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Association with litter size of new polymorphisms on *ESR1* and *ESR2* genes in a Chinese-European pig line

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Abstract – The objective of this study was to search for polymorphisms in the coding region of the estrogen receptors 1 and 2 (*ESR1* and *ESR2*) and to analyze the effects of these variants and the well known intronic *ESR1* *PvuII* polymorphism on litter size in a Chinese-European pig line. We identified five silent single nucleotide polymorphisms (SNP) in the *ESR1* cDNA: c.669T > C (exon 3), c.1227C > T (exon 5), c.1452C > T (exon 7), c.1665T > C and c.1755A > G (exon 8). One pair of these SNP (c.1665T > C and c.1755A > G) co-segregated in the analyzed line, and the SNP c.669T > C showed the same segregation pattern as the *PvuII* polymorphism. These polymorphisms were tested in this study, although the c.1452C > T SNP within exon 7 was not analyzed due to its low informativeness. In the *ESR2* cDNA, one missense SNP was found within exon 5, which caused an amino acid substitution in the coded protein: “c.949G > A (p.Val317Met)” and was tested on sow litter size. Information on 1622 litter records from 408 genotyped sows was analyzed to determine whether these SNP influenced the total number of piglets born (TNB) or the number of born alive (NBA). The polymorphisms *ESR1*: [*PvuII*; c.669T > C], *ESR1*: [c.1665T > C; c.1755A > G] and *ESR2*: c.949G > A showed no statistically significant association with litter size. However, the *ESR1*: c.1227T allele was significantly associated with TNB. The additive substitution effect was estimated to be 0.40 piglets born per litter ($P < 0.03$), and no dominance effects were observed. This SNP could be useful in assisted selection for litter size in some pig lines, as a new genetic marker in linkage disequilibrium with the causative mutation.

estrogen receptor genes / polymorphisms / pig / litter size

1. INTRODUCTION

Conventional selection for litter size in pig breeding schemes has traditionally been inefficient because of the low heritability and difficulties for intense

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selection of this sex-limited trait for which phenotypic measurement is only possible late in life [25]. Marker-assisted selection (MAS) has been proposed as a complementary tool to enhance the rate of genetic improvement of this kind of trait [16]. During the past two decades, the application of techniques of genetic evaluation based on mixed-model methodology on large datasets of pig reproductive records has been successful in achieving moderate genetic changes for prolificacy in dam lines [19, 21]. The use of direct selection of some genes associated with reproductive traits has been recommended to achieve further genetic progress for litter size in pig breeding schemes [23]. However, the genetic tests recommended to the industry programs cannot detect the causative mutations (direct markers) but detect linked markers in a linkage disequilibrium population with these ones (LD markers). As a consequence, the potential extra genetic gains achieved from assisted selection should be lowered [3]. A paradigmatic case is the estrogen receptor 1 (*ESR1*), proposed as a major gene for prolificacy in 1996 by Rothschild *et al.* [24]. The reported association between a polymorphic *PvuII* restriction site in the *ESR1* gene and litter size has been supported by subsequent studies [8, 27, 28], whereas no statistical association between this single nucleotide polymorphism (SNP) and prolificacy was detected in other populations [5, 6, 20]. A meta-analysis of 15 published association studies outlined the high and significant heterogeneity of the *ESR1 PvuII* polymorphism effects estimated among studies performed in diverse pig populations [1]. This inconsistency among results could be attributed to sampling error but also to genotype by environment interaction, linkage, genetic background or population stratification.

