNTP, 10pM of each primer and 0.6 U of *Taq DNA polymerase*. Primer sequences proposed by Munoz *et al.* (2010) was used, the forward primer: 5'-GAG CAA GAT GGA ATG GGT T- 3' and the reverse primer 5'-CTC GGT GTC TGT AAA GGT G- 3'. Conditions were 1 cycle at 94°C for 5 min, followed by 35 cycles (30 sec at 93°C, 45 sec at 56°C and 45 sec at 72°C), followed by 1 cycle at 72°C for 5 min, stopped at 4°C. PCR product, 15µl was digested with 10 U of restriction enzyme *Msp I* and the digested products were resolved in 3% agarose gel. PCR products from different genotypes were sequenced using the forward and reverse primer to detect variations at the nucleotide level. Sequencing was done at a commercial unit and phylogenetic analysis was done using MegAlign Programme of Lasergene Software (DNASTAR).

Allele frequencies were calculated using direct count method and variation of the allelic frequencies among the

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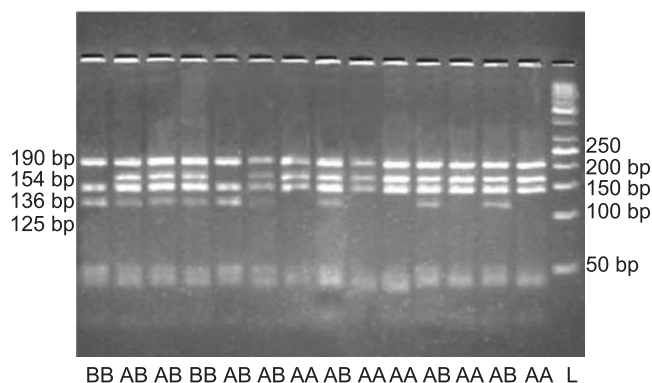


Fig.1. *MspI* digestion pattern of 565bp fragment of RBP4 gene.

and landrace sows, whereas Terman *et al.* (2007) observed 70bp fragments in addition to 190bp, 154bp and 136bp for AA genotype and two fragments, 70bp, 29bp in addition to 190bp, 136bp and 125bp in BB genotypes between exon two and four RBP4 with *MspI* restriction enzyme in Polish large white pigs. Spotter *et al.* (2009) and Dall'Olio *et al.* (2010) also observed polymorphism in the same region of this gene using *MspI* restriction enzyme. From the results and reports, it seems to be the region between exon 2 and 4 of RBP4 gene of pigs showed high degree of variability.

The overall genotype frequency observed in this study was 0.48 for AA, 0.28 for AB and 0.24 for BB. The frequency of AA genotype was 0.58 in crossbred followed by 0.48, 0.28

Table 1. Genotype and allelic frequencies of RBP4/*MspI* polymorphism in pigs

Breed	Genotypic frequency			Allelic frequency		χ^2 value df=2
	AA	AB	BB	A	B	
LWY(98)	0.48(47)	0.26(26)	0.26(25)	0.61	0.39	8.18*
Duroc(25)	0.28(7)	0.40(10)	0.32(8)	0.48	0.52	
LWY×Ankamali(57)	0.58(33)	0.26(15)	0.16(9)	0.71	0.29	
Overall(180)	0.48(87)	0.28(51)	0.24(42)	0.63	0.37	

*Allele frequency differ significantly at (P<0.05). Figures in parenthesis denote number of observations

populations was analyzed by the *Chi*-square test of significance. The association between RBP4 genotypes and reproduction traits was evaluated using least square mixed model analysis of variance for non-orthogonal data (Harvey 1986). Model used was

$$Y_{ijkl} = \mu + G_i + A_j + S_k + R_l + (GR)m + e_{ijklm}$$

where, Y_{ijklm} , phenotypic record of TNB, NWP, LWB and LWV

μ , general mean; G_i , effect of genetic group (i, Yorkshire, Duroc and LWY × Ankamali); A_j , effect parity number (j = 1 to 4); S_k , effect of season of birth (k = 1 to 3); R_l , effect of RBP4 genotypes (l = AA, AB and BB); e_{ijklm} , random error NID (0, σ_e^2).

RESULTS AND DISCUSSION

The PCR-RFLP was performed to detect polymorphism in the 565bp fragment of second to fourth exonic region of the RBP4 gene of pigs. Digestion with restriction enzyme *MspI* showed 3 different genotypes AA (190bp, 154bp and 136bp), AB (190bp, 154bp, 136 and 125bp) and BB (190bp, 136bp and 125bp) in all 3 genetic groups (Fig.1).

Similar band pattern and fragment size using same enzyme were reported in Landrace, Large white Yorkshire breeds by Linville *et al.* (2001), in Polish large white, Polish Landrace, commercial pigs by Korwin-Kossakowska *et al.* (2005). Though, the same 3 band pattern was observed by Rothschild *et al.* (2000) for different genotypes, fragment size was 154bp instead of 190bp for BB genotype. However, Wang *et al.* (2006) reported 2 different fragment size of 136bp and 108bp along with 190bp for BB genotype in French large white

in large white Yorkshire and Duroc respectively. Duroc breed had AB and BB genotypes with frequency of 0.40 and 0.32 respectively. The genotypic and allelic frequencies observed in three genetic groups of pigs are presented in the Table 1.

