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Research Article

Interaction between estrogen receptor and retinol-binding protein-4 polymorphisms as a tool for the selection of prolific pigs

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Abstract

The aim of the present study was to investigate the association of the estrogen receptor (*ER*-PvuII) and retinol-binding protein 4 (*RBP4*-MspI) gene polymorphisms and their interactions with prolificacy in a commercial synthetic pig line reared in Brazil. A total of 10,374 piglet records from 218 sows and 817 litters were used for litter size analysis. Only females with three or four farrowings were included in the analysis. The mean litter size ranged from 5.0 to 19.5 piglets. DNA was extracted from leukocytes by a standard method, and *ER*-PvuII and *RBP4*-MspI polymorphisms were characterized by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The association between alleles or genotypes and reproductive performance was analyzed using a general linear model including the interaction between the *ER*-PvuII and *RBP4*-MspI genotypes. For the *ER*-PvuII gene, the allele frequencies of allele A and allele B were 0.56 and 0.44, respectively. For the *RBP4*-MspI gene, the frequencies of alleles A1 and A2 were 0.29 and 0.71, respectively. The total number of piglets born (TNB), born alive (NBA), or number of mummies and stillborn piglets (NMUM and NSB) per litter did not differ between the various *ER*-PvuII and *RBP4*-MspI genotypes. However, when the *ER*-PvuII and *RBP4*-MspI genotypes were considered together in each sow, TNB and NBA were 1.4 ($p = 0.0026$) and 0.9 ($p = 0.019$) higher in AA/A1 and AB/A1 animals, respectively, than in AA/A2 and BB/A1 animals. Likewise, TNB and NBA were 0.9 ($p = 0.0258$) and 0.8 ($p = 0.0168$) higher in BB/A2 and AB/A2 sows, respectively, than in AA/A2 and BB/A1 animals, but no difference was observed compared to AA/A1 and AB/A1 animals. The results showed larger litter sizes (TNB and NBA) for sows carrying the *ER*-PvuII allele A and the *RBP4*-MspI genotype A1, and for animals carrying the *ER*-PvuII allele B and the *RBP4*-MspI genotype A2. In conclusion, the interaction between genotypes *ER*-PvuII and *RBP4*-MspI is more efficient in the selection of prolific sows than each one of these molecular markers alone.

Key words: swine, marker-assisted selection, retinol-binding protein-4, estrogen receptor.

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Introduction

The main objective of genetic pig breeding, especially sows, is to increase the production efficiency and the final quality of the product. Animals with a high genetic potential for a set of traits are selected as parents for the next generations. In current selection programs, the prediction of genetic merit of selection candidates is based on the evaluation of traits in the candidate animals themselves or in members of the family (Varona *et al.*, 2005). However, this approach is not ideal when the traits are limited by sex, measured in carcass, or related to reproductive efficiency.

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Molecular biology techniques allow identifying and characterizing mutations that have occurred during animal evolution (Dekkers, 2004). Due to the recent advances in molecular genetics, marker-assisted selection offers new opportunities for the identification of differences between individuals, both at the genetic and at the phenotypic level (Vidal *et al.*, 2005). Marker-assisted selection combined with conventional methods is particularly interesting for the selection of traits that cannot be assessed in all candidates. In addition, this approach is more efficient in the case of negatively correlated traits. A first step toward this type of selection was taken with the development of high-density genetic maps. Thus, genetic markers that have been

correctly associated with important genes can be used in selection programs, providing a substantial genetic gain.

