Investigation of candidate genes with potential influence on litter size in two commercial pig cross populations

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List of Abbrevations

a	additive effect
BF	properdin (factor B)
BLUP	best linear unbiased prediction
bp	base pairs
cM	centi Morgan
CYP21	cytochrome P450 steroid 21 hydroxylase
d	dominance effect
D	degree of dominance
DL	Deutsche Landrasse
DNA	desoxyribonucleic acid
Du	Duroc
ESR(1)	estrogen receptor 1
ESR2	estrogen receptor 2
F_1	filial generation 1
F_2	filial generation 2
FUT1	fucosyltransferase 1
g	acceleration of gravity
GPX5	glutathione-peroxidase 5
λ	wavelength
Lc	Leicoma
Lr	Landrace
LW	Large White
M	Mol/ Liter
MAS	marker assisted selection
MHC	major histocompatibility complex

N	number
NBA	newborn alive
PCR	polymerase-chain-reaction
P	parental generation
Pi	Piétrain
QTL	quantitative trait loci
r	coefficient of correlation
RFLP	restriction-fragment-length-polymorphism
SD	standard deviation
SE	standard error
SNP	single nucleotide polymorphism
SSC	sus scrofa chromosome
TNB	total newborn
U	unit
3'UTR	3' untranslated region

1 Summary

1.1 Summary English version

The improvement of desirable traits due to breeding methods in animal farming is still performed by regarding mainly phenotypical selection. Concerning fecundity in swine, this is inefficient and time-consuming due to long generation intervals and high animal numbers, often associated with high costs. However, the improvement of fecundity of sows is of expanding interest for pig producers mainly because improvements concerning feeding regime and housing systems are limited. Furthermore, an improvement of fecundity is also desirable, especially where the achievment potential cannot be exploited because of suboptimal feeding and housing regimes for example in tropical and subtropical regions in order to ensure a sufficient provision of livestock products in the future. Marker assisted selection, employed in conjunction with traditional selection methods is in progress to increase litter size in swine and can accelerate breeding progress. In the past years, researchers began to search for genes underlying reproductive traits in swine. Beside QTL-analyses, in which chromosomal regions can be detected for quantitative traits such as litter size, also the candidate gene approach has been performed by testing one, or several genes directly to associations on fecundity traits. Rothschild et al. (1996) were the first, who published a candidate gene approach with regard to litter size in swine and reported a genetical effect of the estrogen receptor 1 gene (ESR1) for litter size in pigs. Since this time, several candidate genes have been investigated with regard to different fecundity parameters in swine, however, results were often extremely inconsistent.

The aim of this thesis was first to evaluate all chromosomal regions (QTL) and candidate genes with association on fecundity in swine, second, to deduce suitable candidate genes and third, to genotype them by using PCR-RFLP methods in order to investigate them for associations to fecundity in two commercial sow populations.

As phenotypical trait for fecundity, litter size comprising the total number of born and the number of born alive piglets was chosen according to convenience of its measurement and because of great economical importance of this trait. The selection of candidate genes depended on positional, physiological and comparative aspects. Selected candidate genes were properdin (BF), estrogen receptor 2 (ESR2), glutathione peroxidase 5 (GPX5), fucosyltransferase 1 (FUT1) and cytochrome P450, steroid 21 hydroxylase (CYP21). Two

commercial sow populations were used to test the association between gene variants and fecundity. In the first commercial sow farm, 123 out of 447 F_2-sows with at least four litters were selected with extreme high and extreme low total number born piglets for genotyping (two-tail analysis). The high performance group consisted of 61, wheras the low performance group consisted of 62 sows. In the second commercial sow farm, a total of 129 F_1-sows with at least four litters were available for the analyses.

Evaluation of all published data so far showed that there were 54 QTL, which overlap in part, and 10 candidate genes with association to fecundity parameters in swine, respectively. Fecundity parameters were litter size, ovulation rate, uterine capacity, age of puberty, gestation length and number of nipples. SSC8 and to a lower extent SSC7 and SSC15 are the chromosomes on which most QTL have been found so far.

Concerning own results, it can be stated that GPX5 and CYP21 had no effect on litter size in our sow populations. Moderate effects on litter size parameters have been found for the BF and FUT1 genes. However, due to suboptimal animal structures in both commercial sow farms, these results should be considered with caution. For the mutation in exon 5 of the coding region of the ESR2 gene, a significant increase of 0.68 liveborn piglets per sow and litter could be found for the AG genotype in the second sow farm. Due to the pedigree structure, the AA genotype was not detectable. Finally, five novel polymorphisms have been found in the 3' untranslated region of the CYP21 gene by gene sequencing.

Most important conclusions are that the improvement of fecundity in swine is one of the most difficult tasks for geneticists as it is determined by several genes due to several chromosomal regions and candidate genes with association to fecundity in swine. "Fine mapping" of QTL regions is necessary in order to narrow QTL intervalls and the number of potential positional candidate genes. In order to obtain balanced genotype frequencies, planned matings should be performed for candidate gene approaches in the future. High standardization of environmental parameters is strongly required due to low heritability of litter size. Therefore, working with commercial sow farms is limited for further investigations on genetical influences on fecundity in swine. SSC8, the centromeric region of SSC7 and the ESR2 gene on SSC1 should be considered for further investigations in this field.

1.2 Summary German version (Zusammenfassung)

In der Tierzüchtung wird die Eignung von landwirtschaftlichen Nutztieren zur Nutzung gewünschter Merkmale bisher überwiegend nur anhand ihrer phänotypischen Leistung überprüft. Im Hinblick auf Fruchtbarkeitsparameter bei multipaaren Tieren ist dies in der Schweinezucht mit langem Generationsintervall, zuchtorganisatorischem Aufwand, großen Tierzahlen und hohen Kosten verbunden. Die Selektion auf Fruchtbarkeitsmerkmale stellt aber gerade beim Schwein eine aus ökonomischen Gesichtspunkten sehr wichtige Aufgabe der Tierzucht dar. Eine Erhöhung der lebend geborenen Nachkommen als wichtigstem Merkmal der Fruchtbarkeit könnte dazu beitragen, auch in Zukunft eine ausreichende Versorgung mit tierischen Produkten zu gewährleisten. Dies gilt auch, wenn aufgrund suboptimaler Fütterungsbedingungen (z. B. den Tropen und Subtropen) keine Ausschöpfung des Leistungspotentials bzw. keine Verbesserungen möglich sind. Gelingt es zukünftig, ergänzende molekulargenetische Tests im Rahmen einer Marker gestützten Selektion (MAS) zum Einsatz zu bringen, ließen sich durch Vorselektion zeit- und kostenintensive Testverpaarungen der Elterntiere vermeiden. Als molekulargenetisches Verfahren, um entsprechende Gene zu detektieren, bietet sich – neben der QTL-Analyse zum Auffinden relevanter Chromosomenregionen – auch der Kandidatengenansatz an, bei welchem ein Gen direkt auf einen gewünschten Parameter hin untersucht wird. 1996 wurde erstmals eine Assoziation zwischen verschiedenen Östrogen-Rezeptor 1 Gen (ESR1) Varianten und der Wurfgröße beim Schwein publiziert. Seitdem wurden mehrere Kandidatengene auf verschiedene Fruchtbarkeitsmerkmale untersucht, allerdings wurden teilweise auch widersprüchliche Ergebnisse gefunden.

Das Ziel dieser Untersuchung war es daher, erstens eine Zusammenstellung aller bisher publizierten Chromosomenregionen (QTL) und Kandidatengenen mit Assoziation zu Fruchtbarkeitsmerkmalen beim Schwein anzufertigen, zweitens, geeignete Kandidatengene für Wurfgröße beim Schwein abzuleiten, und drittens, diese an zwei kommerziellen Zuchtsauen-Populationen mittels PCR-RFLP Methode zu genotypisieren und mittels biostatistischer Verfahren auf Assoziation zu Fruchtbarkeit zu untersuchen.

Für die Assoziationsstudien wurde als ökonomisch bedeutender und einfach zu messender Parameter der Phänotyp „Wurfgröße“ mit sowohl insgesamt als auch lebend geborenen Ferkel erfasst. Als Kandidatengene wurden aufgrund positioneller, physiologischer und komparativer Aspekte die Gene Properdin (BF), Östrogenrezeptor 2 (ESR2), Glutathionperoxidase 5

(GPX5), Fucosyltransferase 1 (FUT1) und Cytochrom P450 Steroid 21 Hydroxylase (CYP21) ausgewählt. Von 447 F_2-Sauen mit mindestens vier Würfen eines Praxisbetriebes wurden 61 Sauen mit extrem hoher, und 62 Sauen mit extrem niedriger Anzahl insgesamt geborener Ferkel für die Genotypisierung selektiert (two-tail Analyse). Von dem zweiten Praxisbetrieb wurden 129 F_1-Sauen genotypisiert, welche ebenfalls mindestens vier Würfe aufwiesen.

Die Anfertigung der cytogenetischen Karte und die Auswertung der bisher publizierten Ergebnisse ergab 54 zum Teil überlappende QTL und 10 Kandidatengene mit Assoziation zu Fruchtbarkeitsmerkmalen beim Schwein, wie Wurfgröße, Ovulationsrate, Uteruskapazität, Geschlechtsreife, Trächtigkeitsdauer und Anzahl Zitzen. Die meisten QTL wurden bisher auf Chromosom 8 gefunden, ferner sind auch Chromosom 7 und 15 von hervorgehobener Bedeutung.

Hinsichtlich der eigenen Ergebnisse konnten für die Gene GPX5 und CYP21 keine, für die Gene BF und FUT1 geringfügige Assoziationen zur Wurfgröße ermittelt werden, welche aber aufgrund des Tiermaterials nicht als zuverlässig betrachtet werden können. Lediglich für die Mutation im codierenden Bereich des ESR2 Gens konnte ein signifikanter Effekt von zusätzlich 0,68 lebend geborenen Ferkel pro Sau und Wurf zugunsten des AG Genotyps in einer Zuchtsauenanlage festgestellt werden. Der AA Genotyp wurde nicht gefunden. Für das Gen CYP21 wurden mittels Gensequenzierung ferner fünf neue SNPs im 3'-Bereich entdeckt.

Schlussfolgernd kann gesagt werden, dass, Fruchtbarkeit beim Schwein ein außerordentlich schwieriges, und aufgrund bisher zahlreich gefundener QTL und Kandidatengene ein hochpolygen vererbbares Merkmal ist. Um Chromosomenbereiche und die daraus resultierende Anzahl geeigneter positioneller Kandidatengene für Fruchtbarkeit einzugrenzen, stellt das „Fine mapping" für QTL-Analysen eine vielversprechende Strategie dar. Um balancierte Genotypenfrequenzen zu erhalten, wären Anpaarungstests für Kandidatengenansätze von Vorteil. Ferner ist eine hohe Standardisierung des Tiermaterials aufgrund niedriger Heritabilität von ausschlaggebender Bedeutung. Für weitere Untersuchungen im Hinblick auf Assoziation zu Fruchtbarkeit beim Schwein eignen sich daher Praxisbetriebe nur eingeschränkt. Chromosom 8, die Centromerregion auf Chromosom 7, sowie das ESR2 Gen auf Chromosom 1 sollten bei weiteren Untersuchungen verstärkt berücksichtigt werden.

2 Introduction

The improvement of fecundity of sows is of expanding interest for pig producers mainly because improvements concerning feeding regime and housing systems are limited. Furthermore, an improvement of fecundity is also desirable, especially where the achievment potential cannot be exploited because of suboptimal feeding and housing regimes for example in tropical and subtropical regions in order to ensure a sufficient provision of livestock products in the future. It has been also observed, that up to the middle of the nineties in the past century, improvements were first of all made concerning meat quality. This led to a stagnancy and in some cases also to a deterioration of fecundity-performance especially in swine production (Kisner et al., 1995). For example, in Germany, the number of newborn piglets alive (NBA) from the breed "Deutsche Landrasse" decreased from 11.3 at the beginning of the seventies up to the end of the past century to 10.3 (Steinheuer, 2001). As the number of NBA influences the economic success of pig producers, the selection on reproductive traits in swine is of great economic importance. So it can be concluded that an improvement of fecundity in swine is desirable in the future.

In the past, several breeding strategies have been performed in order to test breeding schemas and to exploit heterosis effects concerning the improvement of reproductive traits in swine. However, these breeding strategies were only based on phenotypical measurements and are expensive, time-consuming and difficult to perform. In France, for example, the generation of a special breed, named "Lignée hyperprolifique", based on breeds like "French Large White" and later also on "French Landrace" sows led to an improvement of the NBA piglets and of the number of weaned piglets per sow and year. Table 2.1 gives an overview on the improvements concerning reproductive traits of these sows.

Table 2.1: Reproductive efforts of sows of the breed "Lignée hyperprolifique"

Year	No. of farms	No. of litters	NBA/ litter	Weaned piglets/ sow and year	Interval between farrowings (days)
1970	1025	24979	10.3	16.4	187.9
1975	5077	306136	10.1	18.5	169.4
1980	8681	726658	10.2	20.2	160.2
1985	8564	908863	10.4	21.0	156.3
1990	4800	786757	10.8	22.2	154.0
1995	4153	906227	11.1	23.1	152.8
1997	3940	1016527	11.3	23.8	151.6

In the past years, researchers began to search for genes underlying reproductive traits in swine, and Rothschild et al. (1996) were the first, who reported a genetical effect for litter size in pigs. They investigated the estrogen receptor 1 gene (ESR1) with regard to litter size in pigs and found, that sows with the B allele are superior, leading to more offspring. The first, who reported a quantitative trait locus (QTL) with regard to reproductive traits in swine were Rathje et al. (1997). They could show that on the telomeric end of the long arm on porcine chromosome 8, a chromosomal region is located harbouring one or more genes which underlay for litter size. However, this study lacked in that case that not each chromosome was covered with microsatellite markers, and that markers were in most cases non-equally distributed over the genome, resulting in chromosomes or chromosomal regions for which possible QTL could not been detected. Since this time, many candidate genes and QTL for different reproductive traits have been reported. However, it must be stated that the reported results are often inconsistent. This is probably the reason for not or only unassertive introduction of molecular genetical information into marker assisted selection (MAS) in order to improve fecundity of sows so far. If the causative mutation of a gene is known, it would be easily possible to use it in MAS in order to accelerate genetical improvement. At this time, only the ryanodine receptor gene (RYR-1) test is used in order to eliminate homozygous stress susceptible boars (Wendt et al., 2000). This test, which is used to improve meat quality, was developed in 1991 by Fujii et al. (1991). Until today, no other genetical test is performed in pigs to a considerable extent. With regard to fecundity, the commercial use of genetic markers is at the beginning. The aim is to find genes underlying desirable traits such as litter size, and to establish genetical tests in order to introduce them into MAS for high fecundity in sows.

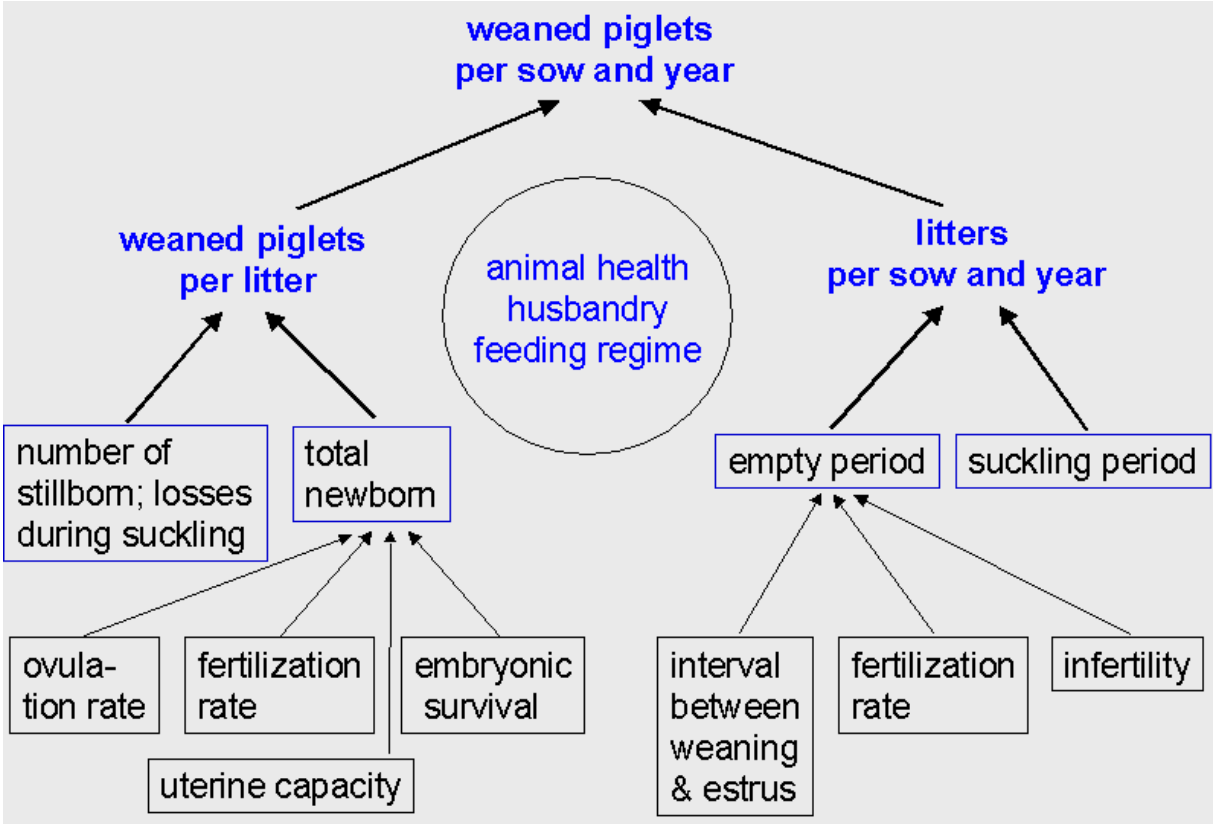
The objective of this doctoral thesis was

1) to review all published literature data on QTL and candidate gene analyses concerning fecundity of sows,
2) to derive suitable candidate genes with potential influence on litter size, and
3) to genotype them and investigate the relationship between genotype and litter size.

For this purpose, firstly, all published articles about QTL and candidate gene assays with regard to reproductive traits of sows were evaluated and compared. Focus was given to the fact that the results and conclusions differed among studies and were often inconsistent. The reasons for this phenomenon were evaluated and improvements for further investigations

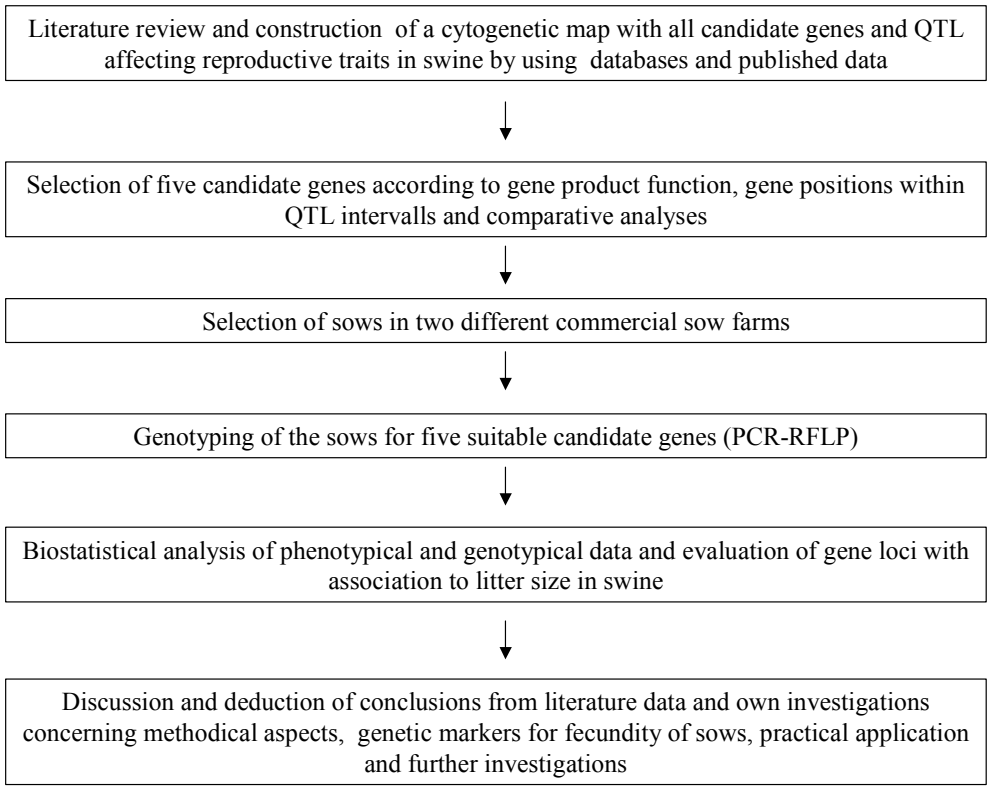
were derived. Furthermore, a cytogenetic map with all known QTL and candidate genes with influence on several reproductive traits was built in consideration of all available published data. Afterwards, suitable candidate genes with potential influence on litter size were chosen according to the following three main criteria: Firstly, physiological functions of the gene product in reproduction processes were consulted. Secondly, allowance was also made for comparative aspects between species, especially genes or chromosomal regions with influence on fecundity from mouse models as multiparous animals were considered (Brunsch, 1999). Thirdly, positional aspects, like genes, which are in a chromosomal region, in which a QTL for reproductive traits in swine has been detected, were included. Additional aspects were the availability of information about genotype and allele frequencies within breeds and information concerning the identification of polymorphisms through suitable laboratory methods, as for example PCR-RFLP. Five appropriate candidate genes with potential influence on litter size of sows were genotyped for known SNPs or sequenced to find new SNPs, and genotype groups were compared concerning their phenotypical performance. Litter size parameters such as TNB and NBA as phenotypical performance were firstly chosen because these parameters are easy to measure. Secondly, it enables us to compare own results with published results because many researchers also focused on this reproductive trait. Furthermore, in multiparous animals, litter size can be seen as the "final product" of reproductive-physiological procedures such as ovulation rate, uterine capacity, fertilization rate and embrionic survival (Figure 2.1). Moreover, litter size basically affects the number of weaned piglets per sow and year, which is the economically most important trait for pig producers.