The moderate frequencies for AA genotype (0.46) were also reported in Polish large white as 0.46 (Terman *et al.* 2007), 0.59 in Xiaomeishan pigs (Fei *et al.* 2008) and 0.53 in German Large white breed (Spotter *et al.* 2009). Low frequency of AA genotype were also observed in German landrace as 0.33 (Spotter *et al.* 2009) and 0.22 in Italian large white (Dall'Olio *et al.* 2010). However, Fei *et al.* (2008) reported very high frequency of 0.90 in Fengjing pigs. Present findings of low frequency for BB genotype (0.24) was in

Table 2. Least square means of reproduction traits with standard error for different genetic groups of pigs

Traits	LWY(98)	Duroc (25)	LWY × Ankamali (57)
	Mean ± SE	Mean ± SE	Mean ± SE
Total number born (TNB)	09.39±0.22 ^b	09.56±0.40 ^b	10.36±0.29 ^a
Number of piglets weaned (NWP)	06.67±0.21 ^b	06.04±1.56 ^b	07.30±0.29 ^a
Litter weight at birth (kg)	11.08±0.30 ^a	11.86±1.41 ^a	11.26±0.36 ^a
Litter weight at weaning (kg)	59.25±1.81 ^c	46.05±3.68 ^b	51.84±2.13 ^a

*Means bearing different superscripts in row- wise differ significantly. Figures in parenthesis are number of observations.

Table 3. Least square means for reproduction traits with standard error of different RBP4/*Msp*I genotype in pigs

Traits	Genotypes with Mean $\pm$ SE (190)			Genotype (df-2)	Error (df=178)	R ² -value (%)
	AA (86)	AB (51)	BB (43)			
Total Number Born (TNB)	09.91 $\pm$ 0.24 ^a	09.59 $\pm$ 0.30 ^a	09.52 $\pm$ 0.35 ^a	01.32	04.77	04.40
Number of Piglets Weaned (NWP)	06.80 $\pm$ 0.22 ^a	06.67 $\pm$ 0.31 ^a	06.88 $\pm$ 0.36 ^a	02.37	04.30	09.90
Litter weight at birth (LWB in kg)	11.60 $\pm$ 0.35 ^a	11.07 $\pm$ 0.36 ^a	10.74 $\pm$ 0.32 ^a	13.11	07.87	03.60
Litter weight at weaning (LWW in kg)	55.05 $\pm$ 1.94 ^a	54.51 $\pm$ 2.27 ^a	55.14 $\pm$ 2.90 ^a	15.29	316.30	09.00

* Means bearing same superscripts did not differ significantly in row- wise. Figure in parenthesis are number of sows.

agreement with the reports made by Terman *et al.* (2007) in Polish large white (0.17), Spotter *et al.* (2009) in German large white breed (0.16) and Dall'Olio *et al.* (2010) in Italian large white pigs (0.25). However, a very low frequency of 0.06 for BB genotype was also observed in German Large white breed by Spotter *et al.* (2009). The observed frequency for AB genotype was 0.28. Terman *et al.* (2007) reported low frequency of 0.37 in Polish large white pigs as noticed in this study. However, moderate frequencies were also reported in German landrace and large white breeds as 0.51, 0.41 respectively by Spotter *et al.* (2009) and 0.53 in Italian large white pigs by Dall'Olio *et al.* (2010). In contrast to the present study, López *et al.* (2006) reported only 2 genotypes in Yorkshire, Landrace sows. Flores *et al.* (2009) and Liu *et al.* (2010) also observed only 2 genotypes AA and AB in Yorkshire, Pelon, Cuino, and Tibet pig breeds. Monomorphic pattern of AA genotype for RBP4 locus were also observed by Fei *et al.* (2008) in Erhualian pigs and Yuan *et al.* (2010) in Large white pigs. Based on the results of this investigation frequency of AA genotype varied from low to medium and the frequency of BB genotype was low in all the 3 genetic groups of pigs.

The overall allelic frequency was 0.63 and 0.37 for A and B respectively and the frequency of A allele in crossbred was high followed by LWY and Duroc breeds. Frequency of B allele was high in Duroc followed by LWY and crossbred pigs. The comparison of allele frequencies between 3 breeds using *Chi*-square test showed significant difference between breeds ($P \leq 0.05$). Similar findings of higher frequency of A allele were observed by Drogemuller *et al.* (2001) in Duroc (0.85), German landrace (0.67) and synthetic line (0.62), Korwin-Kossakowska *et al.* (2005) in Polish large white, landrace and commercial pigs (0.68), Terman *et al.* (2007) in Polish large white (0.65) and Flores *et al.* (2009) in Yorkshire (0.74), Pelon (0.74), Cuino pigs (0.75) and Ji *et al.* (2011) in Hunan Black pig (0.85). The moderate frequency of A allele was noticed by Wang *et al.* (2006) in Landrace (0.57) and French Large white (0.56), Omelka *et al.* (2008) in Large white (0.51) and White meaty pigs (0.52), Zhuoma *et al.* (2008) in Beijing Black Pig (0.53), Spotter *et al.* (2009) in German Landrace (0.57) and Large White Yorkshire (0.56) and Munoz *et al.* (2010) in Tai zumu pigs (0.59). Few reports were also indicated low frequency of A allele in

Landrace and Large White Yorkshire (Linville *et al.* 2001), in synthetic line (Gonçalves *et al.* 2008) and in Italian large white pigs (Dall'Olio *et al.* 2010).

