J. Anim. Breed. Genet. ISSN 0931-2668

ORIGINAL ARTICLE

Detection of novel single-nucleotide polymorphisms (SNPs) in the *CYP21* gene and association analysis of two SNPs for *CYP21* and *ESR2* with litter size in a commercial sow population

B. Buske, I. Sternstein, M. Reißmann & G. Brockmann

Department of Breeding Biology and Molecular Genetics, Agricultural and Horticultural Faculty, Institute of Animal Sciences, Humboldt-University of Berlin, Berlin, Germany

Correspondence
Bernd Buske, Department of Breeding Biology and Molecular Genetics, Agricultural and Horticultural Faculty, Institute of Animal Sciences, Humboldt-University of Berlin, 10115 Berlin, Invalidenstr. 42, Germany.
Tel: +49-30-2093 6449;
Fax: +49-30-3093 6397;
E-mail: bernd.buske@staff.hu-berlin.de

Received: 7 April 2006;
accepted: 3 August 2006

Summary

Altogether 129 F_1 sows from a commercial sow farm with at least four litters were genotyped for the oestrogen receptor 2 gene (*ESR2*) and cytochrome P450 hydroxylase 21 gene (*CYP21*) and investigated for associations on the litter-size parameters: total number born and number born alive. Five novel polymorphisms were found in the 3′-untranslated region for the *CYP21* gene. Genotype and allele frequencies for the *CYP21* (position 3462G > A) single-nucleotide polymorphism (SNP) were 0.434 (GG), 0.504 (AG), 0.062 (AA) and 0.69 (G):0.31 (A), respectively. No association was found between this polymorphism and litter-size parameters. For the *ESR2* gene, the SNP in exon 5 associated with an amino acid substitution MET (allele A) > VAL (allele G) was investigated. Only two genotypes were found leading to allele frequencies of 0.34 (A):0.66 (G). Only number born alive piglets were significantly increased for the AG genotype ($p = 0.034$) with 11.64 piglets per sow and litter in comparison with the GG genotype, leading to only 10.96 piglets per sow and litter. From these data, it can be concluded that the investigated SNP of the *ESR2* gene is associated with the number of live-born piglets in the commercial population considered, and hence could be useful in selection for litter size. Therefore, this gene should be investigated in additional populations.

Introduction

The genetic improvement of litter size in pigs is of increased interest for pig producers because further improvements in feeding regime and housing systems are limited. Up to the early 1990s, improvements were essentially made in growth and carcass traits, for example increases in average daily gain and reduction of backfat thickness, respectively (Merks 2000). This led to a stagnancy, and in some cases even to a decrease of litter size (Kisner *et al.* 1995). As litter size is also an economically important trait for pig producers, it can be inferred that an improvement of fecundity in pigs is desirable for the future. Recently, marker-assisted selection (MAS) in conjunction with traditional selection methods has been implemented to increase litter size in pigs. One marker, which is successfully used in commercial pig production is the oestrogen receptor 1 gene (*ESR1*). Rothschild *et al.* (1996) were the first to observe an association between ESR1 genotypes and litter size in pigs. These observations were confirmed by Short *et al.* (1997). There are also some inconsistent studies with regard to the influence of the ESR1 gene on the fecundity parameters in pigs (Alfonso 2005; Buske *et al.* 2006a). Therefore, additional candidate genes with a potential influence on litter size are studied.

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Concerning oestrogen receptors, there are at least two different classes. Muñoz *et al.* (2004) mapped the oestrogen receptor 2 (*ESR2*) gene at the telomeric end on the q-arm of SSC1 in contrast to the ESR1 gene, which is located at the telomeric end on the p-arm of SSC1. The polymorphism in the coding region of exon 5 in the *ESR2* gene leads to an amino acid substitution (MET > VAL) in the hormone-binding domain, which is critical because of its role as a transcription factor. The *ESR2* gene has been characterized in the rat, mouse and humans, and several studies suggest that this receptor displays a high binding affinity to oestrogens and therefore might be involved in ovarian follicular growth and development at peri-implantation (Kowalski *et al.* 2002). Hence, *ESR2* could also serve as a candidate gene for litter size in pigs.

