Molecular Characterisation of QTLs controlling Fatness in Pigs

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Abstract. This thesis deals with the identification of genes controlling intramuscular fat and backfat thickness. Markers linked to quantitative trait loci (QTL) for these traits in the cross between Meishan x Large White breeds will lead to the identification of the underlying genetic causes. A whole-genome scan revealed significant evidence for five QTLs affecting body composition, of which four were imprinted. Additional markers were typed in this cross to further investigate the regions on pig chromosome (SSC) 2, SSC4 and SSC7. Imprinting analysis revealed a genome wise significant paternally expressed QTL on SSC2. A radiation hybrid (RH) map was constructed for SSC2. Comparison of the porcine RH map with homologous human chromosomes identified four conserved segments between SSC2 and HSA11, HSA19, and HSA5. To improve the existing comparative map for SSC2 and increase the gene density on this chromosome, a porcine BAC library was screened. Sequences from the BACs were compared with sequences in the nucleotide databases to identify homology with other mammalian sequences. For the investigation of the borders of the conserved segments between SSC2 and HSA11 genes located on HSA11 were mapped to SSC2. This resulted in refinement of the borders of the conserved segments and in the detection of a new rearrangement in the comparative map between HSA11 with the porcine genome. Through the use of radiation hybrid maps that include both Type I and II markers, homologous chromosomal locations for QTL of specific traits can be identified in other species. The conservation of genome organisation between pig, man and mouse makes it possible to take advantage of genetically well-characterised species for the selection of candidate genes for the imprinted QTL for backfat thickness on SSC2. Human obesity research can help to determine the direction and give clues for further porcine fat trait related genetic research, but emphasis should be on better understanding of the fat traits in pigs themselves and improvement of the genetic map of the pig.

Stellingen

1. Als een kenmerk eenmaal is gelokaliseerd in een chromosomale regio door QTL mapping, is het identificeren van de onderliggende genen een significant probleem. *Dit proefschrift*

2. Zelfs al levert het humane genetische vetzucht-onderzoek aanwijzingen voor rugspek-dikte en intramusculair vet in het varken, meer nadruk zal moeten liggen op het verbeteren van de kennis van het varkens-genoom en fysiologie van het varken voor volledig begrip over het varkens vet-metabolisme. *Dit proefschrift*

3. Niet alleen genetische veranderingen, maar juist ook epigenetische activiteiten zoals inprenting zorgen voor de mogelijkheid om te reageren op verschillen in de omgeving. *Vrij naar Pennisi, 2001, Dit proefschrift*

4. De opmerkelijke overeenkomsten tussen de mens en de muis voor monogenetisch bepaald vetzucht geven een ontdekkingsbias aan omdat de zoektocht naar de meeste humane mutaties zijn geleid door eerder beschreven muis-mutaties. *Barsh et al., 2000*

5. Iemand heeft pas gelijk als zijn gelijk past bij het gelijk van de mensen in wier gezelschap hij zich bevindt. *Vrij naar J.J. Voskuil, Het Bureau deel 5*

6. Het invoeren van stemplicht geeft naast bijna een verdubbeling van het aantal uitgebrachte stemmen een betere afspiegeling van de samenleving in de politiek.

7. "All animals are equal, but some are more equal than others", gaat zeker op bij het afgeven van verblijfsvergunningen in Nederland.

8. Veel mensen laten zich in hun airconditioned werkruimte onnodig beïnvloeden door het weer buiten.

Stellingen behorende bij het proefschrift van Annemieke Rattink
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Contents

Chapter 1

General Introduction

In recent years, genome research in livestock animals such as cattle, pig and chicken has increased enormously. The discovery of highly polymorphic microsatellite markers in the livestock genomes and the completion of the sequencing of the human genome have a high impact on animal genome research. This has led to the construction of genetic linkage maps in farm animals and subsequently in a large number of linkage studies, with the aim to localise genes involved in all sorts of production traits of these species. Markers linked to these quantitative trait loci will lead to the identification of the underlying genetic causes. Identification of these markers, and more preferably the genes themselves, will allow breeders to select more accurately on important performance traits. In this thesis, the identification of genes controlling fatness in the pig and in particular genes controlling intramuscular fat and backfat is described.

Backfat thickness and intramuscular fat

White adipose tissue is the major energy reserve in higher eukaryotes. Storing energy-dense triglyceride in periods of energy excess and its mobilisation during energy deprivation are its primary purposes (Gregoire et al., 1998, Friedman and Halaas, 1998). Fat is deposited in four different depots in the body; subcutaneous (backfat), visceral, intermuscular and intramuscular fat (Kouba et al., 1999). The two fat depots of greatest interest to pig breeders are backfat, accounting for 60 to 70% of total body fat and intramuscular fat.