Figure 2.1: Factors influencing the number of weaned piglets per litter and litters per sow and year



Because candidate gene assays can be generally carried out in any population (Rothschild et al., 1996), and because no reference families for gene mapping were available, we investigated animals from two different commercial sow farms. Figure 2.2 provides a general outline on the strategies for the analyses contributing to this doctoral thesis.

Figure 2.2: Flow chart about the strategy for finding genes with association to litter size in swine



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3 QTL and candidate genes influencing fecundity in sows: a review

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Abstract

Fecundity in pigs is a trait of major economic interest but low heritability. For the improvement of fecundity, genetic markers for selection are desirable and therefore, several searches for genetic variation influencing fecundity have been performed. The aim of this review is to compare and to evaluate all published QTL analyses and candidate gene approaches concerning reproductive traits in sows. For this purpose, we present a comprehensive cytogenetic map comprising 54 QTL and eleven candidate genes with influence on reproductive traits. The evaluation and comparison of the results showed similarities, but also marked differences among studies. Reasons for different results are multicausal and are due to differences between resource populations, number of evaluated animals, mating systems, measured phenotypical traits and environmental influences. We could show that chromosome 8 and to a lower extend chromosome 7 are the most important chromosomes with regard to reproductive traits in pigs. For further research, fine mapping of the identified QTL regions is necessary in order to confirm and to narrow the most likely chromosomal intervals. Although difficult to perform, an advance would be a standardization of the experimental setup in particular, in respect to the collection of phenotypic data. Furthermore, we suggest to publish the information on further identified QTL and candidate genes as comprehensive and accurate as possible in order to allow a more transparent comparison and collation of the results.

Key words:
Candidate gene approach, cytogenetic map, fecundity, pigs, QTL analysis, reproductive traits

Introduction

The improvement of reproductive traits in livestock species has become of expanding interest especially in pigs, where moderate increases in litter size can equal large gain in profit (Short et al., 1997; Vincent et al., 1998). However, until now, selection programs are almost only based on phenotypical traits which are laborious, expensive and especially in pig production time-consuming. Marker assisted selection (MAS), employed in conjunction with traditional selection methods, could accelerate the rate of change in economically important traits. However, so far only little is known about genetic variability that can be used to improve fecundity by selection of favorite alleles. Two approaches have been pursued to identify genetic markers for reproductive traits: First, genome scans employing anonymous DNA markers like microsatellites have been used to identify quantitative trait loci (QTL) for reproductive traits (Rathje et al., 1997; Rohrer et al., 1999; Wilkie et al., 1999). Second, candidate gene approaches as a direct gene assay have been employed in order to find associations between the gene itself and a phenotypical trait (Rothschild et al., 1996; Drogemuller et al., 2001; Jiang et al., 2001; Linville et al., 2001; Isler et al., 2002; Muñoz et al., 2004). Since 1996 when the estrogen receptor gene has been investigated with regard to influence litter size, several candidate gene and QTL analyses have been performed in order to find genes or regions with an impact on fecundity. However, until now, no gene itself with a causative mutation for such a trait has been detected through linkage analyses. Reasons for this failure are multicausal, e.g. low heritability and the fact that many genes contribute only for a small amount for fecundity parameters. There are two possible ways in which a gene can influence the productive performance: First, mutations in the coding regions of a gene may change the quality of the encoded protein, which is the case if mutations cause amino acid exchange, premature stop of translation or alternative splicing. Second, mutations in regulatory regions of the gene that affect the amount of transcripts of the gene in the cell and thus the amount of produced functional protein with unchanged quality. Concerning linkage and association studies, it cannot be distinguished between these two effects because both may cause measurable changes of the phenotype. However, major effects are often caused by severe changes of the primary protein structure causing a failure of the functional protein with dramatic consequences for the phenotype. Neutral amino acid substitutions as well as mutations in the regulatory regions are expected to cause small effect phenotypical changes. Our review bases only on association studies concerning candidate gene approaches and linkage studies concerning QTL-analyses. Therefore, with linkage or association studies

including candidate gene analyses we cannot distinguish between the two different types of effects. Finally, the aim is to find genes or at least closely linked markers to genes with an impact on fecundity in order to (pre-) select animals with the desirable genotype.

The aim of this article is to review the results of QTL analyses and candidate gene approaches with regard to fecundity in pigs. For this purpose, we present a comprehensive cytogenetic map comprising all published genes and QTL regions with an impact on fecundity. We compared the results, evaluated the reasons for the different findings and finally, give advice and strategies for further investigations.

General aspects of QTL studies and candidate gene approaches for reproductive traits in pigs

In order to detect loci for a special trait, there are in principle two strategies possible: QTL-analysis and the candidate gene approach (Rothschild et al., 2000; Omelka et al., 2001). There is a general debate about which of these two strategies is more powerful in order to detect genes for reproductive traits in pigs.

QTL-analyses

For QTL analyses as an "indirect gene approach" so far, microsatellites have been used because of their high polymorphic structure. Rathje et al. (1997) stated that it is important to select markers that are informative within a specific resource population for a genomic scan. Because at least three generations are required, QTL analyses are time-consuming and costly. Another aspect is, that it is generally accepted that the distance between the genotyped markers should be 20 cM or less, thus, a large number of informative markers is required. The resource populations consisted in crosses between animals of the breeds Large White, Landrace, Yorkshire, White composite and chinese Meishan pigs. In general, animal numbers varied between 114 and 600 genotyped F_2-sows and are rather low in comparison to QTL analyses performed with mice or to some candidate gene approaches performed with pigs. This is due to the fact that QTL analyses are more time-consuming because of the long generation interval in pigs and because animals must be genotyped for many markers contrary to candidate gene approaches, where only one or a few genes are investigated. However, when only teat numbers were investigated, also males were genotyped and their breeding values for teat number were investigated, and hence animal numbers increased (Rohrer 2000;

Hirooka et al., 2001). The aim of a QTL analysis is the identification of genomic regions, which are responsible for a desirable trait. So far, no gene with a causative mutation has been identified, which underlies a detected QTL effect concerning reproductive traits in pigs. The reasons for this failure are multicausal, but in general, identified QTL regions are usually wide, which makes it difficult to define a candidate gene or genes underlying an observed QTL effect. Therefore, QTL fine mapping is necessary to narrow the chromosomal region harboring the QTL and thus the number of potential candidate genes. According to Khatkar et al. (2004), fine mapping of QTL for economic traits is at an early stage in livestock and should be extended in the future. Furthermore, it is generally accepted that in polygenic traits with low heritability such as fecundity in pigs, many genes account only for a small amount of the phenotypic variance. Heritabilities for litter size for example were estimated between 0.06 and 0.15 by Roehe and Kennedy (1995) and between 0.00 and 0.16 by Holl and Robison (2003). Moreover, it is difficult to standardize all environmental influences for a longer period, especially when working with pigs. However, this is strongly required when analyzing genes which contribute only for a small amount to the trait in order to separate genetical from environmental influences. Some further aspects, concerning limitations and methods for QTL analyses and candidate gene approaches with regard to reproductive traits in pigs are presented in Table 3.1.

Table 3.1: QTL analysis in comparison to candidate gene approaches concerning reproductive traits in pigs

Parameter	QTL analysis	Candidate gene approach
Principle	indirect gene assay	direct gene assay*
Costs	high	moderate
Number of genotyped animals	middle (approx. 100 - 600)	extremely different
Accuracy	moderate because of the recombination frequencies	high, when the causative mutation is detected
Limitations	at least three generations are required	in principle not any, each animal of a population can be investigated
Expressiveness	no information concerning: - how many genes underlay the QTL - the causative gene - the favorable allele	high, because of a direct gene assay
Number of identified QTL/ genes	many (54)	several (11)
Probability to find further loci	moderate	moderate
Suitability for use	indifferent, QTL dissipate with further generations	high, when the causative mutation is known
Difficulties	decision, which QTL should be considered for further selection, pleiotropic effects	genotype frequencies are often unbalanced, pleiotropic effects

* strictly speaking only, when a causative mutation is investigated with regard to expected influences on phenotypes

Candidate gene approaches

The proportion of pig genes that have been mapped is still small and consequently, the number of positional candidate genes is limited (King et al., 2003). It is often mentioned that in principle every animal from any population can be investigated. Basically, this is feasible, and many research groups genotyped a candidate gene for a chosen trait at a given standardized F_1- or F_2-population, depending on the breeding system. According to Rothschild et al. (2000) one requirement of the candidate gene approach is to test the gene variants in several populations to detect general effects. In general, the resource populations and the number of tested pigs in different experiments varied to a considerable amount. Some research groups used reference families, some used commercial pig populations, but only a

few of them let the animals house in a commercial farm. Because of differences concerning housing, feeding and other environmental influences, results of these studies are difficult to compare. Rothschild et al. (1996) investigated 161 sows of a synthetic Meishan line and 1079 sows of a Large White synthetic line for variants of the estrogen receptor gene (ESR). Short et al. (1997) extended the number of animals by the use of 4262 sows of Large White based commercial pig lines. An association study for the ESR gene with regard to litter size was also performed by Depuydt et al. (1999) using only 144 sows housed in 3 commercial pig farms. Van Rens et al. (2000) genotyped 79 Meishan x Landrace gilts for the ESR gene and evaluated the sows for luteinizing hormone, estrogen and progesterone. Matoušek et al. (2003) used two elite herds consisting of 178 and 144 Large White sows, respectively, in order to investigate litter size effects for the genes ESR and ryanodine receptor gene 1 (RYR1). For the prolactin receptor gene (PRLR), Vincent et al. (1998) used five populations consisting of Large White, Landrace, Duroc and Large White x Meishan pigs. Animal numbers varied between 261 and 416 sows. Li et al. (1998) investigated the follicle stimulating hormone beta gene (FSHb) with regard to litter size. All pigs in this study descended from nucleus herds in China and consisted of Landrace, Duroc and of a Yorkshire x Erhualian synthetic line. They investigated litter size parameters, and for the first parity, 289 sows could be evaluated, whereas for the fourth parity, only 52 sows were available. This led to only two different genotypes in sows with four litters, compared to sows with only one litter, where all the three genotypes were found. A total of 2545 litter records of 1300 sows from commercial lines consisting of Large White, Landrace and Duroc housed in a genetic nucleus farm, were evaluated for the retinol binding protein 4 gene (RBP4) by Rothschild et al. (2000). The authors concluded that a very large data set is needed to show significant differences for allelic effects in a range of 0.2 - 0.25 piglets/ litter observed in their study. Similar conclusions were drawn by Muñoz et al. (2004). They investigated the ESR2 gene with regard to the total number of born pigs in two Iberian pig lines with 46 and 150 sows, respectively. They were not able to find significant differences between genotypes and proposed a further investigation with more animals. The problem is, especially when animal numbers are low, that it is rather unlikely to obtain balanced genotype frequencies. Often, phenotypic selection over many years leads to loss of rare alleles if they are not strongly linked to the selection aim. Hence, rare genotypes cannot be evaluated with sufficient statistical power. In order to obtain more balanced genotype frequencies, a special mating test is required. This could be achieved by crossing phenotypically extreme different breeds or by crossing animals which are opposed homozygous at the candidate gene locus stating as the

parental-generation. Afterwards, an intercross between the heterozygous F_1-animals or, alternatively, a backcross to the parental breed is performed in order to produce the F_2- or backcross generation that includes all genotypes in expected Mendelian ratios. Then, the sows of the F_2 or backcross generation can be genotyped and phenotyped. A disadvantage is, that similar to QTL-analyses, this procedure is also time-consuming. In principle, a direct candidate gene approach can be recommended when it is known that a gene product has a measurable influence on a phenotypical trait (physiological candidate gene) or that a gene is located in a narrowed QTL region (positional candidate gene) or that the candidate gene has an influence on the phenotypical trait in other species (comparative candidate gene). The more reasons for a candidate gene approach are given, the higher is the probability to detect a real effect for the chosen gene.

Construction of a cytogenetic map harboring QTL and candidate genes for fecundity

In 1997, Rothschild et al. reviewed for the first time approaches to improve fecundity in pigs on the molecular level. More recent reviews in this field were presented by Omelka et al. (2001) and by Rothschild (2004). Alfonso (2005) conducted a meta-analysis concerning the PvuII polymorphism for ESR genotypes for litter size in swine. It has been shown that combining the results across studies can provide a more precise and consensus estimate of the effect of a candidate gene as compared with any single study. The objective of our study was to combine results from QTL mapping and candidate gene approaches to provide a cytogenetic map with all up to date known QTL and candidate genes influencing reproductive traits in pigs. Until now, this has been performed for production and fecundity traits only for single chromosomes, such as for SSC1 (Smith et al., 2001) and for SSC10 (Nonneman and Rohrer, 2003). In order to present a complete cytogenetic map, each publication which reported a QTL or a candidate gene for such a trait was adducted. For the construction of the map, we used all available data on reproductive traits. Reproductive traits were teat number, gestation length, age of puberty, uterine capacity, ovulation rate and litter size parameters comprising the total number of born piglets, the number of born alive piglets and the number of stillborn piglets. The method for the construction of the cytogenetic map was the following: In cases where the cytogenetic position of the observed QTL was given, this region was directly adopted and drawn into the genome map. Most authors, however, gave the position of a QTL in centiMorgan (cM) in a linkage map. Because the presented linkage maps differed among studies, it was difficult to find consensus QTL regions. Therefore we converted the QTL-linked marker positions into a cytogenetic position. For this purpose, first, it was shown

at which marker the highest F-ratio was found or, alternatively, between which markers the confidence interval representing the most likely position of the QTL was observed. The next step was to evaluate the cytogenetic position of these markers by using several databases (listed at the end of this article). When the cytogenetic position of those markers was not evaluable, the closest linked marker(s) to the reported marker(s) was used and its cytogenetic position was examined as described above. When a cM position was given only and the kind of genotyped markers (in most cases microsatellites) referred to other publications, these publications were consulted in order to determine the cytogenetic position as described above. In a few cases, it was impossible to determine any cytogenetic position of a QTL because of the absence of any cytogenetic position of markers. Then, the QTL was added without further localization to a chromosomal region in that way, that a line was drawn below the adequate chromosome. Furthermore, we distinguished between QTL above and below the genome-wide significance threshold of α=5%. The cytogenetic positions of the candidate genes were identified by the use of the same databases and included into our map.

Results of linkage and association studies for subtraits of fecundity

Table 3.2 shows the currently mapped QTL regions with influence on fecundity in pigs with a level of significance at $p<0.05$. Further information concerning chromosome number, crossbreeding system, number of genotyped F_2-animals, recorded trait and the reference are also presented. In Table 3.3 we present all currently known candidate genes with potential influence on reproductive traits. Further information concerning the location on the chromosome as well as the polymorphism, the number of genotyped animals, the observed trait and the reference are also presented.

Table 3.2: QTL for reproductive traits in pigs ($p<0.05$)

SSC	Crossbreeding	No. of genotyped F_2-pigs	Trait	Reference
8, 13, 15	Large White x Landrace	114	Ovulation rate	Rathje et al. 1997
4 8 9	Yorkshire x Meishan	122	Number of stillborn piglets Ovulation rate Gestation length	Wilkie et al. 1999
8	White composite x Meishan	295*	Ovulation rate	Rohrer et al. 1999
6	Göttingen miniature pig x Meishan	143	Litter size	Yasue et al. 1999
10	White composite x Meishan	750	Teat number	Rohrer 2000
1, 7	Göttingen miniature pig x Meishan	265	Teat number	Wada et al. 2000
7 12, 14, 17	Large White/ Landrace x Meishan	249	Total number born; litter 1 Total number born; litter 2	De Koning et al. 2001
8	Yorkshire x Meishan	108	Ovulation rate	Braunschweig et al. 2001
7, 8 9 8, 11 5, 13 11	Large White x Landrace	423	Age of puberty Ovulation rate Teat number Number of stillborn piglets Fully formed piglets	Cassady et al. 2001
2, 10, 12	Large White/ Landrace x Meishan	1173	Teat number	Hirooka et al. 2001
8	White composite x Meishan	600*	Ovulation rate	Campbell et al. 2003
8 8	Large White x Meishan	220	Teat number Number born alive	King et al. 2003

*BC, F_3-, F_4- generation together, followed by a backcross between the F_1- and P-generation

Table 3.3: Candidate genes with association to reproductive traits in pigs

Gene	SSC	Polymorphism	Breed/ crossbreeding[a]	No. of genotyped pigs	Trait[b]	Reference
ESR	1	Intron[c]	(M x SL); LW	161 and 1079[d]	TNB, NBA	Rothschild et al. 1996
		No information	LW	4262	TNB, NBA, Teat number	Short et al. 1997
		No information	CB	262	TNB, NBA	Chen et al. 2000
		Intron	(M x LW)	275	TNB, NBA	Van Rens et al. 2002
		No information	LW	74 and 124[d]	TNB, NBA	Matoušek et al. 2003
		No information	LW	1030	TNB, NBA	Goliášová and Wolf 2004
		No information	LW	226	TNB, NBA	Horogh et al. 2005
		Exon 8	Lr; SL	144	NBA	Depuydt et al. 1999
PRLR	16	No information	LW	400	NBA	Vincent et al. 1998
		No information	M, Lr	261 and 416	TNB, NBA	Vincent et al. 1998
		No information	SL	273	NBA	Drogemuller et al. 2001
		No information	(M x LW)	77	Age of puberty, TNB, NBA	Van Rens and Van der Lende 2002a
		Exon[e]	(M x LR)	55	Ovulation rate, Uterine length	Van Rens et al. 2003
FSHb	2	Intron	(YS x EL)	289	TNB, NBA	Li et al. 1998
		Promotor	LP; DP; Lr	No information	Litter size	Du et al. 2002
RBP4	14	Intron	SL	1300	TNB, NBA	Rothschild et al. 2000
GNRHR	8	3'UTR	(M x LW)	200	Ovulation rate	Jiang et al. 2001

Continuation of Table 3.3: Candidate genes with association to reproductive traits in pigs

Gene	SSC	Polymorphism	Breed/ crossbreeding[a]	No. of genotyped pigs	Trait[b]	Reference
LEP	18	Exon 3[f]	SL	519	> 1. litter TNB, NBA	Korwin-Kossakowska et al. 2002
		Exon 3	Ys; Lr	62 and 170	Litter size, 1-4 parity	Chen et al. 2004b
		Intron 1	Du	246	1. litter	Chen et al. 2004b
LEPR	6	Intron 2, Exon 2, Exon 18	Ys, Du	62 and 246	Litter size, 1-4 parity	Chen et al. 2004a
OPN[g]	8	Intron	SL	519	> 1. litter, TNB, NBA	Korwin-Kossakowska et al. 2002
BF	7	Intron 1	(LW x Lr) x Lc	123	TNB, NBA, 2-4 parity	Buske et al. 2005
FUT1	6	Exon 2	PBP	104	TNB, NBA, 1-6 parity	Horák et al. 2005
EPOR	2	Intron 4	(Ys x Lr x CW x LW)	402	Uterine capacity	Vallet et al. 2005

a: CB = Chinese breeds, CW = Chester White DP = Duli pigs, Du = Duroc, EL = Erhualian line, Lc = Leicoma, LP = Laiwu pigs, Lr = Landrace, LW = Large White, M = Meishan, PBP = Přeštice Black-Pied, SL = Synthetic line, Ys = Yorkshire

b: TNB = Total newborn, NBA = Newborn alive

c: personal communication by M. Rothschild in Van Rens et al. 2002

d: two investigations

e: personal communication by M. Rothschild in Van Rens et al. 2003

f: in: Chen et al. 2004b

g: OPN = SPP1

Teat number

Figure 3.1 shows that most QTL were found for teat number, likely because this trait is easy to measure. Significant QTL for teat number were found on SSC1 and SSC7 (Wada et al., 2000), on SSC2 and SSC12 (Hirooka et al., 2001), on SSC8 (Cassady et al., 2001; King et al., 2003), on SSC10 (Rohrer, 2000; Hirooka et al., 2001), and on SSC11 (Cassady et al., 2001). It is interesting to note that on chromosomes 8 and 10, where two QTL were found independently, the QTL overlap very well. So it can be assumed, that one or more genes are located in this region influencing this trait. Furthermore, putative QTL were found on SSC1 (Rohrer, 2000; Cassady et al., 2001), SSC3 (Rohrer, 2000; Hirooka et al., 2001), SSC6 and SSC7 (Cassady et al., 2001). The analysis of the estrogen receptor gene has shown an influence on teat number (Short et al., 1997). The cytogenetic position of this gene is located within the QTL region on SSC1 with influence on this trait. Teat number plays a significant role when many piglets are born. Hence, the selection on litter size may require the increase of teat number (Hirooka et al., 2001). However, although teat number is easy to measure in both, males and females, it is not likely to include this trait into MAS programs because firstly, there is no major QTL effect and thus, the question arises for which chromosomal region should be selected for in commercial pig production. Secondly, there are other, more important traits affecting reproduction performance in pigs such as ovulation rate and uterine capacity (Bennett and Leymaster, 1989). Moreover, pleiotropic effects between increased teat number and reduced ovulation rate in Meishan pigs have been reported (Rohrer, 2000).