Polymorphisms in genes related to prolificacy and productivity, such as the estrogen receptor polymorphism (*ER*-PvuII) gene (Rothschild *et al.*, 1996), and to higher embryo survival, such as the retinol-binding protein 4 polymorphism (*RBP4*-MspI) gene (Rothschild *et al.*, 2000), have been identified as molecular markers for the selection of more prolific animals. These markers were first investigated in Meishan pigs, which produce about four piglets more per litter (Rothschild *et al.*, 1996). Animals carrying the favorable *ER*-PvuII allele (allele B) produced 1.25 to 1.50 piglets more per litter (Rothschild *et al.*, 1994; Rothschild *et al.*, 1996; Short *et al.*, 1997). In the first studies on the *RBP4*-MspI polymorphism, animals with the favorable genotype produced about 0.50 more piglets and 0.26 more liveborn piglets per litter than those with the unfavorable genotype (Rothschild *et al.*, 2000). However, studies using these markers for the analysis of other populations were unable to demonstrate an advantage of these genotypes (Drogemuller *et al.*, 2001; Isler *et al.*, 2002; Gibson *et al.*, 2002). In addition, the favorable allele (allele B) of the *ER*-PvuII polymorphism has not been observed in lines derived from the Duroc and German Landrace breeds (Short *et al.*, 1997; Drogemuller *et al.*, 2001). In parallel, a low frequency of allele B (0.10) was observed in synthetic pig lines from Germany (Drogemuller *et al.*, 2001).

Prolificacy is a multigene trait of low heritability, and an increase in this trait is determined by various factors. Therefore, a single molecular marker will hardly be efficient in the identification of more productive animals in all populations. The hypothesis of the present study was that the interaction between polymorphisms of two candidate genes, *ER* and *RBP4*, might represent an alternative for the identification of more prolific animals in a larger number of populations. The aim of the present study was to investigate the association of *ER* and *RBP4* gene polymorphisms and their interactions with prolificacy in a synthetic pig line reared in Brazil.

Material and Methods

Animals

A total of 10,374 piglet records from 218 commercial sows belonging to the São Roque farm, municipality of Videira, State of Santa Catarina, and 817 farrowings were analyzed. The animals were selected according to individual history, considering the number of farrowings and prolificacy. Only sows with three or four farrowings were included in the analysis. Based on their mean productivity ± 2 standard deviations, the animals were classified as being of low, medium or high productivity, and blood was collected for DNA extraction, amplification by PCR and characterization of polymorphisms by RFLP (polymerase chain reaction-restriction fragment length polymorphism - PCR-RFLP).

Blood samples were collected by puncture of the jugular vein using an anticoagulant, and genomic DNA was extracted according to the method described by Lahiri and Nurnberger (1991).

For the characterization of the *ER*-PvuII polymorphism by PCR-RFLP, a 120-bp fragment was amplified by PCR, using the following specific primers described by Short *et al.* (1997): forward 5'-CCTGTTTTTACAGTGA CTTTTACAGAG-3' and reverse 5'-CACTTCGAGGGT CAGTCCAATTA-G-3'. The reaction mixture contained PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2 mM of each dNTP, 5 mM of each primer, 0.5 U Taq DNA polymerase and 30 ng genomic DNA, in a final volume of 25 µL. The amplification conditions were: one denaturation cycle at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 45 s and 72 °C for 1 min, and a final extension step at 72 °C for 4 min. The PCR products were digested with PvuII for 2 h at 37 °C in a reaction mixture containing 1.6 µL ultrapure water, 1.5 µL buffer, 1 U of PvuII, and 11.5 µL of the amplified product. The digested products were submitted to electrophoresis on 4% agarose gel containing ethidium bromide at 94 V. The bands were visualized under ultraviolet light, and the size of the fragments was determined by comparison with a known molecular weight marker (100-bp ladder). The results obtained were recorded with an Alpha Digidoc 1000 photodocumentation system (Alpha Innotech Corp., San Leandro, CA). The animals were classified as genotype AA, AB or BB.

For the characterization of the *RBP4* gene polymorphism by RFLP, a 550-bp fragment was amplified by PCR, using the following specific primers described by Rothschild *et al.* (2000): forward 5'- GAGCAAGATGG AATGGGTT - 3' and reverse 5'- CTCGGTGTCTGTAA AGGTG -3'. The reaction mixture, in a final volume of 25 µL, was the same as that used for *ER*-PvuII. The amplification conditions were: one denaturation cycle at 93 °C for 3 min, followed by 40 cycles at 93 °C for 30 s, 56 °C for 45 s, and 72 °C for 45 s, and a final extension step at 72 °C for 5 min. The PCR products were digested with MspI for 12 h in a reaction mixture containing 1.6 µL ultrapure water, 1.5 µL buffer, 1 U MspI, and 11.5 µL of the PCR product. The samples were submitted to electrophoresis on 2% agarose gel containing ethidium bromide at 94 V, and the bands were visualized under ultraviolet light. Fragment size was determined by comparison with a known molecular weight marker (100-bp ladder). The results obtained were recorded with an Alpha Digidoc 1000 photodocumentation system (Alpha Innotech Corp., San Leandro, CA). The two genotypes distinguished as 11 and 22 (Rothschild *et al.*, 2000) were named A1 and A2, respectively.