At birth, lipid storage of the piglet is low (1 to 2%), but a ten times increase is reached at 12 days of age. The cellular development of pig adipose tissue has three successive phases: a dominant hyperplasy between 7 and 20 kg, a hyperplasy and hypertrophy between 20 and 70 kg and a predominantly hypertrophy beyond 70 kg of live weight (Mourot and Hermier, 2001). Backfat is the earliest maturing fat depot, while intramuscular fat is the latest maturing (Kolstad, 2001). It is widely accepted that differences in the amounts of fat and fat distribution exist between and within pig breeds (Kolstad, 2001). In addition, metabolic differences between genetically obese and control pigs are already apparent in the pre-obese state prior to birth when foetuses have only 1-2% body fat.

Selection against backfat thickness to obtain leanness has been very important in pig breeding schemes. Reduced carcass fatness brings benefits in both improved carcass quality and efficiency of production. Reducing overall fatness, however, also influences the other fat depots. The level of intramuscular fat will reduce, which may reduce quality traits such as tenderness, juiciness and taste of the meat (Hovenier, 1993). The optimum range of

intramuscular fat content for pig meat has been suggested to be 2-3% (DeVol et al., 1988). However, the correlation between backfat thickness and intramuscular fat is not undisputed. Kouba and co-workers (1999) state that the development of intramuscular fat and backfat thickness are partially independent and that the development of intramuscular fat may be determined at an early stage, before 20 kg. In their study, intramuscular fat was more developed in genetically lean pigs than in genetically fat pigs. Others, however, claim that the correlation between intramuscular fat content and backfat thickness and carcass growth is negligible (Casteels et al., 1995). This would indicate that different genes are involved in intramuscular fat and backfat development. The identification of these genes and implementation in breeding schemes could lead to increasing intramuscular fat levels in pigs, without negative consequences for backfat thickness and carcass growth.

Quantitative Trait Loci Mapping

Fatness in pigs and other traits like milk production in cows and disease resistance are quantitative traits. Quantitative traits show a continuous distribution of phenotypic values rather than the discrete values observed for a qualitative trait. Quantitative traits are usually controlled by multiple genes and in addition are influenced by environmental factors. Two approaches can be followed for the identification of genes involved in fatness traits in the pig: the candidate gene approach and the genome scan approach.

Based on the physiology and biology of the trait, candidate genes that are expected to influence the trait can be investigated for association with the trait. This approach can be very powerful to detect even loci with small effects. For example, the melancortin-4 receptor *(MC4R)* gene has a role in regulation of body weight in humans and mice. In pigs, a significant association of porcine *MC4R* genotypes with backfat has been reported (Kim et al., 2000). However, for fatness in pigs many candidate genes have already been identified that can be investigated. Many more are present in the genome, but have not yet been investigated because of the present limited knowledge of gene functions. Therefore, it is more convenient to evaluate all these in one genome scan.

In a total genome scan the segregation of a large number of genetic markers across the genome with the phenotypic trait of interest can be traced (Geldermann et al., 1996). A quantitative trait locus (QTL) is defined as a chromosomal region harbouring genes controlling variation in quantitative traits (Andersson, 2001). For QTL mapping no prior knowledge about the biology of the trait is needed. Several genome scans identifying QTLs involved in fatness and growth traits have been reported in pigs (reviewed by Rothschild, 1998). However, once a

trait has been located in a large chromosomal region through QTL mapping, identifying the underlying gene remains a significant problem.

Comparative mapping

The conservation of genome organisation between species makes it possible to take advantage of genetically well-characterised species as man and mouse for the improvement of the less characterised genomes of livestock species. Evolutionary conserved genes often have similar structure and function and important regulatory elements may be conserved even between distantly related organisms whose genomes may have little or no similarity overall (Onyango et al., 2000). However, during evolution chromosomal rearrangements invariably occur, disrupting some of the ancestral linkages. Closely related species usually accumulated fewer rearrangements and therefore have long conserved segments (Nadeau and Sankoff, 1998).

Most existing genetic linkage maps of domestic animals are ill-characterised. They exist mainly of random DNA markers (Type II markers). Type II markers have been used extensively to saturate the genetic maps of various species because of their technical advantages and high degree of polymorphism. However, Type II markers are seldom informative across mammalian orders and therefore are not suitable for the comparison of genetic maps from different species. Conversely, molecular markers for functional genes (Type I markers) conserved across species can be used for this purpose (Caetano et al., 1999).