Beyond the commercial application as LD marker of the intronic *ESR1 PvuII* polymorphism, *ESR1* and *ESR2* have a crucial role in the female biology of reproduction that justify further research on the association of other SNP with litter size. Estrogen is known for its role in the development and functioning of the female reproductive system. The primary mechanism of estrogen's action is *via* binding and modulation of activity of the estrogen receptors (*ESR1* and *ESR2*), which are ligand-dependent nuclear transcription factors expressed at high levels in female tissues critical to reproduction, including the ovaries, uterus, cervix, mammary glands and pituitary gland. Although *in vitro* studies suggest that both *ESR* may play redundant roles, a dissimilar tissue distribution indicates otherwise [15], and their respective functions have been inferred from the use of specific *ESR1* and *ESR2* knockout mouse models [2, 10, 26]. Korach *et al.* [9] and Krege *et al.* [14] generated and studied knockout mice lacking estrogen receptors 1 (1ERKO) and 2 (2ERKO). They reported complete infertility for the 1ERKO mice and fewer and smaller litters

than wild-type mice for the 2ERKO mature females. These researches revealed that *ESR1* is essential for female sexual differentiation, fertility and lactation and *ESR2* is essential for normal ovulation efficiency. In pigs, *ESR1* mRNA levels showed maximal expression at early pregnancy [13], and other studies suggested that the *ESR2* gene is related to estrogenic functions such as maturation of the ovarian follicles as well as the growth and development of peri-implantation embryos [12, 29]. In spite of its critical role in reproductive physiology, the *ESR2* gene has scarcely been investigated as a candidate gene for litter size in pigs. We previously detected a polymorphism at an *Hsp92II* recognition site in the *ESR2* gene in two Iberian pig populations [18]. No statistically significant association between this *ESR2* SNP and litter size was found.

The objective of this research was to perform the first joint study of the porcine *ESR1* and *ESR2* genes focused on the research of the polymorphisms located in the coding region of these two genes, and the evaluation of their respective effects on litter size traits in a composite Chinese-European pig line.

2. MATERIALS AND METHODS

2.1. Animals

The Chinese-European line of pigs used for this study was created in 1994 from 31 Meishan and 3 Jiaying sows inseminated with 32 hyperprolific French Large White boars, and then selected for lean growth along nine generations [17]. We procured the total number of piglets born (TNB) and the number of born alive (NBA) recorded in 5932 litters farrowed from 2397 sows in four breeding nuclei located in France and Spain. A subset of 408 genotyped sows with records from 1622 litters was used in the association study. A summary of all the available information is presented in Table I. Moreover, six animals from the Iberian, Large White and Meishan breeds and two Spanish Wild boars were analyzed to look for new polymorphism in the *ESR2* cDNA.

2.2. Isolation and sequencing of porcine *ESR1* and *ESR2* cDNA

Ovary samples from two Chinese-European sows were collected on liquid N₂ and stored at -80 °C. Total RNA was extracted with the Tri Reagent (Sigma Aldrich Chemie, Madrid, Spain). Reverse transcription (RT) was performed with the Superscript II kit (Invitrogen, Life Technologies, Barcelona, Spain) and random hexamers.

Table I. Number of animals, litters, mean, and standard deviation (SD) of the analyzed litter records.

Animals in pedigree	2742
Sows with records	2397
Herd-year-season	50
Litters	5932*
NBA, number born alive	12.76 (SD = 3.49)
TNB, total number born	13.97 (SD = 3.75)

* 1622 litters from 408 genotyped sows.

Four overlapping *ESR1* cDNA fragments encompassing exons 1–8 were amplified with primers designed from the available nucleotide sequences of the coding region and the 5' untranslated region (UTR) of the porcine *ESR1* gene (Genbank accession numbers: Z37167 and AF034972). The polymerase chain reactions (PCR) were performed in 25 μ L volumes containing 2 μ L of cDNA, standard PCR buffer (75 mM Tris-HCl pH 9.0, 50 mM KCl, 20 mM $(\text{NH}_4)_2\text{SO}_4$), and concentrations of MgCl_2 , dNTP, primers and *Tth* polymerase (Biotools, Madrid, Spain) optimized for each amplified fragment. Amplification conditions were 94 °C for 3–5 min, followed by 40 cycles of 94 °C (30 s), different annealing temperatures for each primer pair (30 s) and 72 °C (45 s), with a final extension step of 8–10 min at 72 °C. The PCR reactions were performed on a PTC-100 thermocycler (MJ Research, Watertown, MA, USA). The RT-PCR products were sequenced twice with different primers using the Dye Terminator Cycle Sequencing kit in an ABI 310 automatic sequencer (Applied Biosystems, Warrington, UK) and aligned with the MegAlign software (Winstar program package). Primer sequences, annealing temperatures and amplicon sizes are given in Table II. The assembled fragments form a 1885 bp sequence that covers the 5'UTR and exons 1–8 (1788 bp).