Gestation length

Wilkie et al. (1999) found one significant QTL for gestation length on the q-arm on SSC9 and two putative QTL on SSC1 and SSC15. The effect of the QTL on SSC9 contributed to a reduction of the gestation length by 3.04 days for the alleles from the Yorkshire founder sows in comparison to the Meishan founder boars. An imprinting effect, however, was not observed. As shown in figure 1, the two putative QTL on SSC1 and SSC15 for gestation length overlap well with the QTL for age of puberty on SSC1 (Rohrer et al., 1999) and ovulation rate on SSC15 (Wilkie et al., 1999), respectively. Because King et al. (2003) observed that sows with shorter gestation periods had higher levels of prenatal survival, this trait has potential to be introduced into MAS.

Age of puberty

Several QTL have been detected for age of puberty. Cassady et al. (2001) detected a suggestive QTL for this trait on the telomeric end of the q-arm on SSC8. A further suggestive QTL was detected on SSC7 by the same authors, but the cytogenetic position of this QTL was not evaluable. Additional putative QTL for this trait have been mapped on SSC1 and SSC10 (Rohrer et al., 1999), and on SSC7, SSC8 and SSC12 (Cassady et al., 2001). It must be noticed that none of the observed QTL overlap each other, thus, it can be expected that for this trait, many different genes are responsible. The prolactin receptor gene (PRLR) on SSC16 as a physiological candidate gene has shown association with age of puberty. Van Rens and Van der Lende (2002a) observed that gilts with the genotype BB were significantly younger at the age of first estrus compared to AA gilts. However, pleiotropic effects with other traits, such as litter size have been observed within the same animals. Therefore, it can be assumed that PRLR will rather not serve as a marker for MAS to reduce the age of puberty.

Uterine capacity, uterine length

Uterine capacity is a major component contributing to litter size in pigs (Christenson et al., 1987; Leymaster and Johnson 1994). Because this trait is a limiting factor for high litter size, researchers focused on it despite the fact that this trait is difficult to measure. That is why less investigations have been performed for uterine parameters in relation to ovulation rate and litter size parameters. For uterine length, Wilkie et al. (1999) found a QTL on SSC5 and SSC7. Rohrer et al. (1999) observed a QTL for uterine capacity on SSC8, but, until now, none of these QTL have been confirmed. Van Rens et al. (2003) investigated the PRLR gene on SSC16 with regard to uterine length and found significant differences between genotypes. Recently, Vallet et al. (2005) found an association between the erythropoietin receptor gene (EPOR) and uterine capacity in two distinct sow populations. They investigated a single nucleotide polymorphism (SNP) in intron 4 of a total of 402 gilts and concluded that selection of the favorable genotype could increase litter size in swine that are not limited in ovulation rate. A further candidate gene for uterine capacity could be the estrogen sulfotransferase gene (STE). It is located on SSC8 and Kim et al. (2002) observed different mRNA expression levels in Meishan and White composite pigs during pregnancy. Furthermore, in cyclic gilts, uterine STE activity increases during the luteal phase of the cycle (Pack and Brooks, 1974).

Ovulation rate

Ovulation rate is surely one of the most important traits for reproduction because it directly influences litter size. Therefore, many studies focused on this trait. Until now, a total of 15 QTL affecting ovulation rate has been observed, most of them on SSC8. The first significant QTL analysis affecting reproductive traits in pigs was performed by Rathje et al. (1997). They found a QTL for ovulation rate at the telomeric end of the long arm on SSC8. For this trait, Wilkie et al. (1999) found a QTL near the centromere and Rohrer et al. (1999) found a QTL at the telomeric end of the short arm of the same chromosome. Thus it can be concluded, that SSC8 likely harbors several genes influencing ovulation rate. Recent studies confirmed and narrowed these regions for the mapped QTL on SSC8. Braunschweig et al. (2001) refined the genetic map by the use of 29 markers and confimed the QTL at the centromere region of SSC8. Campbell et al. (2003) narrowed the region at the telomeric end of the short arm on SSC8 using gene and microsatellite markers within the first 27 cM and confirmed and narrowed the QTL found by Rohrer et al. (1999). Additional significant QTL for ovulation rate were found on SSC9 (Cassady et al., 2001), SSC13 and SSC15 (Rathje et al., 1997). Putative QTL were found on SSC3, SSC9, SSC10, and SSC15 (Rohrer et al., 1999), on SSC4 (Rathje et al., 1997), and on SSC7 and SSC15 (Wilkie et al., 1999). The QTL identified by Rohrer et al. (1999) on SSC15 did overlap both with the QTL identified by Wilkie et al. (1999) and by Rathje et al. (1997).

Concerning candidate gene approaches, Jiang et al. (2001) investigated the gonadotropin-releasing hormone receptor gene (GNRHR) for ovulation rate because of the observation of QTL for ovulation rate on SSC8 and because of the fact that GNRHR is critical in the endocrine regulation of reproduction. This gene is located at 8q11 - q12 near the centromere where Wilkie et al. (1999) and Braunschweig et al. (2001) found a QTL for ovulation rate. A significant association between the C/G substitution in the 3'UTR and the number of corpora lutea at first parity was observed, so it can be assumed that GNRHR could have an effect on ovulation rate in pigs. Van Rens et al. (2003) investigated the prolactin receptor gene (PRLR) on SSC16 for different reproductive traits and found a significantly higher ovulation rate for AA genotypes in comparison to BB genotypes. However, until now, no QTL for this trait has been detected there.

Number of stillborn piglets

Significant QTL for the number of stillborn piglets were found on SSC4 (Wilkie et al., 1999), on SSC5 and SSC13 (Cassady et al., 2001). Until now, no candidate gene has been tested for this region. However, Sun et al. (2002) investigated the POU domain class 1 transcription factor 1 (POU1F1) gene on SSC13 with regard to growth hormone and prolactin concentrations in blood serum of growing sows and found differences among genotypes. As these hormones are critical for the development of mammals, POU1F1 could be a candidate gene concerning the number of stillborn piglets.

Litter size

Measuring litter size as a reproductive trait is easy and the most important trait for pig producers. However, it must be taken into consideration that there is no single gene responsible for litter size itself. This trait is influenced by several traits such as uterine capacity, ovulation rate and embryonic viability (Bennett and Leymaster, 1989). Bennett and Leymaster (1989) concluded that selection for these multiple traits is required in order to attain progress in litter size. Significant QTL regions for litter size are located on SSC8 (King et al., 2003) and on SSC11 (Cassady et al., 2001). Putative QTL regions are located on SSC6 (Wilkie et al., 1999), SSC7, SSC12, SSC14 and SSC17 (De Koning et al., 2001).

The leptin receptor gene (LEPR) could be the gene underlying the QTL effect on SSC6, which has been identified by Wilkie et al. (1999). Chen et al. (2004a) found differences between genotype groups for polymorphisms in intron 2, exon 2 and exon 18 of the LEPR gene for litter size in Duroc and Yorkshire sows. Another region with influence on litter size is located on the q-arm on SSC6, however, no level of significance has been reported (Yasue et al., 1999). In this region, 20 genes, which are homologue to human chromosome 19, were registered including the pregnancy-specific beta-1-glycoprotein (PSG1) as the most probable candidate gene. Korwin-Kossakowska et al. (2002) investigated the osteopontin gene (OPN = SPP1) for litter size on SSC8 due to the known homology between this region in pigs and the ovine chromosome 6, where the high prolificacy gene (FecB) is located. OPN maps to 8q2.5 - 2.7 and is located in the region, where QTL for litter size (King et al., 2003) and ovulation rate (Rathje et al., 1997) have been found. For later parities, significantly more piglets (TNB and NBA) were observed for the osteopontin genotype AA in comparison to the heterozygous and homozygous BB genotypes.

Many investigations have shown that the estrogen receptor gene (ESR), which is located on SSC1 has an influence on litter size in pigs (Table 3.3), but, contrary to these findings, there are also many studies which cannot confirm these results (Rohrer et al., 1999; Drogemuller et al., 2001; Linville et al., 2001; Gibson et al., 2002; Isler et al., 2002; Horák et al., 2005). General debate exist now, whether the analyzed ESR polymorphism has a direct impact on litter size or not. For example, Rothschild et al. (1996) and Short et al. (1997) observed, that the BB genotype is favorable concerning litter size parameters such as TNB and NBA, whereas Goliášová and Wolf (2004) reported, that the AA genotype is favorable with regard to the same litter size parameters. They concluded that ESR allelic effects can differ between populations. Obviously the investigated ESR alleles are linked with different alleles of the causative mutation in different populations. In addition to direct effects, allelic effects may differ between different populations as a result of epistatic interaction with the population specific genetical background. Further reasons for the different findings concerning the ESR gene effects might be for example, that the number of analyzed animals and populations varied between studies considerably. Furthermore, one can assume that environmental influences and genetical background were not the same between studies and such effects could overlay small direct effects of the estrogen receptor gene. Moreover, the investigated polymorphism differed among studies (PvuII and AvaI restriction sites). Whereas most authors investigated the PvuII restriction site in an intron region according to Short et al. (1997), Depuydt et al. (1999) investigated both restriction sites for litter size. Interestingly, an association between litter size and ESR genotypes was only observed for the AvaI restriction site, but not for the former one. Because of the fact that association studies between the ESR PvuII mutation and litter size in pigs differed to a considerable amount, recently, Alfonso (2005) performed a meta-analysis, comprising 15 published studies including 9329 sows. He could show, that finally, the B allele is superior for TNB and for NBA piglets. However, no QTL has been found in the chromosomal region where the ESR gene is located for any reproductive trait, except teat number. From these studies one can conclude that the genetic effect of a possible ESR variant is rather small. This also explains, that in pedigree analyses with small sample sizes no additional QTL on SSC1 has been detected. Furthermore, the ESR gene might not have been polymorphic in the populations involved in the QTL scans. Van Rens et al. (2002) stated that the ESR gene is rather a marker than the causative gene itself. Pleiotropic effects for the ESR gene have also been reported. Van Rens and Van der Lende (2002b) observed, that the favorable allele for litter size appears to be the unfavorable allele for pre-weaning piglet growth.

Muñoz et al. (2004) investigated a polymorphism in exon 5 of the ESR2 gene which is located at the telomere of the q-arm of SCC1 in two Iberian pig populations with 46 and 150 sows, respectively. In this study no statistically significant association between the ESR2 polymorphism and litter size was found. The authors recommended to repeat this association study by expanding sample size.

Other candidate genes with a potential influence on litter size are the follicle stimulating hormone gene beta (FSHb) on SSC2 (Li et al., 1998), the leptin gene (LEP) on SSC18 (Korwin-Kossakowska et al., 2002; Chen et al., 2004b) and the retinol-binding protein 4 (RBP4) on SSC14 (Rothschild et al., 2000). Whereas no QTL effects have been detected for the genomic position of FSHb and LEP, a putative QTL for litter size has been detected on SSC14. As the cytogenetic position of RBP4 is not clearly defined, it is not sure whether the effects of the RBP4 gene might be responsible for the reported SSC14 QTL.

Figure 3.1: Cytogenetic map of the pig with all QTL and candidate genes influencing fecundity

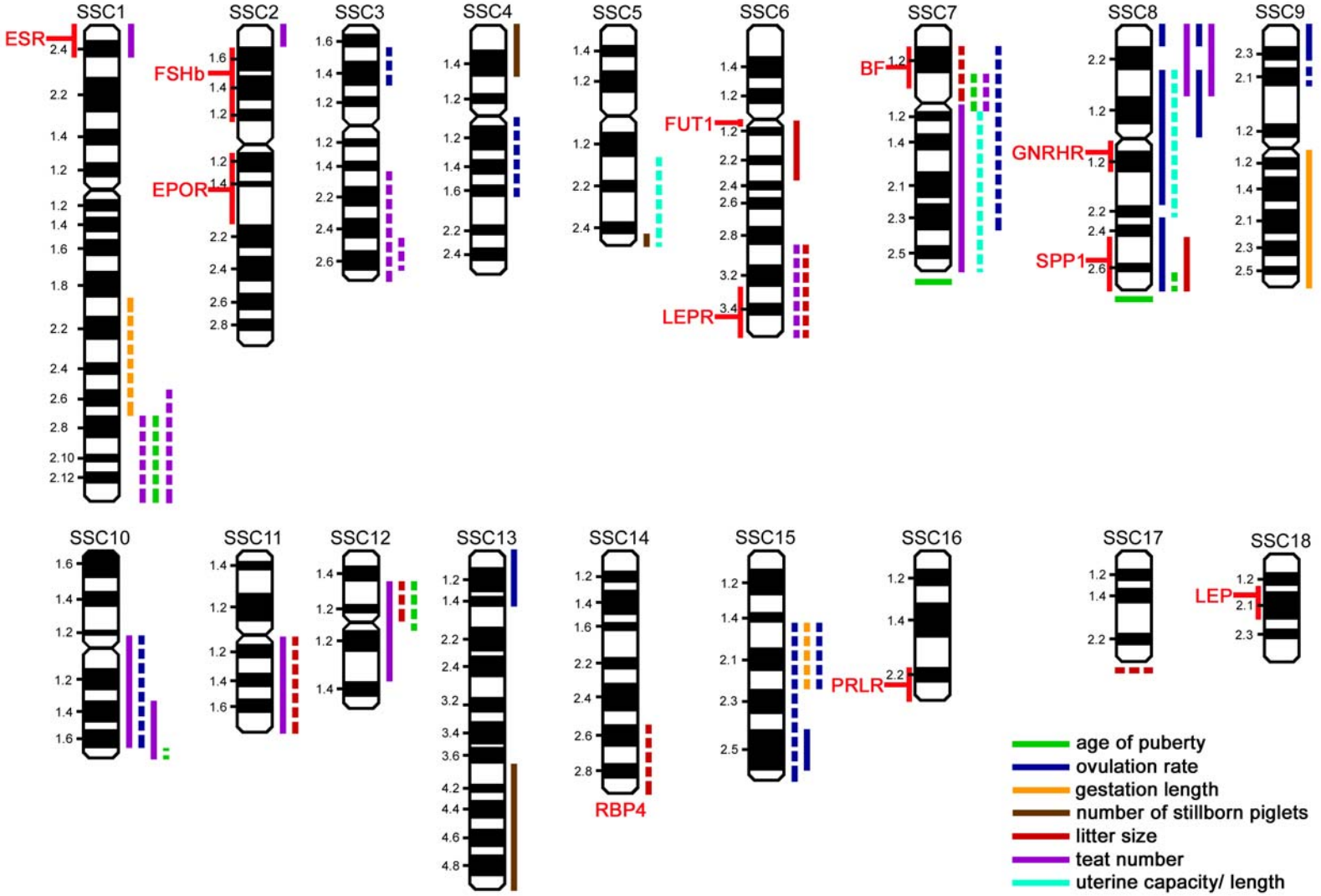

Figure legend:

bold solid lines = level of significance $p<0.05$; dashed lines = level of significance $p>0.05$; cytogenetic positions of the lines at the end of the chromosomes and for RBP4 were not evaluable; BF = properdin; ESR = estrogen receptor; EPOR = erythropoietin receptor; FSHb = follicle stimulating hormone beta; FUT1 = fucosyltransferase 1; GNRHR = gonadotropin releasing hormone receptor; LEP = leptin; LEPR = leptin receptor; PRLR = prolactin receptor; RBP4 = retinol-binding protein 4; SPP1 (OPN) = secreted phosphoprotein 1

Genomic distribution of identified genetic effects

Figure 3.1 shows that all autosomes harbor QTL or candidate genes with impact on fecundity. However, on chromosome 8, and, to a lower extend on chromosome 7 comparatively many QTL for reproductive traits have been identified.

At the telomeric end of the p-arm and the centromeric region of SSC8, the QTL for ovulation rate could be confirmed and narrowed, respectively. At the centromeric region, also a QTL for uterine capacity has been reported. If ovulation rate and uterine capacity have an effect on litter size, we would expect in this chromosomal region also a QTL or at least one gene influencing litter size. However, until now, no genetic effect on litter size has been found in this region. At the telomeric end of the q-arm of SSC8, a QTL for ovulation rate and one for litter size have been reported. As these parameters are in a physiological context, one can speculate that one gene might be responsible for these two traits. A positional candidate gene could be SPP1, which obviously has an influence on litter size (Korwin-Kossakowska et al., 2002).

Similar conclusions can be drawn for SSC7, especially for the centromeric region, where QTL for ovulation rate, number of stillborn piglets and for litter size are located. As these traits are also in a physiological context, one or more genes contributing to the phenotypic variance can be expected. It is interesting to note that exactly at this region the major histocompatibility complex (MHC) is located (Peelman et al., 1996; Ponsuksili et al., 2001). The MHC is a relatively dense gene cluster, which could harbor one or more genes influencing reproductive traits in swine (Vaiman et al., 1998). Within the MHC, the properdin gene (BF) is located, and Buske et al. (2005) found an association between BF genotypes and litter size in a commercial pig cross population. Furthermore, Gautschi and Gaillard (1990) observed influences on litter size of this chromosomal region in targeted mating studies.

Another interesting chromosome is SSC5 because of the overlapping QTL for stillborn piglets and uterine length. If there are many conceptuses, but the uterine length is not long enough at

the same time, the conceptuses cannot develop and thus the number of stillborn piglets increases. SSC6 is particularly interesting because of the economically important QTL for litter size. Recently, Horák et al. (2005) reported an association between the fucosyltransferase 1 gene (FUT1) on SSC6 and litter size in Přeštice Black-Pied sows.

Discussion

Leymaster and Johnson (1994) concluded that selection for high ovulation rate and uterine capacity might produce the greatest response to increase litter size. However, these parameters are difficult to measure simultaneously in the same animals, because both traits are measurable simultaneously only for one parity once in an animal. Until now, Rohrer et al. (1999), Wilkie et al. (1999) and Isler et al. (2002) are the only ones who investigated both traits in the same animals. If ovulation rate and uterine capacity have an effect on litter size, QTL for litter size is expected in the same genomic region as for either QTL for ovulation rate or for uterine parameters, respectively. As several significant QTL were initially found on SSC8, in recent years fine mapping has been started for this chromosome. Consequently, at this time there is a clear bias in favor of investigations on SSC8. In future, fine mapping should be extended on other chromosomes, e.g. on SSC7, where also several QTL effects were found.

During the last years, a large number of reports on QTL and candidate genes with influence on reproductive traits of sows have been published. Inspection of these reports indicates interesting overlapping results among some studies, but also remarkable differences in the location of QTL and in the estimated size and magnitude of the effects of individual QTL and candidate genes. The reasons for this phenomenon are essentially due to differences in the experimental setup. There are differences between resource populations such as different breeds, number of evaluated animals and mating systems, leading to different genotype- and allele frequencies. Differences in genetical background and environmental influences may enhance or inhibit gene effects. Several models for the statistical analyses and the determination of the significance thresholds have been applied. Another aspect is that candidate genes or QTL were investigated with regard to slightly differing traits belonging all to the term "reproduction". As a result, the estimated effect size and the conclusions of the studies varied to a considerable extent. Consequently, there is a need to determine consensus locations of QTL and genes as well as estimates of the effects for the phenotypical traits. The probability that identified effects on fecundity parameters in sows are real effects and typical

for a larger population is higher for chromosomal regions, for which QTL or gene effects have been confirmed in different populations. Chromosomal regions, which have been found in one population only may be caused by population specific seldom allelic variant or might result from multiple statistical tests just by chance. QTL, which have been identified with high genome wide significance threshold of $p < 0.01$ are more likely real effects than suggestive QTL at the chromosome-wide significance treshold of $p < 0.05$. The latter needs confirmation in different populations to show that these are real effects.

As shown in Figure 3.1, so far no QTL effects were identified in chromosomal regions, where potential influences of candidate genes like PRLR, LEP, and FSHb for reproductive traits were found. This could be explained by smaller sample sizes for QTL scans in comparison to candidate gene approaches and the possibility that candidate genes did not have different alleles in segregating pedigrees.