Comparative mapping between the pig and human genome revealed extensive conservation of synteny between the genomes through heterologous chromsomal painting (Rettenberger et al., 1995). Rearrangements in gene orders within syntenic genomic regions were detected between man and pig in comparative mapping studies based on genes mapped on the linkage maps of both species (Johansson et al., 1995). Comparative mapping has advanced by the introduction of somatic cell hybrid (SCH) and radiation hybrid (RH) panels. These panels consist of a set of cell lines that contain a random set of fragments of irradiated porcine genomic DNA in a hamster background. QTLs in pigs are primarily located on linkage maps which contain mainly Type II markers. On the SCH and RH maps Type I and Type II markers are integrated, providing the identification of homologous chromosomal locations for the QTLs in other species.

An introduction to imprinting

Besides the changes in the DNA code itself, a wide range of phenomena influence gene expression at different levels. These so called epigenetic activities include gene regulating activities that do not involve changes to the genetic code and that can be passed on to one or more generations (Pennisi, 2001). In this way, the DNA code itself does not have to be altered for cells to be able to respond to environmental changes. Just as mutations alter DNA, epimutations alter DNA methylation or chromatin patterns (Reik and Walter, 2001). Imprinting is one of the phenomena that can influence gene expression at the level of post-DNA-transcription. Imprinting is defined as genes or whole chromosomes that can be silenced, activated, or even deleted depending on their parental origin (Ohlsson, 1999). Some genes can be kept in active (transcribed) state and others can be held inactive (silenced) in some cell types and during different stages in life. These parent-specific effects can for instance be seen in mule breeding. A mare crossed with a donkey yields a mule, whereas a stallion crossed with a donkey produces a hinny.

In man, approximately 40 genes are known to have imprinting effects. A sizeable number of these genes are involved in fetal growth, where paternally expressed genes enhance fetal growth and maternally expressed genes suppress fetal growth. Clustering of imprinted genes is seen in several regions, for example on human chromosome (HSA) 11p15.5. The expression patterns of the genes in these clusters can differ. Some genes are paternally imprinted, meaning that the maternal allele will be expressed and other genes in that cluster are maternally imprinted, resulting in expression of the paternal allele. The organisation of all clusters is similar: reciprocally expressed genes are located next to each other, genes coding for RNA that is not translated into a protein are present and CpG islands, which function as strong promotors and in addition might serve as replication origins, separate the genes within a cluster (Jones and Takai, 2001; Pennisi, 2001).

For all genes and all clusters, methylation seems to play a key role in imprinting. The addition of methyl groups to the DNA plays a role in silencing of genes and the methylation pattern carries over from one generation to the next. Methyl groups are known to affect protein-DNA interactions, and thus in general repress gene activity (Razin and Shemer, 1999). During two developmental stages the methylation patterns are reprogrammed genome wide. Both in germ cells and preimplantation embryos, reprogramming plays a role in generating cells with a broad developmental potential and in the erasure of the acquired epigenetic information (Reik and Walter, 2001). Failure to methylate DNA correctly has been associated with many human diseases (Robertson and Wolffe, 2000). Animals that miss the enzyme to methylate the DNA

have embryonic developmental problems and methylation is also required for development after birth (Jones and Takia, 2001).

In addition to the methylation of the DNA, chromatine structure, silencers, histons, boundary elements and other chemical modifications are playing a role in the imprinting of genes. Imprinting effects can also be seen in the construction of transgenic animals, where problems in the development of the transgenic animals and abnormalities are observed (Reik and Walter, 2001).

Aim and outline of this thesis

This thesis deals with the identification of genes controlling fatness in the pig and in particular genes controlling intramuscular fat and backfat thickness. Markers linked to QTL controlling intramuscular fat and backfat thickness in the cross between Meishan x Large White breeds will lead to the identification of the underlying genetic causes. Identification of genetic markers linked to separate genes controlling these fat traits would be a major step-forward, enabling animal breeders to select separately for the two traits. De Koning et al. (1999) described the detection of large QTLs for fatness traits on SSC2, SSC4, and SSC7. In Chapter 2 the QTL analysis is described with additional markers on SSC2, SSC4 and SSC7 and imprinting analysis for fatness traits. This imprinting analysis was subsequently extended to the entire genome (Chapter 3). In the project research emphasis was put on SSC2 since the QTL detected on this chromosome is imprinted. Chapter 4 describes the construction of a high-resolution comparative radiation hybrid map of SSC2. To further improve the comparative map of pig chromosome 2 and the homologous regions on the human genome bacterial artificial chromosomes (BACs) located in the QTL region were sequenced and compared with human and mouse sequence databases (Chapter 5). Chapter 6 deals with the refinement of the borders of the conserved segments, and the mapping of additional genes on the porcine genome. Finally, in Chapter 8 the results of this thesis are discussed and implications of this work are explored.