Cytochrome P450 hydroxylase 21 (*CYP21*) has been mapped on SSC7 between the major histocompatibility complex (MHC) class I and II regions (Geffrotin *et al.* 1990, 1991). It was chosen as the candidate gene because several quantitative trait loci (QTL) for reproductive traits such as uterine capacity, ovulation rate and litter size have been detected within this chromosomal region (Buske *et al.* 2006a). From a physiological point of view, 21-hydroxylase deficiency leads to drastic fertility changes in human females (New 1995). A search for QTL for ovulation rate in cattle was performed by Blattman *et al.* (1996). They investigated the ovulation rate by counting corpora lutea over eight to 10 consecutive oestrous cycles and observed that *CYP21* was significantly associated with ovulation rate. Knoll *et al.* (1998) found new single-nucleotide polymorphisms (SNPs) for this gene in several introns by sequencing and developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test for these SNPs. They determined allele frequencies at five SNPs in the pig breeds, Large White, Landrace, Duroc and Piétrain. In their study, allele frequencies differed for most alleles between the different breeds, except for Duroc pigs, in which one of the alleles was always fixed at all the five loci.

However, until now, there has been no study of the association between *CYP21* genotypes and fecundity in pigs. Therefore, the aim of our study was to investigate whether the candidate genes *ESR2* and *CYP21* are associated with litter size.

Material and methods

Preliminary investigations in our animal material showed no polymorphisms for the *CYP21* gene with conventional PCR-RFLP methods developed by Knoll *et al.* (1998). Hence, for this gene it was necessary to search for SNPs by gene sequencing.

Animals

Animals for sequencing

In order to detect new SNPs in the *CYP21* gene, a total of 18 pigs were sequenced; 14 F_2 sows (no full or half-sibs) of a cross between (Large White × Landrace) sows × Leicoma boars from the commercial sow farm 'Polkenberg' (Sachsen, Germany) were used. There were altogether 447 F_2 sows with at least four litters, of which seven with extremely high and extremely low litter size (second up to fourth litter) were selected. The 14 F_2 sows represented the most extreme tails of the phenotypic distribution for litter size of the F_2 generation. Additionally, one Duroc sire and three German Landrace dams from the commercial sow farm 'Schulzendorf' (Brandenburg, Germany) were used for sequencing.

Animals for association study

An association study between gene variants and litter-size parameters was performed with 129 half-sib F_1 sows from the commercial sow farm 'Schulzendorf'. The F_1 sows were the daughters from the sequenced Duroc sire and from 40 German Landrace dams. Litter-size parameters were the number of total number born (TNB) and the number of born alive (NBA) piglets. Each F_1 sow had four or five litters. Both the feeding and housing regime of the F_1 sows was kept constant over the whole experimental period. Diseased sows were excluded from the experiment. Furthermore, it was ensured that boars for producing the fattening piglets were distributed randomly to the F_1 sows to avoid significant paternal influence on litter size. All F_1 sows were inseminated three times per mating with a constant amount of fresh sperm via artificial insemination. DNA was obtained from ear chondral tissue and isolated according to standard methods.

Sequencing of the 3′UTR for the *CYP21* gene

A suitable primer pair was developed using the databases NCBI, Repeat Masker at EMBL and Primer3 Input as well as the publicly available sequence information of the *CYP21* gene (accession number M83939). Primers flanked a small part of exon 10 and the complete 3′-untranslated region (3′UTR). This region was chosen because it is generally accepted that the probability for finding polymorphisms is

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increased for the 3′UTR in comparison with coding regions. The length of the amplified fragment including the complete 3′UTR was 791 bp. Standard PCR was performed in a final reaction volume of 25 μl by using approximately 75 ng of genomic DNA, 1x *Taq* reaction buffer without $MgCl_2$, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM from each primer (Sigma-Genosys, Steinheim, Germany) and 1 U *Taq* DNA Polymerase (Invitek, Berlin, Germany). Primer sequences were: fw: 5′-AGG TAC AGC CTT TCC AGG TG-3′ and rv: 5′-CAA CCT CAA CGG GCT CTA TG-3′. PCR was performed by 35 cycles comprising denaturation at 94°C for 2 min, annealing at 59°C for 30 s and extension at 72°C for 45 s and completed by a final extension step at 72°C for 2 min.