In order to describe and interpret QTL analyses more precisely, Khatkar et al. (2004) gave useful suggestions in their meta-analysis. We extend these suggestions also for candidate gene approaches, particularly with regard to reproductive traits in pigs. Authors should follow the proposed guidelines: (1) For candidate genes, reasons for the selection should be explained, the chromosomal position, the gene identification number and the location of the polymorphism should be reported as well as the equation for the analysis of variance including all fixed effects and covariates. (2) An exact description of the population such as the number of genotyped animals, resource population (race, pedigree, genetical background, inbreeding level), mating regime (insemination regime, artificial or no) and environmental conditions (feeding and housing regimes) should be presented. (3) A detailed description of the measured phenotypical traits, the reason(s) for choosing the traits and in cases of traits such as litter size the number of replications (e.g.: number of litters should be equal for each sow) is desirable. (4) For QTL studies, analytical method, marker map used, number of markers and their distances, QTL map positions and confidence intervals as well as closest markers are to declare. (5) Information concerning the test statistic (level of significance of reported p-values and their description: point-wise, chromosome-, or genome-wide) are necessary. (6) The estimated effect size should be given with standard errors, and if examined, known pleiotropic effects should be reported in detail for both, QTL analyses and candidate gene approaches. A precise recommendation for animal numbers for example is not possible, due to nescience of the size of genetic effects, or due to expected genotype frequencies (for candidate gene approaches). However, two important relations should be provided: Regarding

QTL-analyses and particularly fine-mapping, an extension of marker density for example leads only to more precise results, when at the same time animal numbers are increased (Broman, 2001). Otherwise, additional information about QTL position is poor. Concerning candidate gene approaches, balanced genotype frequencies can lead to reduced animal numbers. Some candidate gene approaches lacked in that way, that one genotype was absolutely not, or only present in low numbers in a population, and therefore, a final conclusion on genotype effects is not feasible. It cannot be excluded, that the absent genotype could lead to phenotypical changes.

Although difficult to perform, a further advance would be a standardization of the experimental setup and methodologies to allow more transparent comparison and collation of results, as it is done for multi-centre clinical trials in human studies for example. Concerning candidate gene approaches, a targeted mating test would be an improvement in order to obtain balanced genotype frequencies. When such an experimental design is not available or feasible, at least the parents of the sows should be genotyped in order to identify, from which parent a desirable allele was inherited. Afterwards, genotype frequencies for a chosen gene should be investigated in a commercial population in order to preselect suitable parents for further genetic analyses in this population.

Conclusions

Fecundity in multiparous animals, especially in pigs, is one of the most difficult and complex traits. The reasons are in addition to low heritabilities, long generation intervals, the polygenic nature of reproductive traits and the strong environmental influences on reproduction processes. The inspection of all evaluated articles indicates similarities among some studies, but also remarkable differences concerning the location of QTL and the estimated size and magnitude of the effects of individual QTL and candidate genes. For further research, the present knowledge on QTL and candidate gene positions and effects should be used efficiently. Fine-mapping of QTL regions should be extended in order to narrow QTL intervals to reduce the number of positional candidate genes with regard to reproductive traits. A combination of fine mapping and candidate gene approaches for promising chromosomal regions is a straight forward strategy. We propose to present the information on identified QTL and candidate genes as accurately as possible and to standardize the methods in order to be able to compare the results among studies more precisely. Furthermore, emphasis should be given on traits which are economically important and preferably easy to measure, and

pleiotropic effects for the chosen genes or QTL should be investigated as accurately as possible. Considering all these facts, selection progress is mainly achieved, when firstly a beneficial polymorphism is detected for a desirable trait and rare genotypes have been found in a commercial breed in order to increase this genotype in the population.

Databases

www.genome.iastate.edu/pig

www.marc.usda.gov/genome/genome.html

www.ncbi.nlm.nih.gov/

www.projects.roslin.ac.uk/pigmap/pigmap.html

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4 Characterization of selected candidate genes for association studies concerning litter size in swine

Five suitable candidate genes were chosen according to positional, physiological, and comparative aspects for genotyping and were investigated for associations to litter size in swine. For this purpose, it was shown, which candidate genes are located within QTL regions for reproductive traits in swine. Emphasis was given for overlapping QTL in certain chromosomal regions among published studies. This was performed by comparing the analogousness for the order of markers in chromosomal regions harbouring QTL in the different published studies with different linkage maps. Because the order of markers differed sometimes among studies due to different family structure of selected animals, the cytogenetic map was also considered. For example, it could be demonstrated, that the centromeric region on SSC7 harbours several QTL for reproductive traits in swine, which could not be evaluated by comparing only porcine linkage maps. Furthermore, suitable genes were also derived according to physiological aspects like gene product function. Additionally, candidate genes for litter size were also derived from investgations on heterosis effects in mouse models (Brunsch, 1999). In order to find homologous chromosomal regions between the mouse and the pig genome, the MGI (Mouse Genome Informatics) database was used. It was evaluated, which chromosomal regions with impact on heterosis on litter size at mice were homologous to porcine chromosomal regions. Information was provided about the nature of homology (for example nucleotide sequence comparison, amino acid sequence comparison, cross-hybridization, conserved map localization), the name and the cytogenetic position of the locus both in mice and in swine. Thereafter, further information about genetic structure, linkage and cytogenetic position, location and nature of polymorphisms as well as applicable methods in order to show the gene variants and genotype and allele frequencies in different breeds were evaluated. For this purpose, databases like NCBI, U.S. PIG GENE MAPPING, pigmap and the MARC-usda database were consulted. Table 4.1 provides an overview about the selected genes.

Table 4.1: Overview on suitable candidate genes for investigations to reproductive traits

Candidate gene (symbol)	Chromosomal localization	Localization of polymorphism	Physiological effect on fecundity	Position within QTL	Comparative aspects
BF	7 1/2p11-p12	Intron 1	+	++	(+)
GPX5	7 p11-p12	Intron ?	(+)	++	0
FUT1	6 q11	Exon 2	+	+	0
ESR2	1 q22-q27	Exon 5	++	(+)	+
CYP21	7 p11-p12	3'UTR	+	++	+

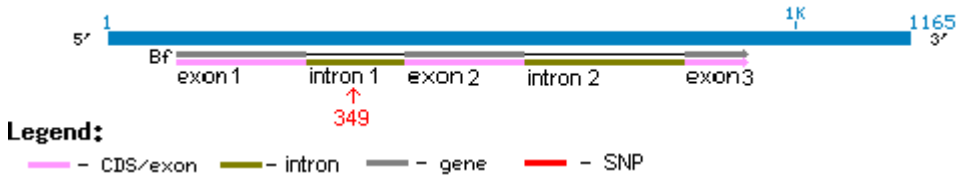
Annotations: ++ = strong effect; + = moderate effect; (+) = possible or weak effect; 0 = no effect observed so far; for detailed description and references see in the text

Properdin (BF)

BF has been mapped on SSC7 at 7 cen (Pinton et al., 2000) and at 7 1/2p11-p12 (Ponsuksili et al., 2001) and was chosen as a suitable candidate gene because of positional, comparative and physiological aspects. It has been shown that the centromeric region of SSC7 harbors multiple QTL for different reproductive traits such as uterine capacity, ovulation rate and litter size. This region comprises the major histocompatibility complex (MHC) class III region, a gene cluster, consisting of the gene BF and other closely neighboring genes (Peelman et al., 1996), which is highly conserved across species (Wu et al., 1995). Altough the chromosomal regions in which QTL for reproductive traits in swine were detected are generally wide, linkage analyses showed, that the MHC class III region lies in, or at least in neighborhood to these regions. Another aspect was, that heterosis effects for litter size in mice have been reported on mouse chromosome 17 (Brunsch, 1999) in a chromosomal region, which is homologue to the centromeric region on SSC7, where the MHC class III region is located. Matsumoto et al. (1997) investigated the gene BF for litter size parameters in mice by generating BF-deficient mice and showed, that BF deficiency *alone* has no major effect on fertility or fetal development. However, in the context of one or more genes derived from a 129 mouse strain, less homozygous offspring for BF deficiency were generated than expected and hence, the authors concluded an effect of this gene on reproduction. Properdin is a 93 kDa single-chain glycoprotein and is mainly synthesized in liver and in extrahepatic sites in endothelial, epithelial and mesenchymal cells (Colten, 1994). Beside immunological effects concerning the alternative pathway, properdin has a physiologically important function in reproductive traits such as uterine epithelium growth (Hasty et al., 1993). Jiang and Gibson (1998)

developed a simple PCR-RFLP method in order to determine genotype- and allele frequencies for a polymorphism in intron 1 of this gene in five breeds. In Landrace pigs as a widely used breed, two alleles, A and B, could be shown. Because this breed was also involved for the production of the sows for this investigation, it was concluded that both alleles could be segregating in our population. Therefore, it would be of value to test possible effects of the gene variants on fecundity. Figure 4.1 provides an overview on the gene structure of the porcine BF gene and the position of the above described polymorphism.

Figure 4.1: Gene structure of the porcine BF gene (Accession number M59240)



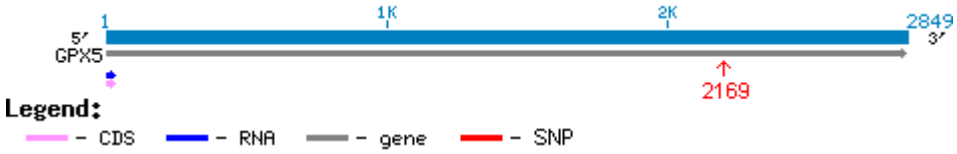
Reference: NCBI database, modified

Glutathione-Peroxidase 5 (GPX5)

GPX5 has been mapped on SSC7 at 7 1/2p12-p11 (Bertani et al., 1999) and was mainly chosen as a candidate gene, because of the fact that it is located in a chromosomal region in which several QTL for reproductive traits such as uterine capacity, ovulation rate and litter size have been detected. Linkage analyses of GPX5 showed that it is also closely linked to the MHC, which has been suggested to have an effect on reproductive traits in swine (Vaiman et al., 1998). It has been manifold demonstrated, that GPX5 is responsible for sperm quality due to the effect to prevent damages of sperm membranes by deleterious effects of lipid peroxidation. The expression of the GPX5 gene is mainly controlled by androgens and testicular factors (Rigaudiere et al., 1992). Hence, GPX5 is likely involved in paternal fertility. However, there are some hints, which let us assume that GPX5 could also be associated with maternal fertility (Bertani et al., 1999). According to Okamura et al. (1997), the GPX5 gene is one of several genes that are expressed in the epididymis. This protein interacts with sperm cells, and therefore, it might play a role in sperm-egg interaction, influencing the number of accessory sperms in the zona pellucida, and thus might have a pleiotropic effect on early embryo viability. Bertani et al. (1999) sequenced a porcine 2849 bp intron fragment of GPX5 and identified overall 16 polymorphisms. They developed a PCR-RFLP test for one of the polymorphisms in order to determine genotype and allele frequencies in 65 animals of eight different pig breeds. Although the number of animals for each breed

was rather low, they were able to find two different alleles in 12 Landrace and 14 Hampshire pigs with nearly balanced genotype frequencies, whereas in the other six investigated breeds, this polymorphism could not be detected. Because the Landrace breed was also involved in the sows of our investigation, it was concluded that both alleles could be present in order to test possible gene effects on maternal fecundity. Figure 4.2 provides an overview on the gene structure of the porcine GPX5 gene and the position of the investigated polymorphism.

Figure 4.2: Gene structure of the porcine GPX5 gene (Accession number AF124818)



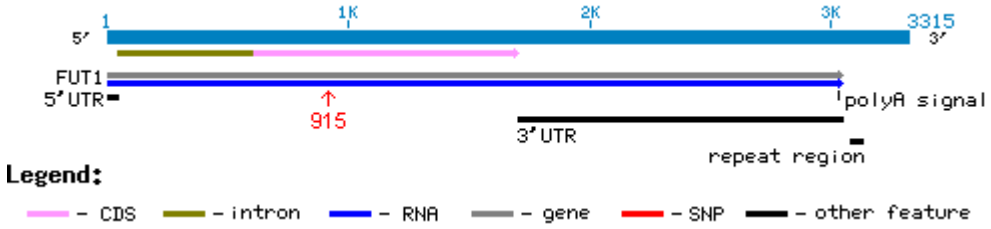
Reference: NCBI database, modified

Fucosyltransferase 1 (FUT1)

FUT1 has been mapped on SSC6 at 6q11 (Vögeli et al., 1996). The gene is located in a chromosomal region, in which a QTL for litter size in pigs was found. The FUT1 gene has been determined as a candidate gene for Escherichia coli F18 (ECF18) receptor locus (Meijerink et al., 1997). Fimbriated Escherichia coli adheres to brush border membranes in the small intestine and causes oedema disease and post-weaning diarrhoea in pigs (Bertschinger et al., 1990). Genetic studies revealed that susceptibility to colonization by an Escherichia coli strain with fimbriae F18 is controlled by a dominant allele, and the resistance by a recessive allele (Bertschinger et al., 1993). Meijerink et al. (2000) demonstrated that FUT1 is expressed in the porcine small intestine and that the susceptibility to ECF18 adhesion appeared to be solely dependent on the activity of FUT1 in intestinal epithelia. Moreover, their genetic and enzymatic studies supported the hypothesis that particularly the single nucleotide polymorphism (SNP) at position 307 in exon 2 is likely important in the synthesis of a structure that enables adhesion of ECF18 bacteria to small intestine mucosa. This SNP leads to an amino acid exchange (ALA → THR), and Klukowska et al. (1999) determined genotype and allele frequencies in five Polish local pig breeds for this SNP. They could show that in two breeds, based on Large White breeds, genotype and allele frequencies were nearly balanced. Because animal health is generally required for high fecundity, the hypothesis was, that FUT1 might also be associated with reproductive traits, such as litter size in pigs. Figure 4.3 provides an overview on the gene structure of the porcine FUT1 gene and the

position of the investigated polymorphism.

Figure 4.3: Gene structure of the porcine FUT1 gene (Accession number U70883)



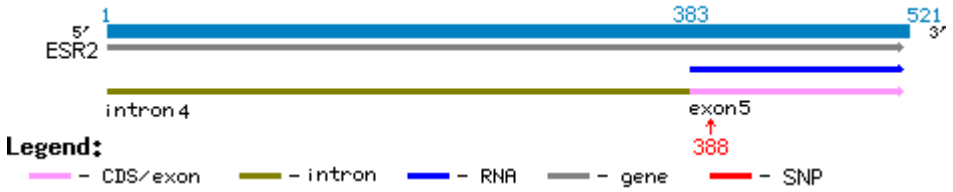
Reference: NCBI database, modified

Estrogen receptor 2 (ESR2)

ESR2 has been mapped on SSC1 at 1q22-q27 (Muñoz et al., 2004). Although in this chromosomal region, only one QTL for the traits "teat number" and "gestation length" has been found, respectively, ESR2 was chosen as a candidate gene mainly due to physiological and comparative considerations. First of all, it is worth to note, that there are at least two different ESR genes. In contrary to the ESR2 gene, which has been mapped on the telomeric region of the q arm, ESR1 has been mapped on the telomeric region of the p arm on SSC1 at 1p24-p25 (Ellegren et al., 1994). From a physiological point of view, steroid hormones and their receptors play an important role in reproductive processes. Cells in target tissues have receptor proteins that specifically bind the hormone during the initial stage in its action. Estrogen is intimately involved with pregnancy and its function is mediated through the estrogen receptor (Rothschild et al., 1996). The main function of estrogen, which is produced from conceptuses, is to establish pregnancy, first of all at the beginning of pregnancy. For this purpose, estrogen must bind to receptors which are located in the uterine epithel cells of the sows (Geisert et al., 1990). Mutations in this protein have been implicated in spontaneous abortion and in human brest cancer (Lehrer et al., 1990). Furthermore, it has been shown, that transgenic male and female mice containing a nonfunctional ESR gene are infertile due to considerable phenotypical changes concerning the gonads, the reproductive system and the mammary glands (Korach, 1994). Additionally, histological abnormalities in the uterus have been observed (Weihua et al., 2000). Up to now, at least 17 studies about the influence of the ESR1 gene on several reproductive traits in swine have been published, but the results differed among most studies. Hence, no other than the ESR1 gene has been discussed controversely with regard to impact on reproductive traits in swine. For the ESR2 gene, to our knowledge, there has been published only one association study concerning reproductive

traits in swine up to now. However, this gene has been characterized in the rat (Kuiper et al., 1996), mouse (Tremblay et al., 1997) and human (Mosselman et al., 1996). Several studies suggest that this receptor displays a high binding affinity to estrogens and therefore might be involved in ovarian follicular growth and development at periimplantation (Kowalski et al., 2002). For the ESR2 gene, a SNP in exon 5 was described by Muñoz et al. (2004). This single nucleotide polymorphism (A → G) leads to an amino acid exchange (MET → VAL), which is critical for its role as transcription factor. This polymorphism can be visualized by the PCR-RFLP method. Muñoz et al. (2004) investigated this polymorphism with regard to litter size in two Iberian pig populations and all the three genotypes could be found. Because a nonsignificant trend influencing litter size has been observed by these authors, we chose this candidate gene for our investigations. Figure 4.4 provides an overview on the gene structure of the porcine ESR2 gene and shows the position of the described polymorphism.

Figure 4.4 Gene structure of the porcine ESR2 gene (Accession number AF164957)



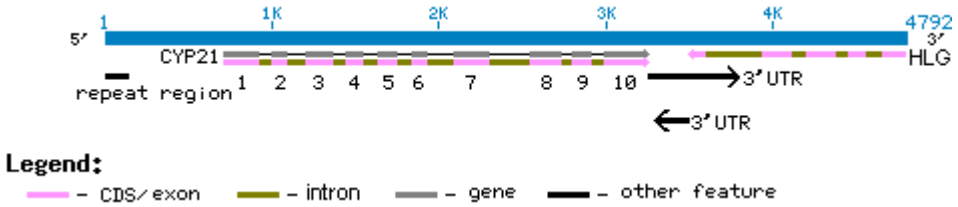
Reference: NCBI database, modified

Cytochrome P450, Steroid 21 hydroxylase (CYP21)

CYP21 has been mapped on SSC7 between MHC class I and II regions by Geffrotin et al. (1990) and Geffrotin et al. (1991) and was mainly chosen as a candidate gene because several QTL for reproductive traits such as uterine capacity, ovulation rate and litter size have been detected within this chromosomal region. From a physiological point of view, 21-hydroxylase deficiency (21-OHD) leads to reduced fertility and virilization in human females due to exceeded androgen production (New, 1995). Furthermore, potentially lethal adrenal insufficiency in humans is characteristic in two-thirds to three-quarters of patients with the classical salt wasting form of 21-OHD. 21-OHD is caused by mutations in the CYP21 gene encoding the steroid-21-hydroxylase enzyme. More than 90% of these mutations result from intergenic recombination between CYP21 and the closely linked CYP21*P* pseudogene. The degree of which each mutation compromises enzymatic activity is strongly correlated with the clinical severity of the disorder (Forest, 2004). A search for quantitative trait loci (QTL) for ovulation rate in cattle was performed by Blattman et al. (1996). They investigated ovulation

rate by counting corpora lutea over eight to ten consecutive oestrous cycles and observed that CYP21 was significantly associated with ovulation rate. Knoll et al. (1998) investigated this gene with PCR-RFLP methods in four different pig breeds: Large White, Landrace, Duroc and Piétrain. They determined allele frequencies at five loci and could show that allele frequencies were mostly balanced at all five loci, except for Duroc pigs, which were in all cases homozygous. Figure 4.5 provides an overview on the gene structure of the porcine CYP21 gene.

Figure 4.5: Gene structure of the porcine CYP21 gene (Accession number M83939)



Reference: NCBI database, modified; HLG = human-like gene

Databases

U.S. PIG GENE MAPPING: www.genome.iastate.edu/pig

Pigmap URL: www.projects.roslin.ac.uk/pigmap/pigmap.html

MARC Table of Contents: www.marc.usda.gov/genome/genome.html

MGI_3.0 (MGD): www.informatics.jax.org/mgihome/MGD/aboutMGD.shtml

NCBI Homepage: www.ncbi.nlm.nih.gov/

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5 Association studies between candidate gene variants and reproduction performance in two active sow populations

5.1 Materials and Methods

5.1.1 Animals

General requirements on the sows

In order to identify genes, which are associated with litter size in the hybrid sows, it was necessary to keep all environmental factors with potential influence on litter size constant. These factors were:

1) standardized feeding- and housing regime of the sows at each farm
2) animal health had to be ensured and sows were not allowed to be in an animal experiment
3) all sows were inseminated by artificial insemination with a constant amount of fresh sperm
4) environmental conditions were standardized in each farm concerning stall climate, housing temperature and housing of the sows in individual pens.