Statistical analysis

The association of alleles *ER*-PvuII and *RBP4*-MspI with genotypes and reproductive performance of sows was analyzed according to the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

where Y_{ij} is the mean number of piglets born alive (NBA), number of mummies (NMUM), number of stillborn piglets (NSB), or total number born (TNB) per litter, μ is the common constant to all observations, α_i is the effect of genotype, β_j is the effect of parity, the letters in parenthesis represent the interaction between factors, and ε_{ij} is the residual effect. The boar effect was not included in the model because the sows were inseminated with pooled doses from several boars. The genotypes and their interactions were compared using the contrast tool of the SAS software. All analyses were carried out with the general linear model (GLM) procedure of the SAS software. Compliance with Hardy-Weinberg equilibrium was evaluated using the GDA program (Lewis and Zaikin, 2001).

Results

For the *ER*-PvuII polymorphism, allele frequencies were 0.56 for allele A and 0.44 for allele B. No significant deviation from the Hardy-Weinberg equilibrium was observed for this locus. The frequencies of the *ER*-PvuII and *RBP4*-MspI genotypes and their interactions are shown in Figures 1 and 2. The mean litter size (of the four parities) ranged from 5.0 to 19.5 piglets per sow per litter (mean of 11.9 ± 2.4), regardless of the genotype (Figure 3).

The total number of piglets born (TNB), number born alive (NBA), number of mummies (NMUM) or number of stillborn (NSB) piglets per litter did not differ among the various *ER*-PvuII and *RBP4*-MspI genotypes when considering either first parity or all four parities (Tables 1, 2 and 3). However, analysis of the interaction between genotypes, excluding the first parity, showed that animals carrying the *ER*-PvuII allele A (genotype AA or AB) and the *RBP4*-MspI genotype A1 produced more piglets

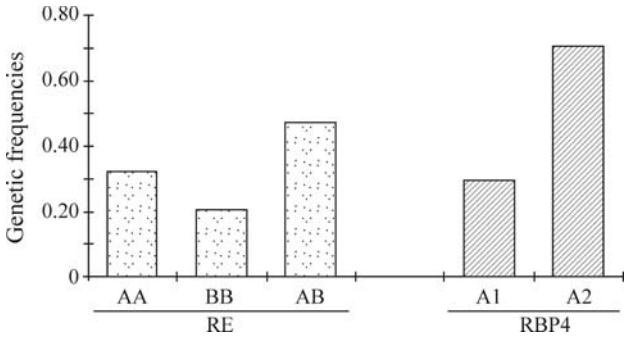

Figure 1 - Genotype frequency of the estrogen receptor and retinol-binding protein 4 gene polymorphisms in sows of a commercial population from Brazil.

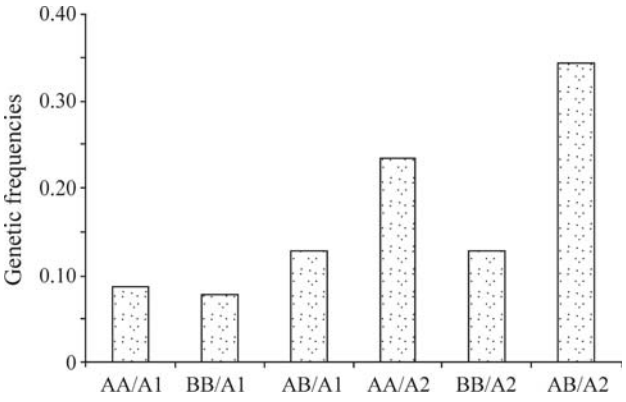

Figure 2 - Frequency of the association between estrogen receptor and retinol-binding protein 4 polymorphism genotypes in sows of a commercial population from Brazil.