Polymerase chain reaction products were loaded on a 2% agarose gel (SeaKem, Rockland, ME, USA). A 100-bp ladder as the length standard was added to each run in order to recognize the correct bands. PCR products were cut from the gel and purified with the JustSpin Gel Extraction Kit (Genaxxon, Martinsried, Germany). Afterwards, DNA concentration and purity were determined by spectral photometry using the Nanodrop photometer (NanoDrop Technologies, Rockland, DE, USA). Sequence PCR was performed in a total reaction volume of 10 μl by using the Big Dye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) with the same primers as described above. The amount of DNA was adjusted to 2 ng/100 bp PCR fragment length. Amplification was performed comprising 25 cycles with 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Afterwards, PCR products were analysed by using a gene sequencer ABI 310 (Applied Biosystems, Foster City, CA, USA). Each sample was sequenced twice in both directions in order to avoid a misinterpretation of base pairs because of weak signals at the end of each sequence. Sequence comparison was performed with the program DNAStar (LaserGene, Madison, WI, USA). Validated SNPs showed either all three expected genotypes or two genotype classes which were confirmed by cleavage with an appropriate restriction enzyme (Table 1).

Table 1 Positions and genotype distributions of SNPs for the *CYP21* gene

	Position[a]	Base pair substitution	Genotype distribution[c]	Restriction enzyme recognition site
1	3462[b]	G/A	8 GG; 10 GA	*Hsp*92II
2	3514	C/T	7 CC; 11 CT	*Hae*III
3	3612	A/T	11 AA; 6 AT; 1 TT	
4	3638	G/A	15 GG; 2 GA; 1 AA	
5	3724	G/A	13 GG; 4 GA; 1 AA	
6	3904	T/C	9 TT; 1 TC; 5 CC[d]	*Pst*1
7	3911	T/C	1 TT; 1 TC; 16 CC[d]	

[a]The position is given according to the sequence for accession number M83939.
[b]Used for our PCR-RFLP test for association analysis.
[c]Genotype distribution of 18 unrelated pigs; only 15 pigs could be genotyped for the sixth polymorphism at position 3904.
[d]Last two SNPs belong to the neighbouring gene.

PCR-RFLP for the CYP21 gene

At position 273 in the PCR product (position 3462 in the GenBank sequence), a restriction site (G > A) for *Hsp*92II enzyme was used for developing a PCR-RFLP test (Figure 1) to analyse all 129 F_1 sows. This restriction site was also chosen because the sire was heterozygous at this SNP. After performing standard PCR as described before, RFLP was performed by incubating the samples in a total reaction volume of 20 μl including 10 μl PCR product, 0.2 μl bovine serum albumin (BSA), 5 U of the enzyme *Hsp*92II and 2 μl appropriate restriction buffer (Promega, Mannheim, Germany) for 4 h at 37°C. Bands were separated and visualized by gelelectrophoresis. Exemplary genotypes are shown in Figure 2. Genotypes could be distinguished because of a 624-bp band (GG), 520 bp and 104 bp bands (AA) and all the bands 624, 520 and 104 bp (AG). An ubiquitary band of 167 bp was also visible.

PCR-RFLP for the ESR2 gene

The investigated SNP of the *ESR2* gene is associated with an amino acid substitution MET (allele A) > VAL (allele G). Genotyping was performed according to the method described by Muñoz *et al.* (2004).

Statistical analyses

Associations of genotypes with TNB and NBA were calculated by analysis of variance. Besides the three genotypes of each gene, seasonal effects (four levels), and litter number (2, 3, ≥4) were also included as fixed effects in the mixed linear model, and the effect of the 40 dams was handled as a random effect. According to most previous investigations by other authors, the first litter was excluded as it is generally accepted that the first litter is significantly smaller and often shows higher variability than the following litters (Buske *et al.* 2006b). All statistical analyses were performed with the SPSS program (version 12.0).