For the control of genetic factors, following requirements should be fulfilled:

1) preferably uniformly genetical origin of the hybrid sows
2) existence of preferably complete pedigree-data
3) existence of at least four litters from each sow

Description of the two selected sow farms

The investigations were performed with sows housed in two different commercial farms: The sow farm "Polkenberg" (Sachsen, Germany) consisted of about 1800 F_2-hybrid sows whereas the sow farm "Schulzendorf" (Brandenburg, Germany) consisted of about 1100 F_1-hybrid sows. Because in commercial pig farms it is unusual to measure piglet and litter weigths just like litter size adjustments, these parameters were not available. The selection criteria in order to genotype the hybrid sows were the following:

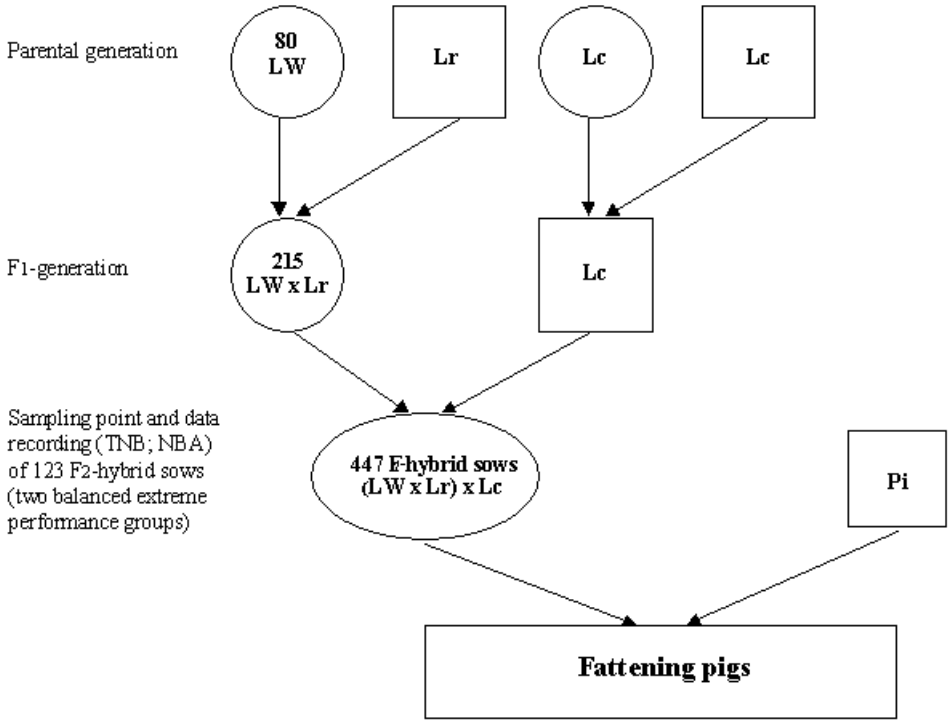
Sow farm "Polkenberg"

In the commercial sow farm "Polkenberg", the investigations were performed with F_2-hybrid

sows of a cross between (Large White x Landrace) x Leicoma. The breeding schema is described in Figure 5.1.1.1.

447 F_2-hybrid sows, which had at least four litters, were stated as the "basic population" out of a total of about 1800 F_2-hybrid sows. Two extreme performance groups were formed according to the number of total newborn (TNB) piglets. The high performance group consisted of 61 sows with at least 14.3 piglets from the second to the fourth litter whereas the low performance group consisted of 62 sows with less than 11.3 piglets from the second to the fourth litter (see also Table 5.2.1.1 on page 58). The first litter was ignored, because in general, first litters are more uneven than the following litters. Standard deviation of TNB piglets from the second to the fourth litter was smaller than 4.5 for each sow. All sows were mated with Piétrain boars. It was ensured that the boars were distributed randomly to the sows of the two extreme performance groups in order to prevent significant paternal influence on litter size. Directly after farrowing, TNB and the number of born alive (NBA) piglets were recorded.

Figure 5.1.1.1: Breeding schema of the commercial sow farm "Polkenberg" (Sachsen, Germany)



Annotations: circle = female; square = male; F_1 = filial generation 1; F_2 = filial generation 2

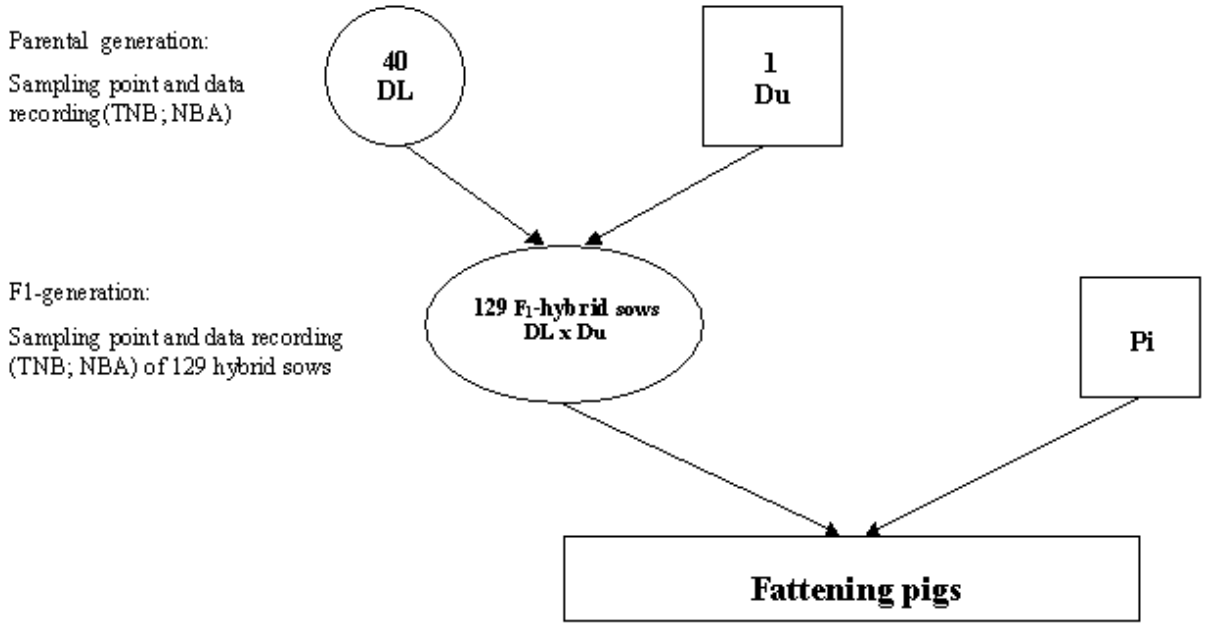
Lc = Leicoma; Lr = Landrace; LW = Large White; Pi = Piétrain

Sow farm "Schulzendorf"

In the commercial sow farm "Schulzendorf" (Brandenburg, Germany), the investigations were performed with F_1-hybrid sows of a cross between German Landrace and Duroc. The breeding schema is described in Figure 5.1.1.2.

129 F_1-hybrid sows had at least four litters. The F_1-hybrid sows, the dams and the sire were genotyped. Directly after farrowing, the total number of born (TNB) and the number of born alive (NBA) piglets were recorded from both, the dams and F_1-hybrid sows. Furthermore, information was available on the age of the first parity, the technician of insemination, the return to estrus of sows, purebreeding of the dams, the boars of the F_1-sows and the season of insemination.

Figure 5.1.1.2: Breeding schema of the commercial sow farm "Schulzendorf" (Brandenburg, Germany)



Annotations: circle = female; square = male; F_1 = filial generation 1

DL = Deutsche Landrasse; Du = Duroc; Pi = Piétrain

5.1.2 Molecular methods

DNA-Isolation

Approximately 0.25 cm^2 ear chondral tissue was punched from each selected sow and stored at –80°C until DNA-isolation. Pinhead-sized samples were incubated with 60 µl Proteinase-K solution (10 mg/ ml) and 1 ml Tris-buffer (pH 8.0) at 55°C over night in a caloric-rotor. Afterwards, samples were centrifugated at 13800 g at 4°C for 10 min. The supernatant was mixed with 500 µl 6M NaCl-solution and centrifugated again at 13800 g at 4°C for 15 min. Precipitation of DNA was performed with 3 ml ice-cold ethanol (100%). DNA was dried and dissolved in HPLC-water. DNA quality and concentration were measured using spectral photometric procedures (Nanodrop, Kisker, Germany). If required, DNA was precipitated again (500 µl sample) with 500 µl ice-cold ethanol (100%) and subsequently rinsed twice with 500 µl ethanol (70%), respectively. Final DNA-concentration for PCR procedures was adjusted to about 50 ng/ µl.

Gene tests for known polymorphisms in the genes BF, GPX5, FUT1 and ESR2

Molecular methods in order to determine genotypes for the BF gene are described in chapter 5.2.1 on page 58 and for the genes GPX5, FUT1 and ESR2 in chapter 5.2.2 on page 68, respectively.

Detection of novel polymorphisms in the gene CYP21 and development of a gene test

The method for the detection of novel polymorphisms at the CYP21 gene as well as the development of a PCR-RFLP test are presented in chapter 5.2.3 on page 81 and 82, respectively.

5.1.3 Statistical analyses

The statistical procedures were performed for each farm separately. The statistical models for the association studies are described in the following publications (chapter 5.2.1, page 59; chapter 5.2.2, page 69; chapter 5.2.3, page 82) in detail. The statistical calculations were performed with the SAS program (version 8.2) and the SPSS program (version 12.0).

Calculation of the genotype and allele frequencies for the candidate genes

The genotype frequencies (in %) were calculated as follows:

$$\text{Genotype frequency (\%)} = (AA/N) \times 100;\ (AB/N) \times 100;\ (BB/N) \times 100$$

Allele frequencies, declared as nondimensional figures for each allele were calculated as follows:

$$\text{Allele frequency (A)} = (2 \times AA + 1 \times AB)/2N$$
$$\text{Allele frequency (B)} = 1\text{-}A$$

N = number of animals

Calculation of additive and dominance effects

The degree of dominance (D) was calculated as follows:

$$D = d/a$$

whereas dominance effects (d) were estimated as the deviation of the heterozygotes from the mean value of the homozygotes and additive effects (a) as the deviation of the mean value from both homozygotes from the superior allele $_{(+)}$. The equations in order to calculate "d" and "a" were the following:

$$d = AB-(AA+BB)/2$$
$$a = AA_{(+)} - (AA_{(+)} + BB)/2$$

The degree of dominance was divided into four classes: D = 0 corresponds for no dominance, D = 1 for complete dominance, 0 < D < 1 for partial dominance and D > 1 for overdominance.

5.2 Results

Overview on the main results concerning association studies

A significant effect of the ESR2 gene on NBA piglets was found in the F_1-hybrid sows (DL x Du). The population of 129 F_1-sows consisted of homozygous GG and heterozygous AG genotypes. No AA genotype was detectable, because the sire was homozygous GG, which showed that in the maternal DL race at least two different alleles were segregating. F_1-sows carrying the A allele had on average 0.57 more TNB and 0.70 more NBA piglets per sow and litter than sows of the GG genotype (Table 5.2.1). As the genotype frequencies for the genes BF and FUT1 were extremely unbalanced in this population, no statement can be given concerning associations between gene variants and litter size. For the CYP21 gene, a novel detected polymorphism in the 3'UTR was tested for associations to litter size in this population, but only a nonsignificant trend in favor of the rare A allele has been observed for both, TNB and NBA piglets, respectively. No effect was found for the GPX5 gene.

Table 5.2.1: Litter size parameters of ESR2 genotypes in F_1-hybrid sows (DL x Du)

Genotype	Sows	Litters	TNB		NBA	
			Mean	SD	Mean	SD
GG	42	162	12.07	2.98	11.06[a]	2.87
AG	87	329	12.64	2.90	11.76[b]	2.69

a,b Means in the same column with different superscripts significantly differ at $p<0.05$.

The analysis of genotype distribution pattern among F_2 hybrid sows (LW x Lr) x Lc with extremely high and low litter size has identified significant differences for the FUT1 gene (Table 5.2.2). The number of sows with the AB genotype was significantly increased in the high performance group in comparison to the low performance group. No significant effects could be attributed to the genes BF, GPX5 and ESR2. With regard to the level of significance of $p<0.05$, TNB and NBA piglets were associated with BF genotypes by analysis of variance. The genotype BB led to 2.64 TNB and 2.11 NBA piglets more than the genotype AA, respectively. The genotype AB was intermediate. However, these results should be considered with caution, as effects are overestimated due to the analysis of extreme phenotype groups.

Table 5.2.2: Genotype distribution of candidate genes in the two litter performance groups

Genotype	BF Performance		GPX5 Performance		FUT1** Performance		ESR2*** Performance	
	Low	High	Low	High	Low	High	Low	High
AA	3	0	4	3	2	1	8	8
AB	13	7	24	17	11*	24*	54	53
BB	46	54	34	41	48*	34*	0	0

* Significantly different at $p<0.05$, Chi-square-test.

** Three sows could not be genotyped for the FUT1 gene.

***In the thesis and publications, except for Arch. Tierz. (2006) is AA = GG, AB = AG and BB = AA.

5.2.1 Analysis of Properdin (BF) genotypes associated with litter size in a commercial pig cross population

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Journal of Animal Breeding and Genetics 122 (2005), 259-263

Summary

Properdin (BF) was investigated as a candidate gene influencing litter size in a commercial pig cross population. The BF gene was chosen because of it's integral role influencing uterine epithelium growth and because several QTL with impact for reproductive traits have been detected near the centromere of porcine chromosome 7. 123 F_2-sows of a cross between (Large White x Landrace) x Leicoma were genotyped using a PCR-RFLP method. The sows were divided into two extreme performance groups, one with a high litter size (n = 61, ≥ 14.3 piglets per litter) and one with a low litter size (n = 62, ≤ 11.3 piglets per litter). Although genotype and allele frequencies were uneven with 2.4% (AA), 16.3% (AB), 81.3% (BB) and 0.11 (A): 0.89 (B), respectively, the A allele was the unfavorable one, leading to less offspring. With regard to the level of significance of $p<0.05$, the total number of born (TNB) and number of born alive (NBA) piglets were associated with BF genotypes. The genotype AA led to 10.55 TNB and 10.00 NBA respectively, whereas the genotype BB led to 13.19 TNB and 12.11 NBA. The genotype AB was intermediate. In future, a systematic mating test is necessary in order to obtain more balanced genotype frequencies. Furthermore it should be taken into consideration that the investigated polymorphism is located in an intronic region and the causative mutation is not clear yet.

Key words:
BF, candidate gene, litter size, pigs, QTL, reproductive traits

Introduction

The improvement of reproduction traits in livestock species has become of expanding interest especially in swine, where moderate increases in litter size can equal large gain in profit (Vincent et al., 1998). However, selection programmes are almost only based on phenotypical traits which are laborious, expensive and especially in pig production time-consuming. Marker assisted selection (MAS), employed in conjunction with traditional selection methods, could accelerate the rate of change in economically important traits. In order to detect loci for a special trait, there are in principle two strategies possible: QTL-analysis and the candidate gene approach (Rothschild et al., 2000). Several QTL regions for reproductive traits in swine have already been detected, among them QTL for uterine length, ovulation rate and litter size near the centromere of porcine chromosome 7. However, until now no gene responsible for litter size has been identified on this chromosome. Furthermore, a putative murine QTL that influences heterosis in litter size was found on mouse chromosome 17 (Brunsch et al., 1998) which is homologue to the major histocompatibility complex (MHC) class III region on porcine chromosome 7 (Peelman et al., 1996). One of the genes in the MHC class III region encodes Properdin. The Properdin (BF) gene has been mapped near to the centromere of porcine chromosome 7 at 7cen (Pinton et al., 2000) and 7 1/2p11-p12 (Ponsuksili et al., 2001). Because Properdin has a physiological important function for reproductive traits such as uterine epithelium growth (Hasty et al., 1993) and on litter size in mice in conjunction with other genes (Matsumoto et al., 1997), Properdin (BF) was selected as a candidate gene for litter size in swine. In most cases, reference families were used for applying either QTL analyses or candidate gene approaches. The aim of this study was to investigate, whether the BF gene is associated with litter size in a commercial sow population and to compare the results with published QTL found for reproductive traits on porcine chromosome 7.

Material and methods

Animals

A commercial pig population was used from a German sow farm (Polkenberg, Sachsen, Germany). 447 sows of a cross between (Large White x Landrace) x Leicoma that had four litters at minimum represented the basic population. Two extreme performance groups were formed according to the number of total born piglets (Table 5.2.1.1): The high performance group consisted of 61 sows with at least 14.3 piglets from the second to the fourth litter

whereas the low performance group consisted of 62 sows with less than 11.3 piglets from the second to the fourth litter. The first litter was ignored, because in general first litters are more uneven than the following litters. All sows were mated with Piétrain boars by artificial insemination with a constant amount of fresh sperm. The feeding and housing regime of the sows were kept constant. Directly after farrowing, the total number of born (TNB) and the number of born alive (NBA) piglets were recorded. DNA was obtained from ear chondral tissue and was isolated according to standard methods.

Table 5.2.1.1: Genotyped sows of two performance groups

		Litter size (second to fourth litter)			
		TNB		NBA	
Performance group	Number of F_2-sows	Mean	range	Mean	range
High	61	15.73	12-24	14.10	9-19
Low	62	10.39	3-14	9.83	3-14

Genotyping

The PCR-RFLP for the Properdin (BF) gene (GenBank Accession no. M59240) was performed according to the method developed by Jiang and Gibson (1998) with modifications. Primer sequences were as follows: Forward: 5' ACT GCT ATG ACG GTT ACA CTC TCC G 3'; reverse: 5' TCC AAG AGC CAC CTT CCT GG 3'. PCR conditions were the following: Approximately 150 ng of genomic DNA was amplified in a final volume of 25 µl containing 0.4 µM of each primer (BioTeZ, Berlin, Germany), 0.2 mM dNTPs, 3 mM $MgCl_2$, 1 x *Taq*-reaction-buffer without $MgCl_2$ and 1 U *Taq* DNA polymerase (Invitek, Berlin, Germany). After denaturation at 94ºC for 2 min, 30 amplification cycles comprising denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 50 s were performed followed by a final 5 min extension step at 72ºC. Subsequently, the PCR fragment was digested with the restriction enzyme *Sma*I (New England BioLabs, Frankfurt, Germany) to show the polymorphism. The amplified fragment was incubated in a total volume of 14.5 µl containing 12 µl of the PCR product, 5 U of enzyme, 1.5 µl restriction buffer at 25ºC for 3 h. Restriction fragments were examined by electrophoresis on 1.5% agarose gel with 1 x TBE buffer. The gels were stained with ethidium bromide and photographed. The PCR-RFLP assay yielded two bands of 237 and 153 bp (genotype AA), a single 390 bp-band

(genotype BB) and all three bands for the genotype AB as it is described by Jiang and Gibson (1998).

Statistical analyses

The statistical analyses were performed with the SAS program (version 8.2). First of all it was investigated, if a special genotype occurs significantly in one of the two performance groups ($p<0.05$). For this purpose, the Chi-square test with the extension of the Fisher's Exact test was used. The latter one was performed in order to determine the exact p-values for the frequencies of the genotyped sows. Furthermore, it was investigated by multivariate analysis of variance including the Duncan test, whether a special genotype has an influence on the traits TNB and NBA. The Duncan test was performed because this test can distinguish significant differences between means for the measured traits even if genotype frequencies are uneven. This test is less conservative than others and is in that cases applicable, when the numbers within class variables are unequal. The following linear model was employed for the analysis of variance:

$$Y_{ijkl} = \mu + gt_i + lno_j + pgr_k + gt(lno)_{ij} + gt(pgr)_{ik} + e_{ijkl}$$

with

Y_{ijkl}	=	observation value for TNB and NBA
μ	=	aggregate mean value
gt_i	=	fixed effect of genotype i
lno_j	=	fixed effect of litter number j
pgr_k	=	fixed effect of performance group k
$gt(lno)_{ij}$	=	fixed effect of genotype i within litter number j
$gt\ (pgr)_{ik}$	=	fixed effect of genotype i within performance group k
e_{ijkl}	=	error

Dominance effects (d) were estimated as the deviation of the heterozygotes from the mean value of the homozygotes and additive effects (a) as the mean value from both homozygotes. The degree of dominance (D) was calculated with $D = d/a$.

Results

Genotype distribution, genotype and allele frequencies

Table 5.2.1.2 shows the distribution of the different genotypes for BF within the two performance groups of commercial (Large White x Landrace) x Leicoma sows.

Table 5.2.1.2: Genotype distribution of the BF gene in the performance groups

Genotype	High performance group (n = 61)	Low performance group (n = 62)	Total n = 123
AA	0	3	3
AB	7	13	20
BB	54	46	100

Among all genotyped sows, the genotype frequencies were 2.4% for AA, 16.3% for AB and 81.3% for BB. The allele frequencies were 0.11 and 0.89 for alleles *A* and *B*, respectively. The genotype distribution between the high performance group and the low performance group showed a p-value ($p = 0.055$) slightly over the level of significance of α=5%.

Influence of the genotype on litter size (TNB and NBA)

Table 5.2.1.3 shows the influence of the genotype on litter size as well as additive and dominance effects for the traits TNB and NBA of the commercial (Large White x Landrace) x Leicoma sows.

Table 5.2.1.3: Influence of BF genotypes on litter size in commercial sows

Genotype	TNB	SD	NBA	SD	Number of litters
AA	10.55a	2.96	10.00a	2.83	9
AB	12.63b	3.58	11.43b	2.86	60
BB	13.19b	3.54	12.11b	3.11	300
a	1.32		1.06		
d	0.76		0.38		
D	0.58		0.36		

a,b Means in the same column with different superscripts significantly differ at $p<0.05$.