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Chapter 2

Fine mapping and imprinting analysis for fatness trait QTLs in pigs

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Abstract

Quantitative Trait Loci (QTL) for fatness traits were reported recently in an experimental Meishan x Large White and Landrace F2 cross. To further investigate the regions on pig chromosome 2 (SSC2), SSC4 and SSC7, a set of 25 additional markers from these regions were typed on 800 animals (619 F2 animals, their F1 parents and F0 grandfathers). Compared to the published maps, a modified order of markers was observed for SSC4 and SSC7.

QTL analyses were performed both within the half-sib families as well as across families (line cross). Furthermore, a QTL model accounting for imprinting effects was tested. Information content could be increased considerably on all three chromosomes. Evidence for the backfat thickness QTL on SSC7 was increased and the location could be reduced to a 33 cM confidence interval. The QTL for intramuscular fat on SSC4 could not be detected in this half sib analysis, whereas under the line cross model a suggestive QTL on a different position on SSC4 was detected. For SSC2, in the half-sib analysis a suggestive QTL for backfat thickness was detected with the best position at 26 cM. Imprinting analysis, however, revealed a genome wise significant paternally expressed QTL on SSC2 with the best position at 63 cM. Our results suggest that this QTL is different from the previously reported paternally expressed QTL for muscle mass and fat deposition on the distal tip of SSC2p.

Introduction

For the mapping of QTLs representing major genes for meat quality traits in livestock a whole genome can be scanned using genetic markers. Over the past few years, several experimental crosses have been used to detect QTLs for fatness and meat quality traits in pigs (Rothschild et al. 1995, Milan et al. 1998, Moser et al. 1998, De Koning et al. 1999). However, the identification of the underlying genes causing the QTL effect still remains a challenging task. Fine mapping of the QTL region in combination with selection of candidate genes is a way to localize and characterize the gene(s) underlying the QTL in question (Fisler et al. 1997).

Previously, we described a total genome scan using an experimental cross between Meishan and Large White (De Koning et al. 1999). The traits examined in that study were backfat thickness (BFT) and intramuscular fat content (IMF). Low BFT levels are desired by both consumers and meat industry. Selection against high BFT levels also decreases IMF levels, which reduces the tenderness and juiciness of the meat. Very high levels of IMF, however,

are not desirable because consumers do not like visible fat within the meat. The study revealed a genome wise significant QTL on SSC7 for BFT as well as suggestive QTLs for IMF on SSC4 and for BFT on SSC2. In this paper, we report the typing of additional markers in the same population (800 animals) to further investigate these QTL regions. The QTL analyses were performed using two different models; the line cross model assuming that the different alleles of the QTL are fixed in the founder lines (De Koning et al. 1999) and a half-sib analysis. Recent publications have revealed a maternally imprinted QTL for muscle mass and fat deposition on the distal tip of SSC2p (Jeon et al. 1999, Nezer et al. 1999). For our study also a model accounting for monoallelic expression was used to analyze the data. The imprinting model was adjusted afterwards to test for maternal or paternal expression.

Materials and methods

Population and traits. A detailed description of the animals used in this study was given previously (De Koning et al. 1999). Briefly, a cross between Meishan and Dutch Large White and Landrace lines was established for detection of major genes (Janss et al. 1997a, 1997b). The F2 design cross consisted of 39 half-sib families, 19 of which were used for this study. The selection of the 19 families was based on their likely presence of a major gene for intramuscular fat content (Janss et al. 1997a). From the 619 F2 offspring, 418 animals had phenotypic data for meat quality traits. In this study the results for BFT and IMF are presented. BFT was measured with the Hennessy Grading Probe between the third and fourth rib of a carcass, 6 cm from the spine. In a sample of *Musculus longissimus* IMF was determined by petroleum ether extraction (Hovenier et al. 1992, De Koning et al. 1999).

Genotyping and map construction. DNA for genotyping was isolated from frozen blood or tissue (spleen, liver). For the total genome scan, 127 microsatellite markers covering the 18 autosomes and the sex chromosomes had been typed on 19 F0 Meishan grandparents, 150 F1 parents and their 619 F2 offspring (De Koning et al. 1999). For this study, an additional 25 microsatellite markers were typed which are located on SSC2, SSC4 and SSC7. The markers used and their map position and allele sizes are summarized in Table 1. Genotyping of the microsatellite markers was done as previously described (Groenen et al. 1996, De Koning et al. 1999). PCR reactions for all microsatellite markers were performed separately in a PTC100 thermocycler (MJ Research) in a final reaction volume of 12 µl. PCR products of different markers were subsequently pooled and loaded on an ABI 373 automated sequencer

(Perkin Elmer Biosystems, Forster City, CA, USA). Fragment sizes were calculated using the GENESCAN 2.1 fragment analysis and GENOTYPER 2.0 software (Perkin Elmer Biosystems, Forster City, CA, USA). Linkage analysis was performed using the CRIMAP software package version 2.4 (Green et al. 1990). Multipoint maps were made using the CRIMAP build option. The FLIPS 5 option was used to check the marker order.