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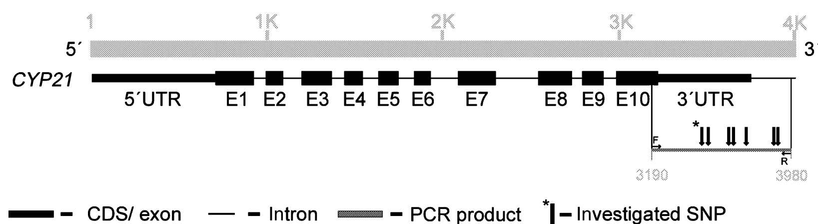

Figure 1 Gene structure of porcine *CYP21* including five novel polymorphisms; reference: NCBI database, modified; last two SNPs belong to the neighbouring gene.

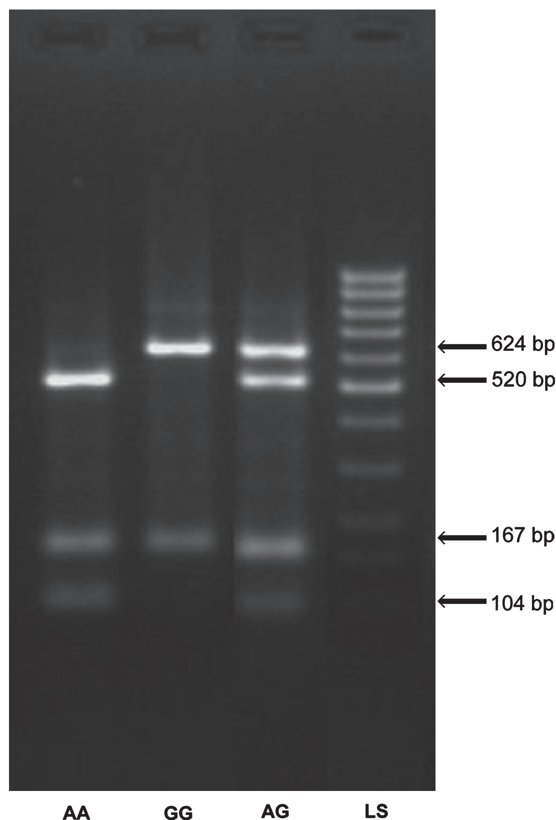

Figure 2 Different genotypes for the *CYP21* (g.3462 G > A) polymorphism by the PCR-RFLP method; LS = 100 bp length standard; 167-bp band is ubiquitary; last band (104 bp) is only weakly visible.

Results

Detection of new polymorphisms in the 3′UTR of the *CYP21* gene and development of a PCR-RFLP gene test

Table 1 provides the positions of seven detected SNPs from which five are located in the 3′UTR. Two of these polymorphisms are located in restriction enzyme recognition sites. At base pair position 3462, the sire of the F_1 sows was heterozygous. Therefore, this polymorphism was used to genotype all the 129 F_1 sows with the PCR-RFLP test. Figure 1 shows the gene structure of porcine *CYP21* including the novel detected polymorphisms. In Figure 2, we present the three different genotypes for the SNP at position 3462 for the *CYP21* gene.

Genotype and allele frequencies for the investigated SNPs for *CYP21* and *ESR2* genes

Table 2 shows the genotype and allele frequencies for the *CYP21* and *ESR2* genes in 129 F_1 commercial sows. Although all genotypes could be found for the *CYP21* gene, there was no AA genotype detectable for the *ESR2* gene because the sire was homozygous GG for this polymorphism.

Associations between genotypes and litter-size parameters (TNB, NBA)

Table 3 shows the results of association analyses between genotypes of the *CYP21* and *ESR2* genes and litter-size parameters TNB and NBA piglets, respectively. For the *CYP21* polymorphism at position g.3462G > A, no significant association with TNB or NBA piglets was found. Concerning the ESR2 gene, the results only indicated a significant association with NBA piglets. The AG genotype led to significantly more liveborn piglets than the GG genotype ($p = 0.034$). In the population studied, an effect size of 0.68 more NBA piglets per sow and litter for the AG genotype was sufficient to detect significant differences between the two genotypes of the 129 F_1 sows comprising 491 litter records.

Table 2 Genotype and allele frequencies for the *CYP21* and *ESR2* genes

		Genotype frequency			Allele frequency	
Gene	n	AA	AG	GG	A	G
CYP21[a]	129	0.062	0.504	0.434	0.31	0.69
ESR2	129	0.000	0.674	0.326	0.34	0.66

[a]Investigated SNP at position 3462 according to the sequence for accession number M83939.