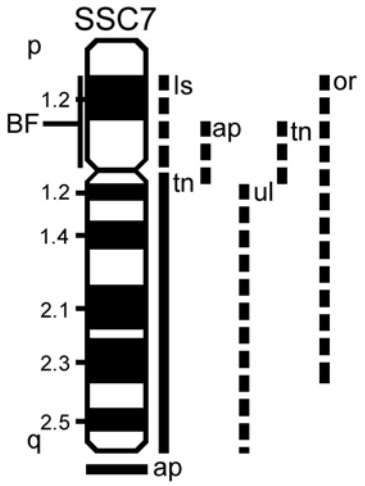
a = additive effect; d = dominance effect; D = degree of dominance

Both, TNB and NBA were influenced significantly by the genotype. In comparison with the A allele, the B allele was the favorable one, leading to more offspring. However, it is also to indicate, that the least squares means including the performance group do not confirm this result. There were dominance effects of 0.76 and 0.38 and additive effects of 1.32 and 1.06 for TNB and NBA, respectively. The degree of dominance was 0.58 and 0.36, and hence, it follows that this can be considered according to the assumption of partial dominance ($0 < D < 1$) for both TNB and NBA, respectively.

Discussion

In Figure 5.2.1.1 we present a compilation of all known QTL regions on SSC7 with an influence on reproductive traits and the cytogenetic position of the gene Properdin (BF). BF has been mapped on the p arm near the centromere to SSC7 and overlaps well with QTL regions for reproductive traits such as litter size (de Koning et al., 2001), ovulation rate (Wilkie et al., 1999) and age of puberty (Cassady et al., 2001).

Figure 5.2.1.1:Cytogenetic map of porcine chromosome 7 showing known QTL affecting reproductive traits in sows



ap = age of puberty, ls = litter size, or = ovulation rate, tn = teat number, ul = uterine length; bold solid lines = genome wide level of significance < 0.05, dashed lines = genome wide level of significance > 0.05; cytogenetic position of the bold line at the end of the chromosome (ap) was not evaluable; BF = Properdin.

Almost all investigations with regard to QTL analyses and candidate gene approaches were performed with reference families. We used a commercial sow population, and therefore it was unlikely to obtain equal allele and genotype frequencies. However, as a result, it can be

assumed that the A allele is the unfavorable one, producing less piglets than the B allele (Table 5.2.1.3). Furthermore, this allele occured more in the low performance group (Table 5.2.1.2); however, the level of significance was not reached. Therefore it seems that the unfavorable A allele has been reduced in this population over years by phenotypical selection. A similar experimental setup with regard to effects of RYR1 and ESR genotypes on the fertility of sows was performed by Matoušek et al. (2003). They investigated two elite herds of Large White sows and observed also unequal genotype and allele frequencies concerning the RYR1 gene locus. Although the number of animals (124 sows) was rather low, the authors were able to find significant differences for the trait TNB, because they evaluated six litters for each sow. Although there are many QTL on SSC7 affecting reproductive traits, so far no gene on this chromosome has been identified that shows an effect on fecundity. Cassady et al. (2001) reported a significant QTL for age at puberty and a suggestive QTL for teat number on porcine chromosome 7. A significant QTL for teat number was also reported by Wada et al. (2000), but at another position. Teat number plays a significant role when there are more piglets than teats. Hence, the selection on litter size may require improvement of teat number (Hirooka et al., 2001). In contrast, although teat number is easily measured in both, males and females, it is not a likely candidate for MAS because firstly, there are a lot of QTL detected for teat number also on other chromosomes and secondly there are more important other traits affecting litter size in swine. Moreover, pleiotrophic effects on several reproductive traits such as ovulation rate in Meishan pigs have been reported (Rohrer 2000). Wilkie et al. (1999) observed two suggestive QTL for uterine length and ovulation rate on porcine chromosome 7. Because these two parameters affect litter size in a considerable amount, Leymaster and Johnson (1994) concluded that selection for ovulation rate and uterine capacity might produce the greatest response in litter size. However, these parameters are difficult to measure, especially in the same animals in which both traits are only measurable for one parity. Until now, only Rohrer et al. (1999), Wilkie et al. (1999) and Isler et al. (2002) investigated both traits in the same animals. When ovulation rate and uterine capacity have an effect on litter size, it can be expected, that there would be also a QTL for traits such as TNB and NBA. For the first litter, de Koning et al. (2001) reported such a QTL on the short arm on chromosome 7 near the centromere which overlaps with the QTL for ovulation rate found by Wilkie et al. (1999). These findings indicate, that in this region could be one gene or more having an effect on litter size, because litter size is directly influenced by ovulation rate. It can be noticed, that the centromere region on porcine chromosome 7 is associated with reproductive traits. Properdin (BF) might be one gene with a special importance for litter size.

However, in this region a lot of other genes can be found belonging to the MHC class III complex, so we are not able to say whether Properdin is the gene leading to different litter sizes itself or if it is only a linked marker for this trait. Furthermore it must be taken into consideration that the investigated polymorphism is located in an intronic region and the causative mutation is not clear yet. In the future, a systematic mating test is necessary in order to obtain more balanced genotype frequencies. Considering all these facts, selection progress is mainly achieved, when firstly a beneficial polymorphism is detected for a desirable trait, and rare genotypes are detected in a commercial breed at the molecular gene level in order to increase this genotype in the population.

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5.2.2 Analysis of association of GPX5, FUT1, and ESR2 genotypes with litter size in a commercial pig cross population

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Summary

The aim of this study was to investigate, if special genotypes of the genes glutathione peroxidase 5 (GPX5), fucosyltransferase 1 (FUT1) and estrogen receptor 2 (ESR2) are associated with litter size in a commercial pig cross population. For this purpose, a total of 123 F_2 sows were divided into two extreme performance groups, one with a high litter size ($n = 61$, ≥ 14.3 piglets per litter) and one with a low litter size ($n = 62$, ≤ 11.3 piglets per litter) and genotyped using PCR-RFLP methods. The Chi-square test was used in order to investigate, if a special genotype occurs significantly more often in one of the two performance groups ($p<0.05$). Whereas no association was found between different ESR2 or GPX5 genotypes with one of the two performance groups, the number of sows with AB genotypes at the FUT1 gene was significantly increased in the high performance group in comparison to the low performance group.

Key words:
Candidate gene, ESR2, FUT1, GPX5, litter size, pigs, reproductive traits, two-tail analysis

Introduction

The genetical improvement of litter size in swine is of expanding interest for pig producers mainly because ameliorations due to feeding regime and housing systems are limited. It has been observed, that up to the middle of the nineties in the past century, improvements were first of all made concerning meat quality. This led to a stagnancy, and even in some cases to a decrease of litter size especially in swine production (Kisner et al., 1995). Therefore, an improvement of fecundity in swine is desirable. Selection programs, however, are almost only based on phenotypical traits, which are laborious, expensive and especially in pig production time-consuming. Since a few years, marker assisted selection (MAS), employed in conjunction with traditional selection methods has been in progress to increase litter size in swine. One marker, which is used in commercial pig production, is the estrogen receptor gene 1 (ESR1), and Rothschild et al. (1996) were the first who observed an association between ESR1 genotypes and litter size in swine. However, so far, there are a lot of inconsistent studies with regard to influence on the ESR1 gene on fecundity parameters in swine (Short et al., 1997, Depuydt et al., 1999, Drögemüller et al., 1999, Kmieć et al., 2002, Gibson et al., 2002, van Rens and van der Lende, 2002, Matoušek et al., 2003, Goliášová and Wolf, 2004, Horák et al., 2005, Horogh et al., 2005, Wang et al., 2006). Therefore, additional candidate genes with potential influence on litter size are studied.

The glutathione peroxidase 5 gene (GPX5) is located on SSC7 in a chromosomal region in which several quantitative trait loci (QTL) for reproductive traits in swine, such as uterine capacity, ovulation rate and litter size have been detected. Linkage analyses of GPX5 showed that this gene is closely linked to the major histocompatibility complex (MHC), which has been suggested to have an effect on reproductive traits in swine (Vaiman et al., 1998; Buske et al., 2005).

The fucosyltransferase 1 gene (FUT1) on SSC6 is also located in a chromosomal region, which is associated with litter size in swine. Actually, the FUT1 gene has been determined as a candidate gene for the Escherichia coli F18 receptor locus (Meijerink et al., 1997) and an association with oedema disease, diarrhea, and thus an association with animal health was severalfold observed. Because animal health is strongly attended with fecundity, FUT1 could also serve as a candidate gene for litter size in swine (Horák et al., 2005).

Concerning estrogen receptors, there are at least two different classes, and Muñoz et al.

(2004) mapped the estrogen 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 for its role as transcription factor. Moreover, the ESR2 gene has been characterized in the rat, mouse and human, and several studies suggest that this receptor displays a high binding affinity to estrogens and therefore might be involved in ovarian follicular growth and development at periimplantation (Kowalski et al., 2002). Hence, ESR2 could also serve as a candidate gene for litter size in swine.

Therefore, the aim of this study was to investigate, whether the candidate genes GPX5, FUT1 and ESR2 are associated with litter size in swine in a commercial population. In most cases, association studies were conducted by using a variable number of sows of a resource population. In contrast, we used sows of two extreme performance groups with high and low litter size (two-tail analysis) which were kept in a commercial sow farm.

Material and methods

Animals

Investigations were performed with a commercial pig population from a German sow farm (Polkenberg, Sachsen, Germany). 447 F_2-sows of a cross between (Large White x Landrace) sows x Leicoma boars that had four litters at minimum represented the basic population. Average litter size of these sows was 13.1 piglets per litter according to the number of total newborn (TNB) piglets from the second to the fourth litter. From this basic population, two extreme performance groups were formed comprising 14% best and 14% worst sows, according to the number of TNB piglets. The high performance group consisted of 61 sows with at least averaged 14.3 piglets per litter from the second to the fourth litter whereas the low performance group consisted of 62 sows with less than averaged 11.3 piglets per litter from the second to the fourth litter (Table 5.2.2.1). The first litter was ignored, because in general, first litters are more uneven than the following litters. About 20% of the 123 selected sows were full-sibs. It was ensured that there was no significant accumulation of these sows in one of the two performance groups. The rest of the sows were half-sibs or unrelated. All sows were mated with Piétrain boars by artificial insemination with a constant amount of fresh sperm. The age of first insemination of the sows, as well as feeding and housing regime

were kept constant. A total of 76 boars were distributed randomly to the sows of both performance groups in order to prevent significant paternal influence on litter size. Directly after farrowing, TNB and the number of born alive (NBA) piglets were recorded. DNA of sows was obtained from ear chondral tissue and was isolated according to standard methods.

Table 5.2.2.1: Litter size parameters of selected and genotyped sows of two performance groups

		Litter size (second to the fourth litter)					
		TNB			NBA		
Performance group	Number of F_2-sows	Mean	SD	Range	Mean	SD	Range
High	61	15.73	2.24	12-24	14.10	2.13	9-19
Low	62	10.39	2.44	3-14	9.83	2.31	3-14

TNB = Total number born; NBA = Number born alive

Genotyping

The gene variants were amplified and distinguished according to standard PCR-RFLP methods with small modifications in reference to the original literature (Table 5.2.2.2). For the PCR, all genes were amplified separately in a total reaction volume of 25 µl containing approximately 75 to 150 ng of genomic DNA, 1 x *Taq* reaction buffer without $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, appropriate amount of $MgCl_2$ (Table 5.2.2.2), and 1 U *Taq* DNA polymerase (Genaxxon, Germany). After denaturation at 94°C for 2 min, 35 amplification cycles comprising denaturation at 94°C for 1 min, annealing at appropriate temperature (Table 5.2.2.2) for 30 s, and extension at 72°C for 40 s were performed followed by a final 5 min extension step at 72°C.

Table 5.2.2.2: Different PCR-RFLP parameters for the genes GPX5, FUT1 and ESR2

Gene Access. no.	Primer sequence (forward and reverse) 5' to 3'	$MgCl_2$ (mM)	Annealing temp. (°C)	Restriction enzyme (U)
GPX5[1]	Fw: TTC ATG TAG AAC TTA TTT CTG	2.0	51	*Hinf1*
AF124818	Rv: TGA CTT ACC CAT TCT TCA G			10
FUT1[2]	Fw: CTG CCT GAA CGT CTA TCA AGA TC	2.5	56	*Hha1*
U70883	Rv: CTT CAG CCA GGG CTC CTT TAA G			2
ESR2[3]	Fw: AAA ATA CTG ATA CCC ACC CCA CAT	2.0	61	*Hsp92II*
AF164957	Rv: CGC CAC ATC AGC CCC ACC AT			5

1,2,3: Original methods in (1) Bertani et al. (1999); (2) Klukowska et al. (1999); (3) Muñoz et al. (2004); GPX5 = Glutathione peroxidase 5; FUT1 = Fucosyltransferase 1; ESR2 = Estrogen receptor 2

After the amplification of the genes, PCR products were incubated with the appropriate restriction enzyme (Table 5.2.2.2) at 37°C for 4 h in order to show the gene variants. DNA-fragments were separated on 2% agarose-gels. Table 5.2.2.3 shows the expected fragment length of the different genotypes for the investigated genes.

Table 5.2.2.3: Fragment length in basepairs of the genotypes for the GPX5, FUT1 and ESR2 genes

Gene	PCR-product (bp)	Fragment length of genotypes (bp)			Remarks
		AA	AB	BB	
GPX5	501	298, 94, 53, 33, 23	298, 234, 94, 64, 53, 33, 23	234, 94, 64, 53, 33, 23	23, 33, 53 and 64 are not visible
FUT1	421	328, 93	328, 241, 93, 87	241, 93, 87	87 and 93 are inseparable
ESR2	218	202, 16	202, 142, 60, 16	142, 60, 16	16 and 60 are not visible

Underlined fragments: main fragments in order to distinguish genotypes

Statistical analyses

Firstly, the correlation between TNB and the number of NBA piglets for each performance group was calculated by using the Pearson's Correlation Coefficient *(r).* Afterwards, it was investigated, if a special genotype occurs significantly more often in one of the two performance groups ($p<0.05$). For this purpose, the Chi-square test with the extension of the Fisher's Exact test was used. The latter one was carried out in order to determine the exact

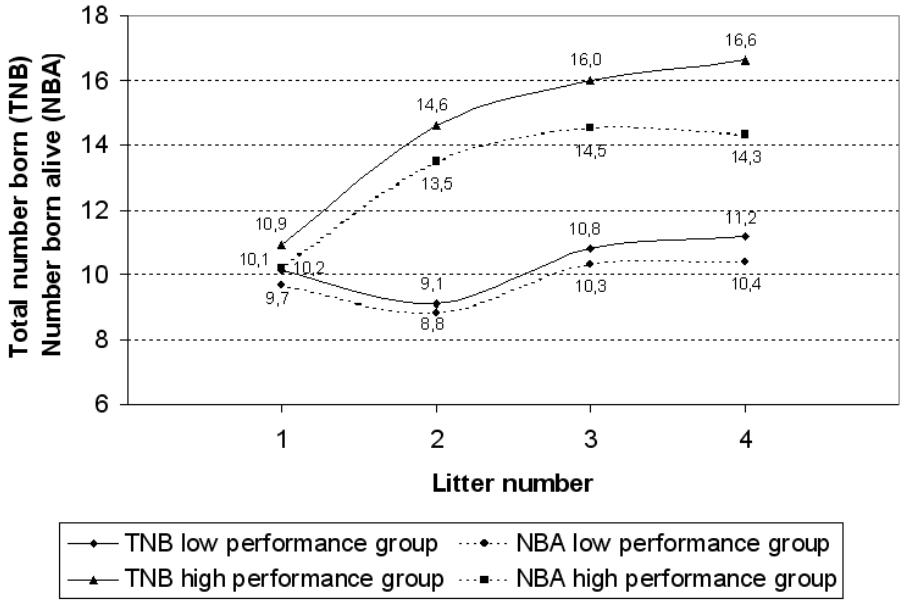
p-values for the expected frequencies of the genotyped sows, when the expected frequencies were smaller than 5 in at least one class. The statistical analyses were performed with the SPSS program (version 12.0).

Results

Development of the TNB and the number of NBA piglets in the two performance groups

Figure 5.2.2.1 shows the development of litter size in the two extreme performance groups of the sows from litter number 1 up to litter number 4. Whereas there is no remarkable difference between the two extreme performance groups for the first litter, both performance groups differed considerably in the following litters for TNB and the number of NBA piglets, respectively.

Figure 5.2.2.1: Development of litter size in dependency of the litter number in the two performance groups



Correlation between the TNB and the number of NBA piglets in the two performance groups

Table 5.2.2.4 shows the mean values for litter size and the coefficients of correlation (*r)* between TNB and NBA piglets for the two extreme performance groups for the litters 1 to 4 and for all 4 litters.

Table 5.2.2.4: Mean values for TNB and NBA piglets and coefficients of correlation *(r)* between TNB and NBA piglets for the litters 1 to 4 for sows in the two performance groups

	F_2-sows (N=123)									
	Low performance group (N=62)					High performance group (N=61)				
	TNB		NBA			TNB		NBA		
Litter no.	Mean	SD	Mean	SD	*r*	Mean	SD	Mean	SD	*r*
1	10.1	3.13	9.7	3.10	0.972	10.9	3.02	10.2	2.91	0.924
2	9.1	2.79	8.8	2.68	0.971	14.6	2.16	13.5	1.87	0.720
3	10.8	2.06	10.3	1.86	0.929	16.0	2.06	14.5	2.26	0.662
4	11.2	1.91	10.4	1.95	0.860	16.6	2.26	14.3	2.17	0.577
1-4	10.3	2.63	9.8	2.53	0.950	14.5	3.23	13.1	2.89	0.845
2-4	10.4	2.44	9.8	2.31	0.938	15.7	2.24	14.1	2.13	0.652

The level of significance for *r* is always $p<0.01$

TNB = Total number born; NBA = Number born alive

Genotype and allele frequencies and genotype distribution

The genotype and allele frequencies for the GPX5, FUT1 and ESR2 genes over all genotyped sows are presented in Table 5.2.2.5.

Table 5.2.2.5: Genotype and allele frequencies over all genotyped sows

Gene	N	Genotype frequency			Allele frequency	
		AA	AB	BB	A	B
GPX5	123	0.057	0.333	0.610	0.22	0.78
FUT1	120	0.025	0.292	0.683	0.17	0.83
ESR2	123	0.130	0.870	0.000	0.57	0.43

For the genes GPX5 and FUT1, expected genotype frequencies corresponded well to the observed allele frequencies according to Hardy-Weinberg equilibrium, however, this was not the case for the ESR2 gene. According to the nearly balanced allele frequencies, one could expect, that there should be about 25% animals with the homozygous BB genotype, but no animal with this genotype was observed. Table 5.2.2.6 shows the distribution of the different genotypes for the GPX5, FUT1 and ESR2 genes in the two performance groups of the

commercial F_2-sows. Only for the FUT1 gene, the genotype distribution between the low and the high performance group was significantly different. Sows with the heterozygous AB genotype occurred significantly more often in the high performance group, whereas sows with the BB genotype occurred significantly more often in the low performance group.

Table 5.2.2.6: Genotype distribution of the GPX5, FUT1 and ESR2 genes in the two performance groups

Genotype	Gene GPX5 Performance group		FUT1 Performance group		ESR2 Performance group	
	low	high	low	high	low	high
AA	4	3	2	1	8	8
AB	24	17	11*	24*	54	53
BB	34	41	48*	34*	0	0
N/ Performance group	62	61	61	59	62	61
N (Total)	123		120**		123	

* significantly different ($p<0.05$), Chi-square-test, **3 sows could not be genotyped for the FUT1 gene

Discussion

In Figure 5.2.2.1 we present the development of litter size in the two extreme performance groups. Because the selection of the sows for the two performance groups based only on the litters two to four, it is interesting to note that for the first litter, litter performance was almost equal for both, the low and the high performance group. A reason for this unexpected result is probably - provided that the age of first insemination was kept constant - that the development of sows has not been finished at the age of first insemination. Therefore, genetical effects on phenotypical fecundity parameters cannot be seen entirely until the sows are not completely full-grown. This observation is important for further investigations, particularly, when gilts are investigated for reproductive traits such as uterine capacity or ovulation rate. For example, Isler et al. (2002) observed an increased ovulation rate in sows from 17.22 corpora lutea for the first litter up to 19.92 for the third litter. The conclusion of non-genetical effects for a phenotypical trait, which does not differ at an early stage of development could be false because of confusion with unmaturity of the organism and the general physiological status of the animal, which hides a potential genetic predisposition.

Because the selection of sows based only on TNB piglets, it was interesting to evaluate the correlation between TNB and the number of NBA piglets in the two performance groups of high and low litter size (Table 5.2.2.4). The expectation was, that in general, the correlation between both traits was high, and, that the correlation in the high performance group should be less than in the low performance group. These expectations were fulfilled. However, there was a strong trend for lower correlations between TNB and the number of NBA piglets in higher litter numbers particularly for the high performance group without any exception. An explanation for these results is probably that sows with a high potential of farrowing piglets are more susceptible to losses during farrowing or directly before, also due to non-genetical effects.

GPX5 has been mapped on SSC7 at 7 1/2p12-p11 (Bertani et al., 1999) and was mainly chosen as a candidate gene because many QTL for fecundity parameters such as litter size (de Koning et al., 2001), ovulation rate (Wilkie et al., 1999) and age of puberty (Cassady et al., 2001) were observed in this chromosomal region. Both, Vaiman et al. (1998) and Buske et al. (2005) speculated of a physiological influence of this chromosomal region on fecundity parameters by the MHC class III region, however, in our population, no association between litter size and a special genotype of GPX5 was observed. From a physiological point of view, GPX5 is mainly involved in sperm quality. Therefore, GPX5 is likely involved in paternal than in maternal fertility.