A similar procedure was used for sequencing the coding region of the *ESR2* gene. Four other overlapping cDNA fragments were amplified with primers designed from the pig *ESR2* cDNA sequence (AF164957), covering exons 1–8. Technical details can be found in Muñoz *et al.* [18]. The assembled fragments cover 1561 bp of the *ESR2* gene coding region (1581 bp), between the positions 17 and 1571. This nearly complete *ESR2* cDNA was re-sequenced in six pigs from the Iberian, Large White and Meishan breeds and in two Spanish Wild boars.

Table II. Primer sequences, annealing temperatures, and amplicon sizes used for sequencing and genotyping the *ESR1* gene.

Name	Primer sequences	Annealing Temp. (°C)	Size (bp)	Exon
ESRa-F	5'-TAACCGCGGGCTGTGCTCTTCTTC-3'	63	510	5'UTR-Exon1
ESRa-R	5'-CCGCTCGGCTCATTCTCCAGGTAA-3'			
ESRb-F	5'-GCCGCAGCTCTCGCCCTTCCT-3'	63	501	Exons 1–4
ESRb-R	5'-ACCGCTTCATTCCTGCCCTCTCCA-3'			
ESRc-F	5'-GGGGGATACGGAAGACC-3'	57	551	Exons 4–6
ESRc-R	5'-ACGGAAGCGAGATGATGTAGC-3'			
ESRd-F	5'-TGTGTGCGAGGGAATGGTGGAG-3'	58	540	Exons 6–8
ESRd-R	5'-TCAGATTGTGGTGGGGAAGTT-3'			
EX5F	5'-CCTCCATGATCAAGTGCATCTTCT-3'	55.5	138	Exon 5
EX5R	5'-CAGCCAGGTCACCTACTGTCCAG-3'			
EX5pyr	5'-TTTGCTCCTAACTTGCT-3'			Exon 5
EX7F	5'-GGAGTGTACACGTTTCTGTCC-3'	55	184	Exon 7
EX7R	5'-TCATGTGCCTGAAGTGAGAC-3'			
EX7pyr	5'-TCCTGGACAAGATCACA-3'			Exon 7

2.3. SNP identification and genotyping

The alignment of the obtained *ESR1* cDNA sequences and the reference sequence Z37167 showed three SNP within exons 3, 5 and 7, respectively and two SNP within exon 8. The polymorphisms detected in exons 5 and 7 were genotyped on genomic DNA samples by pyrosequencing and the first polymorphism detected in exon 8 by PCR-RFLP *AvaI* [4], respectively. Pyrosequencing was performed in a PSQ96 system (Pyrosequencing AB, Uppsala, Sweden). PCR primer sequences (EX5F-EX5R and EX7F-EX7R) and two pyrosequencing primers (EX5pyr and EX7pyr) were designed and are listed in Table II. The previously described intronic *PvuII* polymorphism was genotyped on genomic DNA samples to identify alleles *A* and *B* following the published PCR-RFLP protocol [27]. The *PvuII* polymorphism and the SNP detected in exon 3 co-segregated as well as both SNP in exon 8.

The alignment of the obtained *ESR2* cDNA sequences from diverse genetic origins and the reference sequence AF164957 revealed two SNP within exons 2 and 5, respectively. The SNP detected in exon 5 introduces a polymorphic *Hsp92II* restriction site, and was genotyped on genomic DNA samples by a PCR-RFLP protocol previously described [18].