Least square means for total number born, number of piglets weaned, litter weight at birth and litter weight at weaning of different genetic groups are presented in table 2 and for different RBP4 / *Msp*I genotype are presented in Table 3.

The sows carrying homozygote AA had produced higher number of 0.32 and 0.39 piglets compared to AB and BB. Sows with AA genotype also had better LWB of 0.53kg and 0.86kg compared to AB and BB. However no statistical significance was observed. Similar result of nonsignificant increase in TNB for sows with AA genotype was reported by Korwin-Kossakowska *et al.* (2005) in Polish Large White, Landrace and commercial pigs. However, significant effect of A allele on TNB observed by Rothschild *et al.* (2000) commercial lines of pigs, Omelka *et al.* (2008) in White meaty pig breed and Ji *et al.* (2011) in Hunan black pig. Significant increase in TNB for sows with AA genotype was reported by Sun *et al.* (2009) in Shandong local pig breeds and foreign pig breeds as 0.59 and 0.72 more than BB homozygotes respectively. López *et al.* (2006) also made similar report for AA genotype on TNB and NBA to the extent of 0.5 in Yorkshire-landrace sows. Authors, also reported significant additive (0.24) and dominant effect (0.31) of A allele on number born alive in German Landrace breed (Spotter *et al.* 2009), additive effect of 0.42 in Tai zumu pig breed (Munoz *et al.* 2010). In contrast to present study,

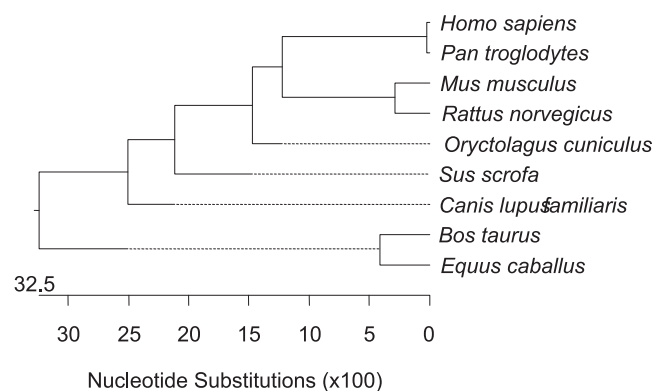


Fig. 2. Phylogenetic tree based on nucleotide sequences of swine RBP4 gene in different species.

significantly higher TNB was also reported by Terman *et al.* (2007) in Polish Large White pigs, Zhuo-ma *et al.* (2008) in Beijing Black Pig. Wang *et al.* (2006) also reported a non significant higher number of +0.40 piglets at birth for BB genotypes in French large white and Landrace sows. Similar nonsignificant effect of B allele on the litter weight at birth and weaning was observed by Zhang *et al.* (2010) in hybrid Chinese pigs. However, Korwin-Kossakowska *et al.* (2005) found a significant effect of B allele on the litter weight at birth and weaning in Polish Large White, Landrace and commercial lines of pigs. In contrast to present study, López *et al.* (2006) found significantly higher weight at birth of 0.6kg and weight at weaning of 2.6kg for sows with AA genotype in Yorkshire-landrace pigs.

Sequence analysis revealed four intronic and one exonic single nucleotide polymorphism (SNP) in RBP4 sequences. The identified SNPs were at intron 2c.111+47T>C, exon 3c.156G>A and intron 3c.248+15G>T, c.248+16A>G and c.249–63G>C. The nucleotide substitutions in exon lead to BB genotype. Munoz *et al.* (2010) also reported one SNP at intron 2, three in intron 3 and one in exon3 as in the present study with an additional SNP at intron 3. However, Rothschild *et al.* (2000) observed only one SNP at intron 3. Korwin-Kossakowska *et al.* (2005) and Spotter *et al.* (2009) had also reported intronic polymorphism at RBP4 locus of swine. The *Sus scrofa* RBP4 565bp fragment gene exhibited 91.8% identity with that of canine, 91.6% with that of rabbit. The sequence identities with chimpanzee and human were 82.5 and 82.1% whereas rodents (mouse and rat) had 78.2 and 77.2% identity. The cattle (*Bos taurus*) and equine had least identity with 43.0 and 41.6% respectively. It is inferred that the RBP4 locus of swine had shown a high degree of homology with higher mammals followed by rodents. Phylogenetic tree based on RBP4 nucleotide sequences showed evolutionary nature of mammals (Fig 2).

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