QTL analyses. Analyses were performed according to De Koning et al. (1999). Two types of analyses were used. First a half-sib analysis design following Knott et al. (1996) in which 19 paternal half-sib families were tested, assuming no fixation of the QTL alleles in the founder lines. Secondly, a line cross analysis (Haley et al. 1994) was carried out where additive and dominance effects were estimated under the assumption that the two founder lines are fixed for the different alleles at the QTL of interest.
Under the line cross model it was also possible to test for imprinting. First an additional imprinting term was fitted, as suggested by Knott et al. (1998). If this was significant the model was modified to test whether there was maternal or paternal expression (i.e. paternal or maternal imprinting, respectively). In this reduced model, only a paternally or maternally expressed effect was fitted. In addition, if imprinting was detected a test to determine whether the reduced imprinting model fitted the data better than the line cross model was performed.

Information content and significance thresholds. Under all models used the information content is proportional to the variance of the estimators that are used in the regression analyses. For the line cross model, when the variance of the estimator of the additive effect plus twice the variance of the estimator of the dominance effect at a given position is large, it indicates good marker information in that area. The information content has a maximum of 1.125 when the marker is typed in all offspring, of which 75% is homozygous and the marker is fully informative (Knott et al 1998), but in this study is scaled to vary between 0 and 1. For the half-sib analysis a similar procedure was followed (Spelman et al 1996). Two significance levels were used in this study. The first one is suggestive linkage where one false positive is expected to occur in a total genome scan (Lander and Kruglyak, 1995). Since the porcine genome is about 21 Morgan the threshold for suggestive linkage is often very close to the chromosomewise 5% threshold. The most stringent threshold is genomewide significance where a 5% risk of a false positive is assumed in a genome scan (Lander and Kruglyak, 1995). Both thresholds were derived empirically by permutation tests. For details see De Koning et al. (1999) and Churchill and Doerge (1994). Bootstrapping under the relevant

genetic model was applied to determine the confidence intervals (C.I.) for the observed QTLs. The best test statistic from each of 10000 bootstrap replicates was stored and sorted in descending order to define the 95% cut-off point. Tests for segregation distortion were performed following Knott et al 1998, using a two-sided T-test.

Results

For SSC2, SSC4, and SSC7, four, eight and thirteen additional markers were typed, respectively. The number of informative meioses of these markers are mentioned in Table 1. In combination with the microsatellite markers previously typed in the total genome scan this resulted in 13, 16 and 22 markers on SSC 2, 4, and 7, respectively and an average marker spacing of 14 cM on SSC2 and 10 cM on SSC 4 and SSC7. Adding four additional microsatellite markers in the proximal part of SSC4p extended the length of the entire chromosome by 40 cM.

Marker	Size range (bp)	Chr.	Map position[a]	Inf. meisoses
SW256	92 - 118	2	25	474
SW1450	187 – 217	2	43	748
SW2513	192 – 216	2	52	506
SW1686	139 – 160	2	62	966
SW2404	131 – 178	4	1	883
SW2509	177 – 198	4	13	826
SW2547	99 – 133	4	34	578
SW835	220 – 245	4	44	607
SW2049	226 – 238	4	50	84
SWR73	166 – 190	4	63	572
SWR2179	127 – 158	4	75	901
FABP4	250 - 280	4	88	1076
SW1369	134 – 157	7	52	1010
LTA	170 – 220	7	58	808
SW2019	127 – 143	7	60	763
SW1856	174 – 198	7	62	954
SWR946	97 – 122	7	73	573
SW859	101 – 118	7	80	553
SWR1928	94 – 111	7	88	969
SWR1806	210 – 222	7	101	749
SW2002	106 – 115	7	130	402
SWR773	138 – 159	7	143	745
SW2537	158 – 182	7	160	640
SW1303	134 – 158	7	181	948
SW1816	94 – 107	7	203	394

Table 1. *Size range of alleles and map position calculated of markers used in this study.* [a]*Map positions in Haldane cM*

New maps were constructed and compared to the maps published by Archibald et al. 1995 and Rohrer et al. 1996. No discrepancies with the published maps were found for SSC2. For SSC4, the best order was SW2509-SW2547-S0301-SW835-SW2049, which is different from the reported order: SW2509-S0301-SW835-SW2547-SW2409. For SSC7, different orders in two regions of the chromosome were found. On the p-arm of SSC7 where the QTL for BFT is postulated, the best order, SW1928-SW175-SW352-SWR1806, is not in agreement with the reported order SW1928-SWR1806-SW175-SW352. On the q-arm of SSC7 where the suggestive QTL for IMF is located, the best order is S0212-SW764-SW1303-SW1816 whereas the reported order is S0212-SW1816-SW764-SW1303.