A chromosomal region for association with litter size in swine is located at the centromeric region on the q-arm of porcine chromosome 6 (Yasue et al., 1999). FUT1 has been mapped on SSC6 at 6q11 (Vögeli et al., 1996) and is located in this region. At first, however, the FUT1 gene has been determined as a candidate gene for Escherichia coli F18 receptor locus (Meijerink et al., 1997). The single nucleotide polymorphism (SNP) at position 307 in exon 2 is strongly associated with oedema desease and diarrhea in swine, and therefore, with animal health. Because animal health is generally required for high fecundity, the hypothesis was that FUT1 might also be associated with reproductive traits, such as litter size in pigs. Recently, Horák et al. (2005) investigated this SNP of the FUT1 gene with regard to litter size at 104 sows of the local autochthonous Czech breed. They investigated up to 6 litters for each sow and observed that the heterozygous AB and the homozygous BB genotypes were the favorable ones for the traits TNB and the number of NBA piglets. Their hypothesis was, however, that the AA genotype should be beneficial on fecundity parameters due to the

resistance to Escherichia coli F18 infection of this genotype. Because we also found that the AB genotype was favorable on litter size parameters, a pleiotropic effect of the FUT1 gene cannot be excluded. However, it is more likely that not the FUT1 gene itself but rather another closely linked gene could be responsible for the observed results. According to Vögeli et al. (1996), a distorted linkage disequilibrium between those genes can lead to such results, when just the beneficial genotype for resistance to Escherichia coli F18 infection is linked with another adjacent gene with unfavorable genotypes for litter size parameters.

For the ESR2 gene, Hardy-Weinberg equilibrium was not fulfilled because of the lack of any homozygous BB genotype (Table 5.2.2.5). One explanation could be that genotyped animals were selected F_2-sows. In selected populations, deviations of genotype frequencies from the Hardy-Weinberg equilibrium should be expected for loci with impact on traits under selection (Goliášová and Wolf 2004). Because of the lack of any homozygous BB sows in our population, a completing statement concerning a potential association between ESR2 genotypes and litter size is strictly speaking not feasible. An explanation of our results could be that the BB genotype has been excluded from this population over years. To our knowledge, up to now, there is only one association study concerning the influence on litter size of the ESR2 gene (Muñoz et al., 2004) and no significant association was found by these authors, probably due to low animal numbers.

An additional improvement of the presented two-tail analysis would be, to extend litter size characterization by including a fifth or even a sixth litter in order to improve the reliability of phenotypes, but under our experimental conditions, this was not possible. Even if it is rather unlikely that sows of the high performance group show few offspring in the following litters or reversely, this cannot be excluded even if environmental parameters were highly standardized. In such a case, the grouping of the selected sows could be false for a few individuals, leading to problematic results with regard to an association between the frequencies of genotypes in one of the two performance groups. Therefore, a conclusion of the methodical point of view concerning two-tail analyses is generally, to characterize the phenotype as accurately as possible as it is easily feasible for carcass and meat quality parameters, for example.

In a two tail analysis, one is looking for dissimilarities for genotype distribution between two performance groups with regard to a special phenotype. However, if commercial sow populations are used, as it was the case in this study, there is often the problem that one

genotype is not, or only present with extremely low animal numbers. Therefore, a comparison between rare genotypes cannot be evaluated with sufficient statistical power. Under such circumstances, statements concerning associations between phenotype and genotype are problematically. The more animals for a certain genotype are available, the better is the expressiveness for an association between genotype and phenotype. For further research a planned mating test would be an improvement in order to obtain more balanced genotype frequencies in the basic population (Rothschild et al., 1996). If there is a strong association between genotype and phenotype, a significant imbalance for genotypes between the two performance groups (tails) can be expected. However, planned matings are hardly imaginable when cooperating with commercial sow farms, so, in our opinion, it is essential to revert to populations, which are kept under controlled and standardized laboratory circumstances, or, even better to revert to resource populations. Considering all these facts, selection progress is mainly achieved, if firstly a beneficial polymorphism is detected for a desirable trait, and secondly, rare genotypes are detected which improve the performance in order to increase this genotype in the population.

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5.2.3 Detection of novel SNPs in the CYP21 gene and association analysis of two SNPs for CYP21 and ESR2 with litter size in a commercial sow population

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Summary

Altogether 129 F_1-sows from a commercial sow farm with at least four litters were genotyped for the estrogen 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 3462 G>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 to 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 liveborn 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.

Key words:
Association analysis, candidate gene approach, CYP21, ESR2, litter size, pigs, SNP

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 1990's, 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 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) employed 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 estrogen receptor 1 gene (ESR1). Rothschild et al. (1996) were the first who observed 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 regards to the influence on the ESR1 gene on fecundity parameters in pigs (Alfonso 2005; Buske et al., 2006a). Therefore, additional candidate genes with a potential influence on litter size are studied.

Concerning estrogen receptors, there are at least two different classes. Muñoz et al. (2004) mapped the estrogen 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 transcription factor. The ESR2 gene has been characterized in the rat, mouse, and human, and several studies suggest that this receptor displays a high binding affinity to estrogens and therefore might be involved in ovarian follicular growth and development at periimplantation (Kowalski et al., 2002). Hence, also ESR2 could 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; Geffrotin et al., 1991). It was chosen as a 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 ovulation rate by counting corpora lutea over eight to ten 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 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 on 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 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 x Landrace) sows x Leicoma boars from the commercial sow farm "Polkenberg" (Sachsen, Germany) were used. There were altogether 447 F_2-sows with at least four litters, out of which seven with extremely high and extremely low litter size (second up to fourth litter), respectively, 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 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 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, 1 x *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.

PCR products were loaded on a 2% agarose gel (SeaKem, Rockland, ME, USA). A 100 bp ladder as 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 using the Big Dye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) with the same primers as described above. DNA amount 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 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 due to weak signals at the end of each sequence. Sequence comparison was performed with the program DNAStar (LaserGene, USA). Validated SNPs showed either all three expected genotypes or two genotype classes which were confirmed by cleavage with an appropriate restriction enzyme (Table 5.2.3.1).

PCR-RFLP for the CYP21 gene

At position 273 in the PCR product (position 3462 in the Genebank sequence), a restriction site (G>A) for *Hsp92II* enzyme was used for developing a PCR-RFLP test (Figure 5.2.3.1) to analyze all 129 F_1-sows. This restriction site was also chosen because the sire was heterozygous at this SNP. After 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 BSA, 5 U of the enzyme *Hsp92II* and 2 µl appropriate restriction buffer (Promega, Germany) for 4 h at 37°C. Bands were separated and visualized by gelelectrophoresis. Exemplary genotypes are shown in Figure 5.2.3.2. Genotypes could be distinguished due to 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) with small modifications (chapter 5.2.2 on page 68 and 69).

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 show higher variability than the following litters (Buske et al., 2006b). All statistical analyses were performed with the SPSS program (version 12.0).

Results

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

Table 5.2.3.1 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 bp 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.

Table 5.2.3.1: 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	*Hsp92II*
2	3514	C / T	7 CC; 11 CT	*HaeIII*
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]	*Pst1*
7	3911	T / C	1 TT; 1 TC; 16 CC[d]	

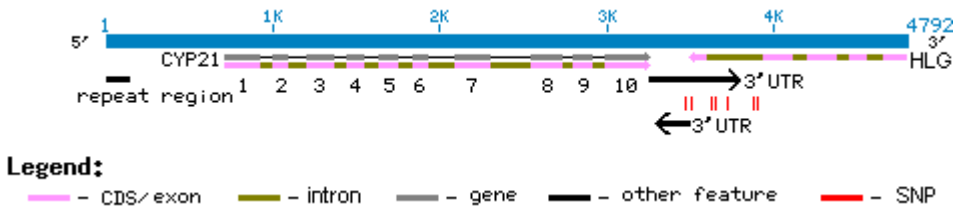
[a] The position is given according to the sequence for Accession number M83939

[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 neighboring gene

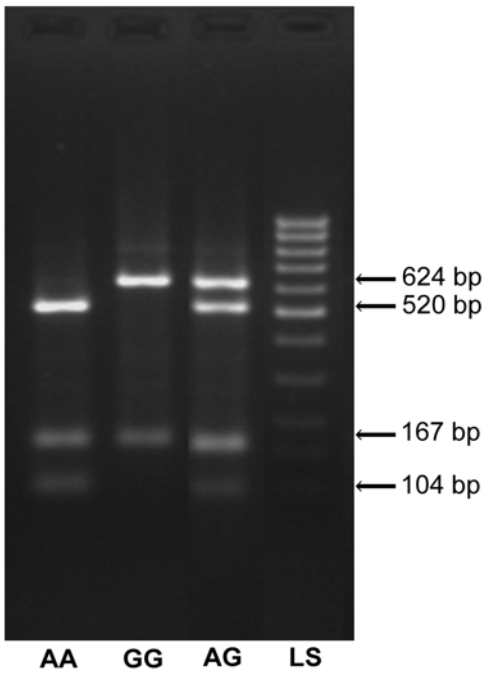
Figure 5.2.3.1:Gene structure of porcine CYP21 including five novel polymorphisms



Reference: NCBI database, modified; HLG = human-like gene; Last two SNPs belong to the neighboring gene

In Figure 5.2.3.2 we present the 3 different genotypes for the SNP at position 3462 for the CYP21 gene.

Figure 5.2.3.2: Different genotypes for the CYP21 (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

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

Table 5.2.3.2 shows the genotype and allele frequencies for the CYP21 and ESR2 genes in 129 F_1-commercial sows. Whereas 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.

Table 5.2.3.2: Genotype and allele frequencies for the CYP21 and ESR2 genes

Gene	N	Genotype frequency			Allele frequency	
CYP21*		AA	AG	GG	A	G
	129	0.062	0.504	0.434	0.31	0.69
ESR2		AA	AG	GG	A	G
	129	0.000	0.674	0.326	0.34	0.66

* Investigated SNP at position 3462 according to the sequence for Accession number M83939

Associations between genotypes and litter size parameters (TNB; NBA)

Table 5.2.3.3 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.3462 G>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 studied population, 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 5.2.3.3: 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*	LS Means TNB (≥ 2. litter) *	SE	LS Means NBA (≥ 2. litter) *	SE
CYP21**						
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 Means in the same column with different superscripts significantly differ at $p<0.05$. SE = Standard error; TNB = Total number born piglets; NBA = Number born alive piglets

* For each sow, second up to fourth or fifth (when available) litter was analyzed. First litter was ignored.

** Investigated SNP at position 3462 according to the sequence for Accession number M83939

Discussion

CYP21 was chosen as a 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 associations 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 physiological aspects of estrogenes. Muñoz et al. (2004) investigated this polymorphism in two Spanish pig breeds (46 Torbiscal and 150 Guadyerbas sows, respectively) 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) with 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 to 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 *PvuII* 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 to 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 x AB, AB x AB and AB x 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 on 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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6 General discussion and conclusions

Because the literature overview and most results of the investigated candidate genes for fecundity in swine have been already discussed in the previous chapters (chapter 3 and 5.2.1 to 5.2.3), here, I will focus the general discussion on methodical aspects, concerning phenotypical traits for fecundity, animal structure and chosen candidate genes. Furthermore, I will give suggestions for further research as well as for practical applications of the presented results.

Litter size as a trait for fecundity in swine

Fecundity in swine is one of the most difficult traits for geneticists. This is due to three reasons:

First, heritability for fecundity is extremely low (in comparison with meat quality and milk yield parameters, for example).

Second, fecundity is influenced by many genes, which have direct additive, dominant or overdominant effects, which likely have epistatic interaction, and which interact also with the environment. Therefore, it is difficult, to separate direct effects of the investigated gene from interaction effects.

Third, standardization of environmental parameters is difficult to perform over the whole experimental period. The experimental period is time-consuming due to the long generation interval in pigs (in comparison with investigations with mice for example).

Litter size, characterized by the subtraits comprising TNB and NBA piglets per sow and litter was chosen as phenotypical trait because of the convenience of its measurement and because of major interest of pig producers in this trait. Litter size is directly based on fecundity traits such as ovulation rate and uterine capacity, which are, however, much more difficult to measure. Other fecundity traits like gestation length or teat number have not the economic value as litter size, and therefore, were not considered in our investigations. Some research groups focused also on the number of weaned piglets per sow and litter as one of the most important economic traits for pig producers, however, in my opinion, this parameter depends on many nongenetical effects, leading to confusions between genetical and environmental effects. It is generally accepted, that the two main reasons for losses after birth are due to

crushing and diarrhea, which are frequently based on suboptimal housing management and suboptimal feeding regime, respectively. Moreover, litter adjustments *between* sows are not always recorded, so it cannot be distinguished between own and foreign piglets, when piglets died during rearing for example. Because these three factors are often insufficiently measurable and therefore consequently lead to incorrect results, measuring this fecundity parameter was renounced. For these reasons, we have decided to focus on TNB and NBA piglets as most useful phenotypical traits, which can be recommended for further investigations.

Critical consideration of family structure and selection of the sows

General aspects concerning the use of "commercial sow farms"

In order to investigate genes with association to fecundity in swine, sows of two different commercial sow farms were used. The fact that the genes with potential influence on litter size were tested in two different sow populations is in principle beneficial due to better establishment of possible effects of such loci with influence on fecundity (Rothschild et al., 1996). However, the question arises, if commercial sow populations or even commercial sow farms – which is indeed not the same - are the best way for testing genes with potential influence on fecundity parameters. One requirement for testing those genes is a standardization of environmental factors such as housing and feeding regime and management system over the whole experimental period. In my opinion, this is better feasible under laboratory circumstances in comparison to commercial sow farms. Commercial sow farms partially suffer under extreme cost pressure, leading often to spontaneous changes in feeding regime and breeding strategies during the experimental period. Additionally, sows with low litter sizes in beginning matings are often excluded from further matings, even if they are healthy. For both, QTL-analyses and candidate gene approaches, however, it is of great importance to compare genotypes and phenotypes of sows with high *and* low fecundity, and if sows with low fecundity are not existing in the population, a possible genetical reason for low fecundity cannot be found. Just for traits with low heritability, it is important to characterize the phenotype as accurate as possible. Therefore, it is not correct to predict low litter sizes for further "non-happening" litters, when such sows were excluded from the farm due to low litter sizes in first or second matings. Another aspect is, that a planned mating test, which is desirable for candidate gene approaches and obligatory for QTL-analyses, is almost impossible when working with commercial sow farms. Regarding QTL-analyses, fecundity

parameters were investigated in offspring of phenotypically extremely diverse founder populations, which were in many cases preselected for extreme phenotypes over generations for desirable traits (Rathje et al., 1997; Cassady et al., 2001). In the case of candidate gene approaches, also commercial sow populations were investigated. Either, this was performed under controlled housing conditions in experimental farms (Rothschild et al., 1996; Short et al.,1997) or, in a few cases, also commercial sow farms were used (Depuydt et al. 1999). Altogether, concerning candidate gene approaches, working with commercial sow farms can only be recommended, if at least strong standardization of environmental parameters can be ensured and exclusion of inefficient sows can be inhibited. Finally, an improvement of candidate gene approaches would be a planned mating test in to obtain balanced genotype frequencies as described by Rothschild et al. (1996). At first, they genotyped the parental generation for the ESR1 gene variants. Afterwards, planned matings of the schema AA x AB, AB x AB, AB x BB were performed. In such a case, in my opinion, working with sows, which are housed in an experimental station, is necessary. Researchers who would like to investigate associations between genotypes and litter weigths as phenotypical trait should work with experimental stations because in commercial sow farms, this parameter is in most cases not recorded.

Commercial sow farm "Polkenberg"

Concerning the sow farm „Polkenberg", a two-tail analysis was performed comprising 61 sows for the high and 62 sows for the low performance group including the second to fourth litter for each sow. The high performance group consisted of sows with at least 14.3 piglets per litter averaged from the second to the fourth litter; whereas the low performance group consisted of sows with less than 11.3 piglets per litter averaged from the second to the fourth litter. In order to select the sows, the phenotypical criteria for fecundity "TNB" was preferred versus the trait "NBA". This was due to additional nongenetical effects for the latter trait such as unexpected stress during farrowing, for example, which can lead in some cases to stillborn piglets. Because for pig producers, the criteria of NBA piglets is more important than the number of TNB piglets, in future, both parameters should be included for genetical selection experiments. One proposal for future experiments is, to furnish each sow with an index, which is based on a desired ratio between TNB and NBA. Afterwards, selection of sows can be easily performed by means of increasing or decreasing indices. It depends on the researcher, which emphasis should be performed. In my opinion, the criteria "weaned piglets per sow and

litter" as a genetical determined fecundity trait needs extremely high standardization of environmental parameters as described above, and therefore, is more difficult to perform.

The basic idea of the strategy to select two extreme performance groups from the complete sow population was -provided that all environmental parameters were kept constant over the whole trial period- that the gene variants of candidate genes with association for litter size should be significantly different between these two extreme groups. Advantages of this strategy are the relatively simple experimental setup and the low number of tested animals. To our knowledge, this approach has not been performed to test candidate genes for fecundity parameters in swine, so far. However, our results showed that this –at first view– simple and promising experimental setup has also some disadvantages due to the following reasons: First of all, regarding the selection of sows by only three litters per sow, it cannot be excluded, that sows with high litter size at the beginning matings show low litter sizes in following litters (or the other way around). It follows that reliable sorting of animals according to their previous existing litter performance cannot be warranted. Finally, associations between genotype and phenotype cannot be indicated reliably, too. For the selection of the sows into one of the two extreme performance groups, it was our intention, to include six litters from each sow, but this time-consuming step was not feasible under our possibilities. An investigation of additional sows, followed by exclusion of sows with extremely inconsistent litter sizes was also not possible under our experimental conditions because the owner of the sow farm changed his livestock completely due to unsatisfying operating results (which is somewhat irreproducible; for details see chapter 5.2.1). Therefore, our study lacked, secondly, on low animal numbers from which we selected sows with extreme litter performance. But, even if animal numbers were twice as high as initially planned for both performance groups, in principle, only a Chi-square-test would be applicable in order to determine significant differences of genotype frequencies between the two extreme performance groups. For example, we investigated the association of the properdin gene variants and litter size (Buske et al., 2005) and found no association between genotypes and phenotypes *within* each of the two performance groups. This is probably due to the fact, that within a performance group, litter size for three matings was relatively constant, and small differences between sows in the same performance group cannot be calculated by means of analysis of variance (or using any other statistical method) when performance groups consisted of only 61 or 62 sows and coevally, genotype frequencies are extremely unbalanced. Additionally, heritability for fecundity is low, and reproductive traits are inherited by many genes, which may have their own effects, but which also can

interact. Thus, under our experimental conditions, animal numbers were too small to find real effects between genotypes and litter size parameters. Therefore, the question arises, whether a candidate gene approach leads to more reliable results if a complete population is investigated without selecting sows by extreme litter size. An additional disadvantage is, that even if there are significant differences between genotype frequencies in the two performance groups, as it was the case for the FUT1 gene, how can be excluded, that no other, closely linked gene to the FUT1 gene is responsible for fecundity? The question, why a special sow occurs in the low or in the high performance group, cannot be completely answered. In fact, we think that a sow belongs to a special performance group because this sow and others have for one gene the same genotype, which is associated significantly with increased or decreased litter size. But can we really conclude, that this investigated gene influences litter size, if we only investigate one, ten, or even 100 genes, even if it was carefully thought about the choice of selected candidate genes? It could be for example, that some sows belong only in the low or high performance group because they have a special genotype for an uninvestigated gene, but underlying the effect. All things considered, in my opinion, a two-tail analysis as an association study can be recommended most notably, if the phenotype is completely recorded. Otherwise, a two-tail analysis is rather applicable as a screening than an association study. The problem of selecting suitable sows as well as the right number of sows for genotyping (for example 10, 15 or 25%) in each group of the basic population remains difficult. It depends on effect sizes and genotype frequencies of the candidate genes, standardization of environmental parameters, and on the size of the complete basic population.

With the most extreme sows in litter performance (seven sows of each performance group), seven new polymorphisms in the 3'UTR of the CYP21 gene were detected. An advantage of selecting phenotypically extreme animals of a population is, to increase the chance to detect new polymorphisms by comparative gene sequencing, in comparison to genotype the same number of animals by non regarding phenotypical aspects. After the detection of new SNPs, a PCR-RFLP test for the *Hsp92II* restriction site has been developed to test this polymorphism successfully in the commercial sow farm "Schulzendorf".