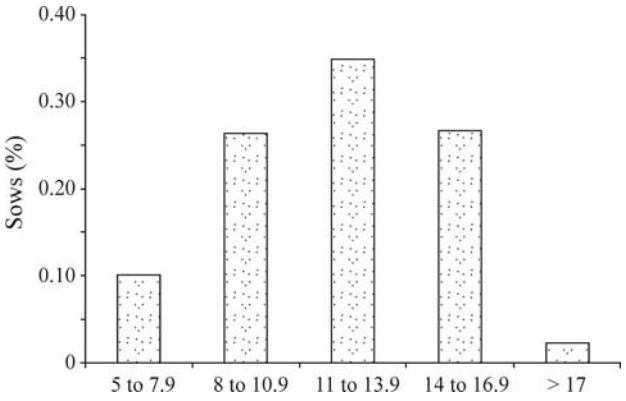

Figure 3 - Distribution of animals according to the mean number of piglets per parity, considering a total of four parities, in sows of a commercial population from Brazil.

(AA/A1 = 13.6 ± 0.5; AB/A1 = 13.6 ± 0.4). Similarly, animals carrying the *ER*-PvuII allele B and the *RBP4*-MspI genotype A2 also presented high productivity (BB/A2 = 13.5 ± 0.4; AB/A2 = 12.9 ± 0.3). In contrast, the mean litter size was smaller for sows with the AA/A2 (12.2 ± 0.3) and BB/A1 (12.3 ± 0.6) genotypes compared to the other genotypes ($p < 0.05$). Thus, the combination of genotypes *ER*-PvuII and *RBP4*-MspI resulted in a mean 1.4-fold increase in TNB ($p = 0.0026$) and a 0.9-fold increase in NBA ($p = 0.0190$) per litter for animals with the AA/A1 and AB/A1 genotypes compared to AA/A2 and BB/A1 sows. Sows with genotypes BB/A2 and AB/A2 produced on average 0.9 ($p = 0.0258$) and 0.8 ($p = 0.0168$) more TNB and NBA, respectively, than AA/A2 and BB/A1 animals, but did not differ from sows with the AA/A1 and AB/A1 genotypes (Table 4).

Discussion

The main finding of this study was the interaction between gene polymorphisms *ER*-PvuII and *RBP4*-MspI and their influence on prolificacy in pig populations, in which

Table 1 - Association of estrogen receptor (*ER*-PvuII) and retinol-binding protein 4 (*RBP4*-MspI) genotypes with mean number of piglets born alive (NBA), number of mummies (NMUM), number of stillborn piglets (NSB), and total number born (TNB) per litter in the four parities.

Genotype*	Parity n	NBA (Mean ± SE)	NMUM (Mean ± SE)	NSB (Mean ± SE)	TNB (Mean ± SE)
ER-PvuII					
AA	258	11.5 ± 0.2	0.2 ± 0.0	0.8 ± 0.1	12.5 ± 0.2
AB	390	11.8 ± 0.2	0.3 ± 0.0	0.7 ± 0.1	12.8 ± 0.2
BB	169	11.7 ± 0.3	0.2 ± 0.0	0.8 ± 0.1	12.7 ± 0.3
RBP4-MspI					
A1	242	11.8 ± 0.2	0.3 ± 0.0	0.9 ± 0.1	12.9 ± 0.3
A2	575	11.6 ± 0.1	0.3 ± 0.0	0.7 ± 0.0	12.6 ± 0.2

*Animals were classified according to genotype into AA, BB (homozygous) or AB (heterozygous) for *ER*-PvuII and A1 or A2 for *RBP4*-MspI (see Material and Methods for details).

Table 2 - Association between estrogen receptor (*ER*-PvuII) genotypes and mean number of piglets born alive (NBA), number of mummies (NMUM), number of stillborn piglets (NSB), and total number born (TNB) per litter at first parity and for all other parities.