The information content for all three chromosomes in the half-sib analysis, line cross analysis and imprinting analysis exceeded 50%. The information contents are lower for the line cross than for the half-sib analysis, resulting from a correction for scaling between 0 and 1, and the use of parental genotypes in the line cross analyses. The thresholds for the specific imprinting models were higher than the thresholds for the standard line cross.

	Half-sib analysis			Line cross analysis					Imprinting analysis			
							Estimates					
Chromosome	F ratio	P-value[1]	Location	F ratio	P-value[1]	Location	Additive[2]	Dominance[3]	F ratio	P-value[1]	Location	Comparison[4]
BFT												
2	2.69[a]	0.0057	26	4.95	0.08	59	1.13 (0.36)	0.036 (0.51)	13.58[b]	0.0022	59	3.58
4	1.13	NS	136	3.48	NS	137	-0.19 (0.41)	-1.77 (0.68)	2.45	NS	75	-4.45
7	3.43[b]	0.0[c]	57	26.2[b]	0.0[c]	57	-2.35 (0.33)	-0.05 (0.50)	22.20[b]	0.0[c]	55	-26.95
IMF												
2	0.53	NS	52	4.55	0.078	13	-0.17 (0.08)	-0.29 (0.14)	3.09	NS	51	-5.90
4	1.92	NS	59	6.02[a]	0.036	98	0.24 (0.07)	-0.07 (0.10)	5.69[a]	0.047	95	-6.19
7	0.97	NS	176	3.94	NS	53	-0.12 (0.07)	0.23 (0.10)	5.20	NS	167	-2.64

***Table** 2. Half-sib, line cross and imprinting analyses for chromosome 2, 4, and 7. [a] Indicates significance at the suggestive level, [b] indicates significance at the genome wise level , [c] test statistic not exceeded during 10,000 permutations, [1] genome wise p-value, [2] additive effect is the effect of the Meishan allele estimated as half the difference between the two homozygous. [3]the dominance effect is the estimated deviation from the mean of the two homozygous genotypes. [4] test for Imprinting vs. non-imprinting NS means not significant. The estimates are in mm. Backfat and percent intramuscular fat content.*

On SSC2, in the half-sib analysis a suggestive QTL for BFT is detected which has its best position at 26 cM (Table 2). Only three families with a test statistic higher than 15 mainly contributed to this effect (unpublished results). Under the line cross model, the QTL for BFT on SSC2 has its best position at 59 cM, but does not reach the suggestive significance level (Figure 1). Typing of the additional markers in the QTL region decreased the 95% confidence interval of the QTL detected in the line cross model from 65 cM to 57 cM. Table 2 also shows the results of the imprinting analyses. To test for significance, imprinting analyses were compared to the line cross model without imprinting. The QTL on SSC2 shows maternal imprinting, which means that only the paternal allele will be expressed in the offspring, resulting in the observed QTL effect (Figure 1). This model with imprinting fits the data better than without imprinting, because more variance is explained using this model. The best position for this QTL is exactly the same as the position of the QTL in the line cross analysis (59 cM), the 95% C.I. is similar to the line cross: 61 cM. No QTLs for IMF were detected on SSC2.

The suggestive QTL for IMF on SSC4p (S0227-S0301 bracket) that was previously detected using the half-sib analysis could not be supported in the half-sib analysis of this study (Table 2). The suggestive QTL for IMF on SSC4 in the S0001-S0073 interval that was reported in the line cross analysis of De Koning et al. (1999), however, is again detected in this study, but only one additional marker (FABP4) was typed in this part of the chromosome (Figure 2). No imprinting effects were found for IMF on SSC4.