Commercial sow farm "Schulzendorf"

The F_1-sows of the commercial sow farm "Schulzendorf" that we have analysed descended from only one Duroc sire and 40 German Landrace dams which had a high degree of relationship. Such a family structure can only be recommended for candidate gene approaches

if the dams are heterozygous or show at least two different genotypes at the chosen candidate genes and *coevally,* if the sire is heterozygous at these genes. Whereas the first requirement is rather probable due to the relatively high number of 40 dams, it is difficult to fulfill the latter condition. This is because of using relatively different inbreed lines for the parental generation in order to exploit heterosis effects in the F_1-generation. So it can be expected that when only one sire serves as the parental generation, this sire is homozygous at many loci. Our study suffered in that way, that for the genes BF, FUT1, ESR2 and GPX5 the sire was homozygous, so it was impossible, to obtain all three possible genotypes in the F_1-sows. Furthermore, for the BF and FUT1 genes, the two genotypes which were found in the F_1-generation were extremely unbalanced distributed, leading to allele frequencies of 0.97 (A) : 0.03 (B) and 0.04 (A) : 0.96 (B), respectively. If the two remaining genotypes do not lead to differences in phenotypical performance, a final conclusion about an association between genotypes and litter size parameters is not feasible. It could be for example, that another gene variant of this gene would lead to phenotypical changes. In this context, it is worth to mention that when genotypes for a potential candidate gene influencing quantitative traits are the same in a phenotypical different population, this gene does not contribute to phenotypical differences in *this* population. But one cannot exclude that this gene has no influence on the phenotypical trait at all or in another population. Therefore, for the candidate gene CYP21, we sequenced the 3'UTR to find SNPs particularly in the founder sire of the F_1-sows. The sire was heterozygous for five polymorphisms, of which two showed a restriction site for developing a PCR-RFLP test. If the founder sire is heterozygous for such polymorphisms, one can expect, that genotype frequencies of the F_1-sows are more balanced. Coevally, genetical background remains the same because of using only one sire as founder. Contrary to the BF, GPX5, ESR2 and FUT1 genes, all the three genotypes have been detected for the CYP21 gene. Genotype frequencies of the F_1-sows were 43.41% (GG), 50.39% (AG), and 6.20% (AA) in comparison to genotype frequencies of their dams with 60.98% (GG), 36.59% (AG), and 2.44% (AA), which led only to little displacement in favour of the "A" allele.

Critical consideration concerning the assortment of investigated candidate genes

For this study, the genes BF, FUT1, ESR2, GPX5 and CYP21 were chosen as candidate genes with potential influence on litter size in swine. All these genes were derived from physiological, comparative and positional aspects as described before. Concerning the concrete assortment of the selected candidate genes, also information about genotype and allele frequencies determined in other populations and methodical aspects were considered.

However, these reasons were of little importance. Even if candidate genes are selected from physiological, comparative and positional aspects, in my opinion, there is no possibility to provide a "ranking list" for genes, which might influence fecundity parameters in swine, resulting from the complexity of this trait. However, it can be stated that the more reasons for a candidate gene are provided, the higher is the probability to detect genes with real effects on fecundity. In particular, confirmed or even narrowed QTL regions, genes with known effects in other (mammalian) species and precise information about gene products are valuable. Until now, associations between genotypes and several fecundity parameters in swine were found for at least 11 candidate genes. Concerning QTL analyses, 54 QTL regions, which overlap in part, have been mapped in swine with association to several fecundity parameters, so far (Buske et al., 2006). Provided that each QTL harbors at least one gene with influence on fecundity, one can estimate that there are many genes underlying for fecundity. The main subject for researchers, however, is to find genes with rather "large effects", which can be introduced into MAS.

Further candidate genes and chromosomal regions with potential influence on litter size

In Table 6.1, additional potential candidate genes for female fecundity in swine are presented, for which no association study has been published, so far. Beside the deduction of candidate genes from comparative, positional and physiological aspects, also results from expression studies were included. The presented candidate genes refer to authors who proposed these genes due to at least one of the four criteria. Because this is only an assortment of candidate genes, one can expect that many genes contribute to this complex trait, even if not all of these genes will show phenotypical differences for fecundity in swine if one examines a special population.

Table 6.1: Additional candidate genes with potential influence on female reproduction in swine

Gene	SSC	Physiological aspects	Comparative aspects	Positional aspects	mRNA expression[1]	Reference
PSG1	6	+	0	++	0	Yasue et al. 1999
LHB	6	++	+	+	(+)	Nilson et al. 2000
Lhx3	1	+	+	+	(+)	Smith et al. 2001
SF1	1	(+)	(+)	+	0	s.a.
STE	8	+	0	+	++	Kim et al. 2002
POU1F1	13	(+)	+	+	+	Sun et al. 2002
PIP5K2A	10	0	0	+	0	Nonneman and Rohrer 2003
ITIH2	10	(+)	0	+	+	s.a.
AKR1C2	10	(+)	0	+	0	s.a.
GAD2	10	0	0	+	0	s.a.
AREG	8	++	0	+	+	Kim et al. 2003
MADH1	8	0	+	++	0	Kim et al. 2003
BMPR-IB	8	+	+	+	+	Kim et al. 2003
MAN2B2	8	(+)	0	++	0	Campbell et al. 2003
RGS12	8	(+)	0	(+)	+	s.a.
PRL	7	++	+	++	(+)	Spencer et al. 2005

Annotations: ++ = strong effect; + = moderate effect; (+) = possible or weak effect; 0 = no effect observed so far; 1 = mRNA expression only in porcine tissues (for example uterine epithelium) with regard to fecundity parameters

AKR1C2 (3-hydroxysteroid dehydrogenase, type III), AREG (amphiregulin), BMPR-IB (bone morphogenetic protein receptor IB), GAD2 (Glutamate decarboxylase 2), ITIH2 (Inter-α-globulin inhibitor heavy chain), LHB1 (Luteinizing hormone beta 1), LHX3 (LIM homeodomain transcription factor), MADH1 (= Smad1; Mothers against decapentaplegic homolog 1), MAN2B2 (Alpha mannosidase), PIP5K2A (Phosphatidylinositol-4-phosphate-5-kinase II alpha), POU1F1 (= Pit-1; pituitary-1 transcription factor), PRL (Prolactin), PSG1 (Pregnancy-specific beta-1-glycoprotein), RGS12 (Regulator G signaling protein 12), SF1 (Steroidogenic factor 1), STE (estrogen sulfotransferase)

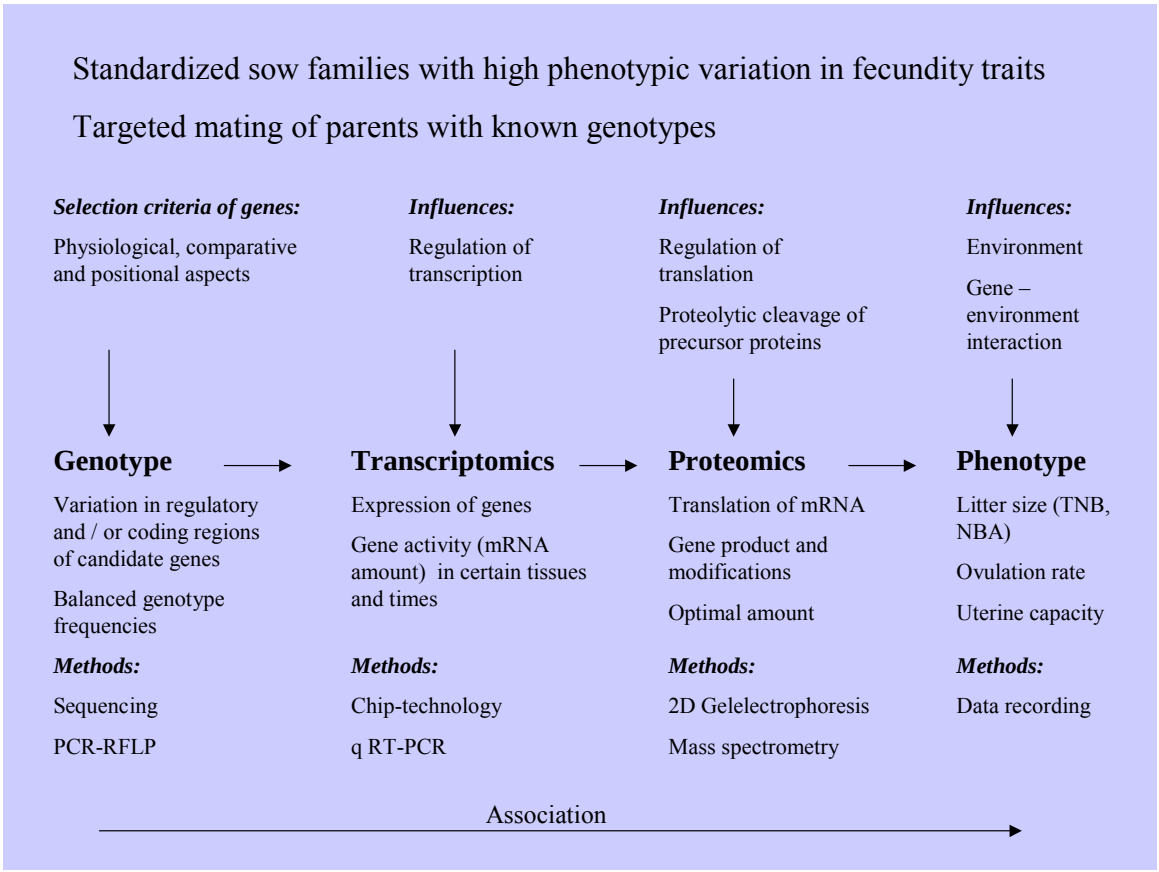
Promising chromosomal regions harboring candidate genes with influence on reproductive traits in swine are besides nearly the complete SSC8 also the centromeric region on SSC7 comprising the MHC.

Strategies for further research concerning genes with influence on fecundity in swine

An improvement of fecundity parameters in farm animals by phenotypical performance tests and classical quantitative genetical methods, in particular the involvement of the BLUP (best linear unbiased prediction) procedure is time-consuming, expensive and inefficient for traits of low heritability. Therefore, the introduction of molecular methods based on candidate gene approaches and QTL analyses for fecundity traits is desirable. However, finding of major genes and implementation into MAS remains difficult because of low heritability, polygenic structure of reproductive traits and deficient phenotype definition (Wolf, 2004). As a new strategy, "systematical gene activity analysis" includes investigations at the level of mRNA (Transcriptomics) and proteins (Proteomics). These methods are in early beginning in farm animals. In Figure 6.1, such a strategy is presented for the improvement of litter size in swine. Association studies lack in that case, that even if an association has been found between a genotpye of a candidate gene and the recorded phenotype, there is no evidence of the gene underlying the effect. It could be, that strong linkage occurs between the investigated gene and a neighboring gene with the causative effect. Furthermore, even if environmental parameters are strongly standardized, phenotypical variation could be also due to posttranslatorical modification of proteins, such as cleavage, or biochemical modifications for which additional genes are responsible, and not the primary candidate gene itself. Another strategy for detecting fecundity genes could be the use of monozygotic twins. Altough monozygotic twins are genetically equal, it is possible to identify genes underlying fecundity parameters when all environmental parameters are strongly standardized, except one, like for example the feeding regime. When then phenotypical parameters for fecundity differ (e.g. ovulation rate or uterine capacity) and the expression profiles of one or several genes in the selected tissues change, one could expect, that this/ these gene(s) has/ have an influence on the phenotypical trait. In a next step, association between different genotypes in such genes and phenotypes can be investigated under strongly standardized conditions in another population. A strategy to elucidate the function of candidate genes is to generate transgenic animals. Functional tests are usually performed in model animals, especially in mice. The improvement of livestock by genomic modification to improve fecundity is rather unlikely from the point of view of our nowadays knowledge. We still do not know key genes for the improvement of fecundity in pigs. Furthermore, transgenesis rates achieved by microinjection of DNA into higher mammals are extremely low. New, efficient methods in gene transfer, based on the use of lentiviral vectors improve the efficiency and offer the possibility to use

this method for key genes directly in farm animals (Hofmann et al., 2003).

Figure 6.1: Strategies for further investigations of candidate genes for fecundity



Practical application of (the investigated) genes in order to improve litter size in swine

The improvement of litter size in swine due to MAS of a single gene (variant) can lead to important economical gains for pig producers. This fact was demonstrated for the first time with the calculations performed by Short et al. (1997) for the ESR1 gene. The authors calculated that assuming an initial gene frequency of 0.25 in parent females, an additive effect of 0.4 piglets/ litter per copy of the B allele, 2.3 litters x sow^{-1} x $year^{-1}$, and a marginal economic value of $30 per additional piglet, then, for a 1000 sow farm the potential value of fixing the ESR1 B allele amounts to an additional gain of $20.700 per year (0.75 x 0.4 x 2.3 x 30 x 1000), or over $20 per sow^{-1} x $year^{-1}$. Furthermore, they concluded, that this value well exceeds testing costs and offers an improvement over traditional selection methods. From this example, a general formula can be developed. The economic value (Y) for fixing a beneficial allele for litter size in a sow farm can be calculated by the equation:

Y = *a b c d e*

whereas

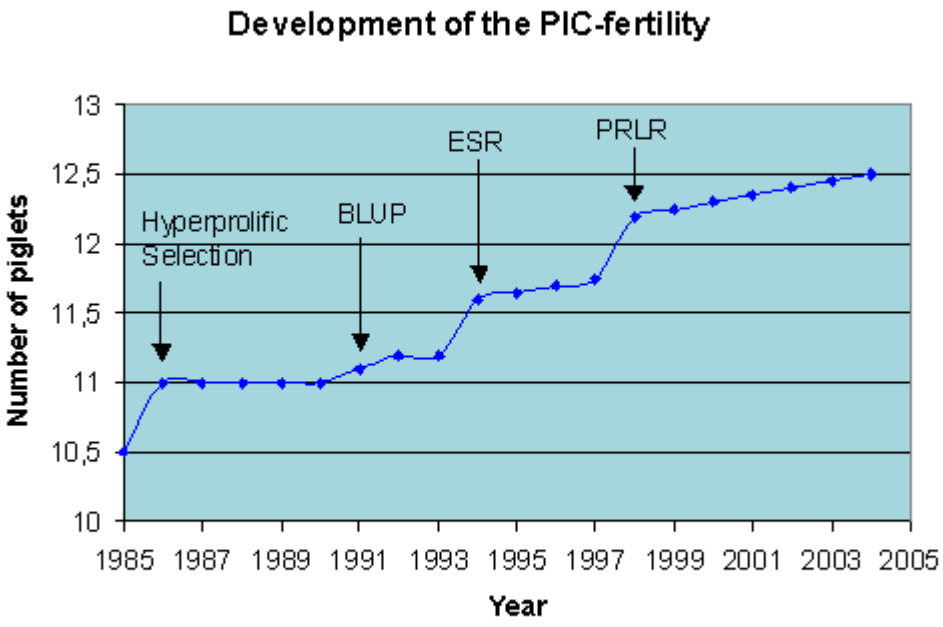
a = 1 – frequency of the favorit allele in the commercial sow population (current state),
b = additive effect,
c = number of litters per sow and year,
d = actual value of a piglet,
e = number of (hybrid) sows, which produce fattening piglets.

Easily can be demonstrated, that the higher all parameters are, the higher is the economic value "Y". It is worth to note that "*a*" is high only, if a beneficial allele (or genotype) is rare in the founder population. Thus, breeding progress is highly achieved, when a beneficial polymorphism is detected in a candidate gene for a desirable trait (here litter size), and the beneficial genotype is rare in the commercial population, which shall be improved by increasing this genotype. In this context, it is important to note that it is inefficient to genotype permanently sows, which are used to produce the fattening piglets. Under commercial and economical aspects, the benefit results from genotyping their parents (or even grand-parents, depending on the breeding system). Using the genotype information of parental strains, the best mating strategy can be deduced to generate a sow population, in which the desirable genotype is fixed. Noncarriers of the favorite allele are excluded from further matings.

Since a few years, the "PIC Group" has permanently introduced some gene tests into MAS. Examples are the ESR1 and PRLR genes for improving litter size in swine (Figure 6.2), which were both examined at the Iowa State University under the reseach group of M. Rothschild (Rothschild et al., 1996; Vincent et al. 1998). Great advertisement has been made by the "PIC Group": "There is no alternative to the PICmarqTM technology concerning fecundity. Surely, it is possible to use fertile breeds by nature, however, the identification of very fertile individuals is, with traditional methods, nearly impossible. Since many decades, litter size is recorded in order to improve fecundity, but with only poor success. Also including the BLUP procedure, leading by all means to higher accuracy, is only still an "estimation" (PIC: Neue Technologien, 2004)." Before introducing a gene into MAS, it is important to test gene effects in different populations and to evaluate pleiotropic effects. For the PRLR gene, an additional beneficial effect has been observed in that way, that not only litter size increased for the

desired genotype, also weaning weigths of the piglets increased, too. Therefore, such a marker is very useful for MAS. Recently, the FUT1 gene has been introduced into MAS in order to improve animal health due to resistance to diarrhea. According to the "PIC Group", no pleiotropic effects have been observed so far, however, according to Horák et al (2005) and to own investigations, the homozygous genotype for resistance leads to fewer offspring than the heterozygous genotype in nonresistant sows. Anyway, all facts considered, it could be useful, to introduce the ESR1 and RRLR genes as well as the FUT1 gene into MAS. Besides the sows, which are resistant to diarrhea, also their fattening piglets profit from animal health depending on the genotype of the FUT1 gene of the sire. A possible pleiotropic effect on decrease of litter size could be compensated by introducing *at the same time* the beneficial gene variants of the ESR1 and PRLR genes into MAS. Finally, the sow farm manager or the owner must decide, if and which gene(s) should be considered for selecting pigs of the parental-generation, regarding also housing, feeding and management aspects.

Figure 6.2: Litter size development of sows of the PIC Group



Reference: PIC: Neue Technologien, 2004, modified

Other genes, which are introduced into MAS by the "PIC Group", are the MC4R (melanocortin 4 receptor) gene for a better feed conversion ratio, decreased fat depth and a more favorable lean to fat ratio. However, coevally, the reduced average daily gain leads to a longer fattening period, so pig producers must decide, which strategy is the best for their sow farm. Besides the selection on desirable RYR1 (ryanodine receptor gene 1) genotypes to eliminate stress susceptibility in fattening pigs, the PRKAG3 (protein kinase gamma 3) gene is also used for improving meat quality due to enhancement on pH_{24} value, higher water binding capacity and meat colour.

Concerning the own investigations, a farm-specific recommendation of introducing one of the five tested genes can be given for the commercial sow farm "Schulzendorf". There is promising potential for the ESR2 gene for increased litter size, although no association was observed in the sow farm "Polkenberg" for this gene. Also the BF gene, or a closely linked gene of the MHC to improve litter size in pigs should be used for additional investigations. For the FUT1 gene, further investiagations are useful in other populations to assess genotypes with litter size more reliable and to test pleiotropic effects between animal health due to resistance to diarrhea and litter size in swine. GPX5 and CYP21 were not associated with litter size, however, it must be stated that GPX5, and especially BF and FUT1 genotypes were distributed extremely unbalanced in the sow farm "Schulzendorf". Associations between CYP21 gene variants and litter size parameters in the sow farm "Polkenberg" were not measured.

Conclusions

1. The improvement of fecundity in swine is one of the most difficult tasks for geneticists, but is of great economic interest.

2. So far, no causative mutation has been found for direct influence on litter size in swine.

3. Altogether, 54 QTL (which overlap in part) and 11 candidate genes (which are mostly located within QTL) have been found for associations with fecundity parameters in swine until now. Therefore, one can conclude that reproductive traits are inherited polygenetically.

4. Many of these QTL are located on SSC8 and at the centromeric region on SSC7 and therefore, these chromosomal regions are promising regions with influence on fecundity.

5. Regarding candidate gene analyses, genotype frequencies were often distributed extremely unbalanced both in our, and in many other published studies. Therefore, rare genotypes could not be evaluated with sufficient statistical power with regard to reproductive traits in swine.

6. Concerning practical applications, at least two genes have been introduced successfully into MAS in order to increase litter size in swine, however, the question arises, which additional markers or genes, derived from either QTL or candidate gene approaches should be considered for introduction into MAS (many QTL for one and the same phenotypical trait have been found, marker distance is often relatively wide, pleiotropic effects have been observed).

7. Sequencing of phenotypically extremely different sows was suitable to find new polymorphisms in the CYP21 gene and hence can be recommended to find polymorphisms also in other genes.

8. The ESR2 gene was associated with increased litter size (NBA) in the commercial sow farm “Schulzendorf” and therefore could serve as a potential candidate gene for litter size.

9. Selected sows with high litter size and low litter size after first matings showed both extremely low litter size at first mating. Therefore, phenotypical differences cannot be seen at an early stage of live in some fecundity traits. This is a hint that some genes with influence on fecundity are activated in a later period of live, probably after first pregnancy.

Implications for further investigations and practical application

1. The phenotypical trait “litter size” comprising the nmber of TNB and NBA piglets is suitable, however, at least five litters should be recorded for each sow in order to record the phenotype reliable. Concerning association studies by using two-tail analyses, this is notably necessarily to avoid selection mistakes.

2. Standardization of the experimental setup is strongly required for both candidate gene approaches and QTL-analyses as well as their description as accurately as possible in order to be able to compare effect sizes more precisely. A planned mating test for candidate gene approaches would be a progress in order to obtain more balanced genotype frequencies.

3. Concerning QTL analyses, fine mapping is desirable in the future. A combination of fine mapping and candidate gene approaches is a straight forward strategy.

4. Beside SSC8, the MHC on SSC7 is a chromosomal region with high potential for harboring candidate genes concerning fecundity in swine and is suited for further investigations including “fine mapping” strategies.

5. Beside the ESR2 gene, also the genes BF and FUT1 should be tested in additional populations to investigate the relationship between genotype and phenotype more reliable.

6. Implementation of major key genes for fecundity into MAS is economically expedient and desirable to accelerate the breeding value, however, pleiotropic effects should be respected. Emphasis should be given on traits which are economically important and easy to measure.

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