2.4. Statistical analysis

The following univariate repeatability animal model was used to estimate genetic parameters for NBA and TNB:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}_1\mathbf{u} + \mathbf{Z}_2\mathbf{p} + \mathbf{e}$$

where $\mathbf{y}$ included the 5932 litter size records (NBA and TNB), $\boldsymbol{\beta}$ the systematic effects of parity order (6 levels), genetic line of the litter's sire (Chinese-European or Landrace) and herd-year-season, and $\mathbf{u}$ and $\mathbf{p}$ the polygenic and permanent environmental random effects, respectively. Heritabilities ($h^2 = \sigma_u^2/\sigma_y^2$) and coefficients of permanent environmental effects ($p^2 = \sigma_p^2/\sigma_y^2$) estimated from these data were 0.13 ± 0.05 and 0.09 ± 0.04 for NBA, and 0.14 ± 0.06 and 0.11 ± 0.05 for TNB, respectively. Similar models were used for the association analyses fitting in $\boldsymbol{\beta}$ the *ESR1* or *ESR2* SNP genotypic effects, and assuming the estimates of variance components as true values. The additive effect (a) of each SNP was measured as half difference between the estimated effects of homozygous genotypes, and the dominance effect (d) as the deviation of the heterozygous genotype effect from the mean effect of the homozygous genotypes. Computations for all these analyses were performed using the VCE-5 software [11].

3. RESULTS

In *ESR1* in samples from Chinese-European pigs, we detected five silent SNP within exons 3 (c.669T > C), 5 (c.1227C > T), 7 (c.1452C > T) and 8 (c.1665T > C and c.1755A > G). In the *ESR2* in samples from Iberian and Chinese-European pigs, one silent SNP was detected within exon 2 (c.486A > G) in samples from the Meishan breed, and moreover one missense SNP within exon 5 (c.949G > A).

A total number of 408 Chinese-European sows with litter size records, 19 of their sires and 78 of their dams, were genotyped for the above quoted SNP identified in both genes. The distribution of the numbers of sows and litters by genotype and the allelic frequencies are provided in Table III. The *ESR1* *PvuII* and c.669T > C polymorphisms co-segregated in this line in the combinations [A; c.669T] and [B; c.669C]. The c.1665T > C and c.1755A > G *ESR1* allelic variants co-segregated in the combinations c.[1665C; 1755G] and c.[1665T; 1755A]. No co-segregation was observed between the c.1227C > T or c.1452C > T polymorphisms within exons 5 and 7, respectively and other SNP identified in the *ESR1* gene. Finally, the c.1452C > T polymorphism

Table III. Number of sows (*n*) and litters (*N*) by genotype, and allelic frequencies of *ESR1* and *ESR2* genes.

ESR1						ESR2					
[PvuII; c.669T > C]			c.1227C > T			[c.1665T > C; c.1755A > G]			c.949G > A		
Genotype	<i>n</i>	<i>N</i>	Genotype	<i>n</i>	<i>N</i>	Genotype	<i>n</i>	<i>N</i>	Genotype	<i>n</i>	<i>N</i>
[A;T][A;T]	56	222	CC	116	433	[C;G][C;G]	200	774	AA	25	92
[A;T][B;C]	180	699	CT	208	868	[C;G][T;A]	166	661	AG	155	575
[B;C][B;C]	167	683	TT	81	308	[T;A][T;A]	39	174	GG	228	955
Allelic frequencies											
[A;T]	0.36		C	0.54		[C;G]	0.70		A	0.25	
[B;C]	0.64		T	0.46		[T;A]	0.30		G	0.75	

Table IV. The results of the association study of *ESR1* and *ESR2* genotypes with litter size: estimated means by genotype, and additive (*a*) and dominance (*d*) effects for the number of piglets born alive (NBA) and the total number born per litter (TNB). Standard errors are reported between brackets.