The QTL for BFT detected in the half-sib analysis on SSC7 has a slightly higher F ratio compared to the previous half-sib analysis by De Koning et al. (1999). However, the most likely position of the QTL could be narrowed to the S0064 – S0102 interval by adding eight markers to this region (Figure 3). In the line cross analysis, the QTL for BFT on SSC7 was significant at the 5% genome wise level with a test statistic of 17.95 (p= 0.0) in the total genome scan (De Koning et al. 1999). With the inclusion of the additional eight markers in this line cross analysis an even higher test statistic of 26.20 (p= 0.0) is reached. Additionally, the 95% C.I. decreased from 45 cM to 33 cM, and is now located in the interval between SW1369 and S0102. The best position is at 57 cM; similar to the best position found in the half sib analysis. This QTL has an additive effect of 2,33 mm backfat (see Figure 3). The QTL for BFT on SSC7 is also significant at the 5% genome wise level under an imprinting model, but explains significantly less variance than under the standard model. For IMF, no significant QTLs and no imprinting effects were found on SSC7.

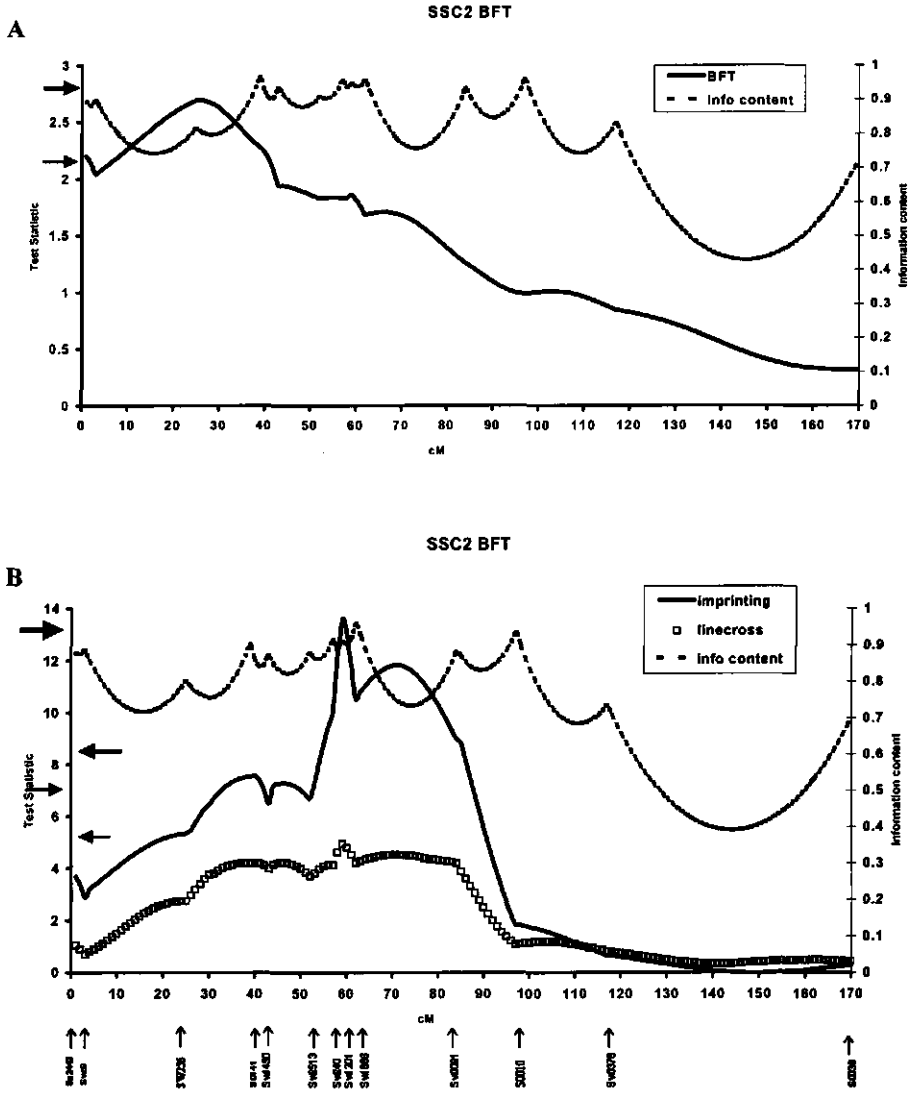

Figure 1. *QTL analyses for BFT on SSC2. A: half-sib analysis, B: line cross and imprinting analysis: Y-line indicates line cross analysis, solid line indicates imprinting analysis, dotted line is the information content. Small arrow indicates the suggestive treshold, the large arrow the genome wise significance treshold*