Genotype*	n	NBA (Mean ± SE)	NMUM (Mean ± SE)	NSB (Mean ± SE)	TNB (Mean ± SE)
First farrowing					
AA	70	11.3 ± 0.4	0.6 ± 0.1	0.3 ± 0.1	12.2 ± 0.4
AB	103	11.0 ± 0.3	0.7 ± 0.1	0.3 ± 0.1	12.1 ± 0.3
BB	45	11.0 ± 0.5	0.6 ± 0.1	0.1 ± 0.0	11.7 ± 0.5
2nd, 3rd and 4th farrowings					
AA	188	11.5 ± 0.2	0.8 ± 0.1	0.2 ± 0.0	12.6 ± 0.3
AB	287	12.0 ± 0.2	0.7 ± 0.1	0.3 ± 0.0	13.1 ± 0.2
BB	124	12.0 ± 0.3	0.9 ± 0.1	0.3 ± 0.1	13.1 ± 0.3

Animals were classified according to genotype into AA, BB (homozygous) or AB (heterozygous) for the *ER*-PvuII system.

Table 3 - Association between retinol-binding protein 4 (*RBP4*-MspI) genotypes and mean number of piglets born alive (NBA), number of mummies (NMUM), number of stillborn piglets (NSB), and total number born (TNB) per litter at first parity and for all other parities.

Genotype*	n	NBA (Mean ± SE)	NMUM (Mean ± SE)	NSB (Mean ± SE)	TNB (Mean ± SE)
First farrowing					
A1	64	11.3 ± 0.4	0.7 ± 0.1	0.3 ± 0.1	12.3 ± 0.4
A2	154	11.0 ± 0.3	0.7 ± 0.1	0.2 ± 0.0	11.9 ± 0.3
2nd, 3rd and 4th farrowings					
A1	178	12.0 ± 0.3	1.0 ± 0.1	0.3 ± 0.1	13.3 ± 0.3
A2	421	11.8 ± 0.2	0.7 ± 0.1	0.3 ± 0.0	12.8 ± 0.2

*Animals were classified according to genotype into A1 or A2 for *RBP4*-MspI (see Material and Methods for details).

the favorable genotypes (*ER*-PvuII allele A and *RBP4*-MspI genotype A1) were not related to TNB and NBA per litter when analyzed separately. *ER*-PvuII allele B (Rothschild *et al.*, 1996; Short *et al.*, 1997) and *RBP4*-MspI genotype A1 have been associated with increased prolificacy in pig populations. However, some studies failed to demonstrate association between these polymorphisms and prolificacy (Drogemuller *et al.*, 2001; Isler *et al.*, 2002), which might be due to possible different linkage phases between the marker alleles and the mutation responsible for the effect in different lines, notably in the case of the *ER*-PvuII polymorphism that is located on an intron.

In the population studied, animals carrying the *ER*-PvuII allele B were only more prolific when associated with the *RBP4*-MspI genotype A2, whereas sows with the *ER*-PvuII allele A presented a larger mean TNB and NBA when associated with the *RBP4*-MspI genotype A1. The effect of this interaction between genotypes *ER*-PvuII and *RBP4*-MspI on the increase in prolificacy demonstrates the need for the establishment of various molecular markers for

Table 4 - Association of combinations of estrogen receptor (*ER*-PvuII) and retinol-binding protein 4 (*RBP4*-MspI) genotypes with mean number of piglets born alive (NBA), number of mummies (NMUM), number of stillborn piglets (NSB), and total number born (TNB) per litter in the four parities.

Genotype*	n	NBA (Mean ± SE)	NMUM (Mean ± SE)	NSB (Mean ± SE)	TNB (Mean ± SE)
First farrowing					
A/A1	47	11.3 ± 0.5[a]	0.4 ± 0.1	0.8 ± 0.1	12.5 ± 0.4[a]
AA2-BB1	68	11.0 ± 0.4[a]	0.2 ± 0.1	0.5 ± 0.1	11.7 ± 0.4[a]
B/A2*	103	11.0 ± 0.3[a]	0.2 ± 0.1	0.8 ± 0.1	12.0 ± 0.3[a]
2nd, 3rd and 4th farrowings					
A/A1	133	12.2 ± 0.3[a]	0.3 ± 0.1	1.1 ± 0.1	13.6 ± 0.3[a]
AA2-BB1	181	11.3 ± 0.3[b]	0.2 ± 0.0	0.8 ± 0.1	12.2 ± 0.3[b]
B/A2	285	12.1 ± 0.2[a]	0.3 ± 0.0	0.7 ± 0.1	13.1 ± 0.2[a]