ESR1						ESR2					
[PvuII; c.669T > C]			c.1227C > T			[c.1665T > C; c.1755A > G]			c.949G > A		
Genotype	NBA	TNB	Genotype	NBA	TNB	Genotype	NBA	TNB	Genotype	NBA	TNB
[A;T][A;T]	0.02 (0.36)	0.08 (0.39)	CC	-0.50 (0.34)	-0.81* (0.36)	[C;G][C;G]	0.33 (0.39)	0.48 (0.42)	AA	0.02 (0.49)	-0.26 (0.53)
[A;T][B;C]	-0.09 (0.24)	-0.10 (0.26)	CT	-0.30 (0.29)	-0.57 (0.32)	[C;G][T;A]	0.15 (0.39)	0.16 (0.41)	AG	-0.02 (0.24)	0.00 (0.26)
[B;C][B;C]	0.00	0.00	TT	0.00	0.00	[T;A][T;A]	0.00	0.00	GG	0.00	0.00
<i>a</i>	0.01 (0.18)	0.04 (0.19)		-0.25 (0.17)	-0.40* (0.18)		0.17 (0.20)	0.24 (0.21)		0.01 (0.25)	-0.13 (0.26)
<i>d</i>	-0.10 (0.24)	-0.14 (0.25)		-0.05 (0.22)	-0.17 (0.24)		-0.02 (0.26)	-0.08 (0.27)		-0.03 (0.30)	0.13 (0.32)

* ($P < 0.05$).

deviations from the genotype c.949GG, were not significantly different from zero (Tab. IV).

4. DISCUSSION

First reports regarding the *ESR1* *PvuII* SNP effects on litter size [24, 27] stated the favorable additive effect of the *B* allele to be the largest in pig lines of Meishan origin (1.4 piglets per copy) and intermediate in Large White based lines (0.4 piglets per copy). However, Rohrer *et al.* [22] failed to detect an effect of the *ESR1* *PvuII* SNP on ovulation rate and uterine capacity in a multi-generation Meishan $\times$ White composite experimental population. Also, Gibson *et al.* [6] found no detectable association of this SNP with sow productivity traits in a Meishan $\times$ Large White F_2 intercross. In a recent study performed on records from 1250 Czech Large White sows and 3600 litters, the *B* allele was found to be disadvantageous to the *A* allele for prolificacy [7]. The results of the present study also failed to confirm significant effects of the *ESR1* *PvuII* SNP on litter size in a composite Chinese-European line. It can also be outlined that this negative result cannot be attributed to a low sample size of the experiment. The number of animals and records of each genotypic class allows the detection of additive effects of at least 0.4–0.5 piglets per litter (Tabs. III and IV).

The main result of this paper is the estimation of a positive effect on litter size of a new detected SNP (c.1227C > T) located in the coding region of the *ESR1* gene, with a favorable effect of the c.1227T allele (0.4 piglets per copy) on the total number born per litter. However, this polymorphism is a silent change, and therefore is not likely to be the causative mutation of a positive effect on litter size. One feasible explanation could be that this SNP is in linkage disequilibrium with the causative mutation, as it probably occurs in other pig populations with the *ESR1* *PvuII* polymorphism. All the results obtained in this study concerning the *ESR1* gene allow us to discard the coding region of this gene as the possible location of the causative mutation. The c.1227C > T polymorphism could be useful as a LD marker to select boars in the breeding nucleus of this composite line among full-sib males with the same estimated breeding value for litter size. However, the effective implementation of a LD-MAS program based on this SNP requires careful measurement of its possible phenotypic effects on growth and carcass traits, continuous monitoring and re-evaluation of its effects on litter size [3].

Whereas all the SNP detected in the coding region of the *ESR1* gene are silent polymorphisms, the c.949G > A SNP identified within exon 5 of the

ESR2 gene is a non-conservative substitution. This alteration involves the replacement of the non-polar amino acid, valine, by the polar sulfur containing amino acid, methionine, which could modify the secondary and tertiary structure of the protein due to the different ability of these amino acids to form hydrogen and di-sulfide bonds. The potential deleterious effect of the mutation p.Val317Met located in the ligand-binding domain is suggested by its conserved amino acid position among some mammalian species (*Rattus norvegicus*, Genbank accession number: Q62986, *Mus musculus*: O08537 and *Bos taurus*: Q9XSB5). Although all these data support the possible biological relevance of this amino acid change, we found no detectable association between the *ESR2* c.949G > A SNP and the litter size traits, and this polymorphism can be discarded as a direct or LD marker in this composite Chinese-European pig line.

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