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Table 3 Associations between genotypes and litter-size parameters (TNB, NBA) for the SNPs of *CYP21* and *ESR2* genes of 129 commercial F_1 sows

Gene	No. of sows	No. of litters[1]	LS mean TNB (≥2 litter)[1]	SE	LS mean NBA (≥2 litter)[1]	SE
CYP21[2]						
AA	8	31	13.06	0.64	12.00	0.59
AG	65	246	12.54	0.26	11.44	0.24
GG	56	214	12.22	0.29	11.30	0.27
ESR2						
AG	87	329	12.60	0.25	11.64[a]	0.23
GG	42	162	12.02	0.32	10.96[b]	0.30

[a,b]LS mean values in the same column with different superscripts significantly differ at $p < 0.05$.

[1]For each sow, second up to fourth or fifth (when available) litter was analysed. First litter was ignored.

[2]Investigated SNP at position 3462 according to the sequence for accession number M83939.

SE, standard error; TNB, total number born piglets; NBA, number born alive piglets.

Discussion

CYP21 was chosen as the candidate gene because several overlapping QTL have been found at the centromeric region on SSC7 (Buske *et al.* 2006a) in which *CYP21* is located (Peelman *et al.* 1996). Furthermore, an association between *CYP21* and ovulation rate has been found in cattle (Blattman *et al.* 1996). Because the 129 F_1 sows in our population were daughters from only one sire, the strategy was to search for heterozygous SNPs in the sire to preferably obtain three genotypes in the F_1 sows. For this purpose, the restriction site at position g.3462 in the 3′UTR was used to design a PCR-RFLP test. Polymorphisms in the 3′UTR can lead to differences in mRNA stability, and therefore, to differences in the phenotype. Such polymorphisms occur relatively often, and thus, were chosen for the *CYP21* candidate gene. The idea of investigating F_1 sows descending from only one sire was to minimize the variation in genetic background. However, no significant association on any litter-size parameters was found. To our knowledge, up to now, there has been no published study concerning the association between *CYP21* genotypes and litter-size parameters in pigs.

The SNP in exon 5 of the ESR2 gene was chosen for our association study because of the physiological aspects of estrogenes. Muñoz *et al.* (2004) investigated this polymorphism in two Spanish pig breeds (46 Torbiscal and 150 Guadyerbas sows) and found no significant association on TNB piglets. Our study was not complete in a sense that no sows with the GG genotype were found, so a calculation of additive and dominance effects was impossible. This was due to the sire being homozygous GG. Therefore, a comparison between the results obtained by Muñoz *et al.* (2004) and our results remains difficult. However, in our study, 0.68 more liveborn piglets were observed for sows with the AG genotype. The difference was statistically significant in comparison with sows with the GG genotype. Up to now, no QTL for litter-size parameters in pigs has been reported in the chromosomal region, in which *ESR2* is located, as it is also the case for the *ESR1* gene (Buske *et al.* 2006a).

One of the most important and earliest publications concerning candidate gene approaches for fecundity in pigs concerns the *Pvu*II restriction site of the *ESR1* gene by Rothschild *et al.* (1996). These authors detected additive effects of about 0.5 piglets for the B allele compared with the A allele for both TNB and NBA piglets in synthetic PIC lines with Large White ancestory; 1079 sows with a total of 1912 litter records were evaluated in their study. Because these authors performed a planned mating test by crossing parents of AA × AB, AB × AB and AB × BB genotypes, they were able to obtain nearly balanced genotype frequencies in the F_1 sows. Balanced genotype frequencies are important to detect gene effects with sufficient statistical power. Therefore, to evaluate the effect of *ESR2* genotypes on litter-size parameters more reliably, an improvement would be to perform an additional investigation with planned matings to obtain all genotypes in balanced frequencies in the sow population. Furthermore, a population-wide candidate gene approach with a larger data set would be desirable in order to avoid overestimation of effects, which can occur by chance in association analyses with comparatively low animal numbers. As the investigated polymorphism in the ESR2 gene is located in an exon region and because our results are promising, *ESR2* can be considered in future analyses as a potential genetic marker to increase the number of piglets born alive.

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