*Animals were classified according to genotype into AA, BB (homozygous) or AB (heterozygous) for *ER*-PvuII and A1 or A2 for *RBP4*-MspI (see Material and Methods for details). A/A1 = animals carrying *ER*-PvuII allele A (genotype AA or AB) and *RBP4*-MspI genotype A1; AA2-BB1 = animals homozygous for *ER*-PvuII (genotype AA or BB) and A1 or A2 *RBP4*-MspI genotype; B/A2 = animals carrying *ER*-PvuII allele B and *RBP4*-MspI genotype A2.

[ab]In the same column values with different letters are significantly different at ($p < 0.05$).

the selection of productive traits of low heritability that involve candidate genes related to the trait to be selected. In the case of selection based on TNB and NBA, several factors should be taken into account, such as the number of ovulations, gamete and embryo viability, body and nutritional condition, uterine capacity, and competence for pregnancy recognition (Flint *et al.*, 1983; Wu *et al.*, 1989; Chen and Dziuk, 1993; Soede *et al.*, 1994; Ferguson *et al.*, 2003; Soboleva *et al.*, 2004). The relevance of *ER* and *RBP4* in the physiology of early pregnancy led to the choice of these genes as candidates for the study of the association with prolificacy in pigs. Estrogen and its receptors are involved in the regulation of hormones which are responsible for the control of follicular dynamics (Soboleva *et al.*, 2004), pregnancy recognition (Flint *et al.*, 1983), and reduction in embryo mortality (Soede *et al.*, 1994). On the other hand, retinol and its binding proteins play a crucial role in embryo and fetal development, with both excess retinol, which exhibits teratogenic effects, and deficient retinol exerting an influence (Lefebvre *et al.*, 2005). Embryo loss has been shown to be reduced and litter size and piglet birth weight to be increased in pregnant sows injected with β-carotene or vitamin A (Coffey and Britt, 1993; Chew, 1996). In addition, retinol and its binding proteins are probably involved in the reduction of embryo mortality. Increased expression of the *RBP4* gene in the endometrium is observed in sows between day 10 and day 12 of gestation, demonstrating the importance of vitamin A and its transport proteins in the establishment of pregnancy (Harney *et al.*, 1993).

The allele and genotype frequencies observed in the present sample suggest that these markers were not used as a tool in the selection of the animals of this population. *ER*-PvuII allele B is present in some highly prolific pig breeds such as Meishan and Large White/Yorkshire pigs (Rothschild *et al.*, 1996). The population studied is a commercial line derived from the crossing of various breeds. This population showed a high frequency of *ER*-PvuII allele B (0.44), and one third of the animals presented *RBP4*-MspI genotype A1 (0.29). Since only two *RBP4*-MspI genotypes were considered, similar allele frequencies were obtained for *ER*-PvuII and *RBP4*-MspI. This fact also demonstrates that animals of the commercial line studied were influenced by prolific ancestors, such as pigs of Chinese breeds used in the crosses that gave origin to the breed.

Since the animals studied were not chosen randomly but based on the history of the last four parities, a distribution according to the mean number of piglets per litter was observed, with the number of piglets ranging from 5.0 to 19.5 in the four farrowings (total of 817 farrowings). The objective of this sampling was to include representative animals of different productivity classes, in order to detect an association or an interaction between genotypes and TNB and NBA.

The interaction between *ER*-PvuII and RBP4-MspI alleles might be more efficient in the selection of more prolific animals than each molecular marker alone. Both the sows carrying the *ER*-PvuII allele A and the *RBP4*-MspI genotype A1 and those with the *ER*-PvuII allele B and the *RBP4*-MspI genotype A2 produce more piglets and more live piglets per litter.

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