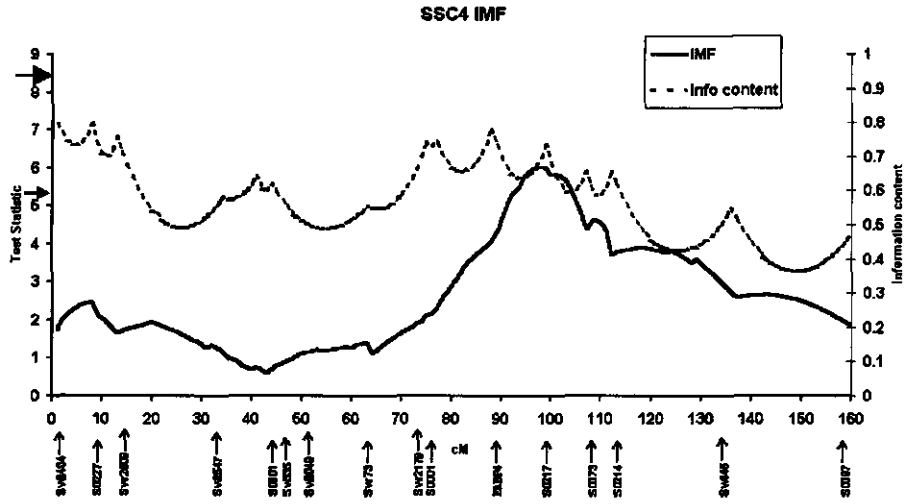

Figure 2. *Line cross analysis for IMF on SSC4. Line cross analysis with additional markers. Solid line indicates the QTL analysis. The dotted line is the information content. Small arrow indicates the suggestive treshold, the large arrow the genome wise significance treshold.*

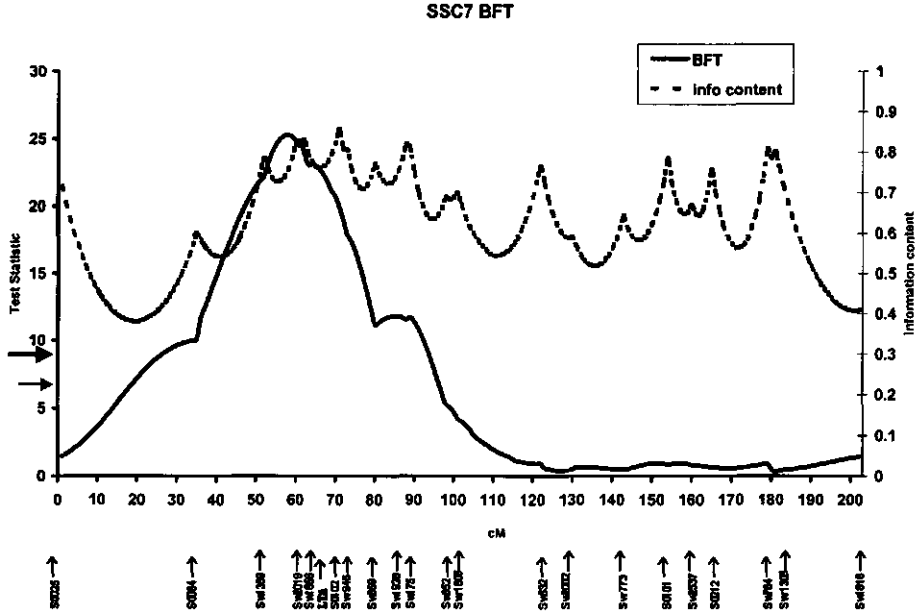

Figure 3. *Line cross analysis for BFT on SSC7. Line cross analysis with additional markers. Solid line indicates the QTL analysis. The dotted line is the information content. Small arrow indicates the suggestive treshold, the large arrow the genome wise significance treshold.*

Discussion

Discrepancies in locus order on SSC4 and SSC7 were observed. In this study a total of 578, 749 and 856 informative meioses (Table 1) were used to predict map positions for the markers SW2547, SWR1806 and SW764, respectively, whereas Rohrer et al (1996) for these markers had respectively 77, 52 and 158 informative meioses to identify the most likely order of loci. The observed orders were tested using the FLIPS5 procedure in CRIMAP (lod = 4). Based on our data, the orders observed in this study for SSC4 and SSC7 are at least 10000 times more likely than the orders reported by Rohrer et al (1996).

The presence of the BFT QTL on SSC7 was approved in the half-sib analysis as well as across families and narrowed down to a smaller interval of 33 cM. In the line cross analysis for BFT on SSC7 the most likely position of the QTL is now located in the SW1369 – SW2019 region. In this part of the chromosome, QTLs for fatness and growth traits have been reported in several studies (Rothschild et al. 1995, Milan et al. 1998, Moser et al. 1998, Rohrer et al. 1998 and Wang et al. 1998, De Koning et al 1999). For SSC7, typing 8 additional markers in the QTL region resulted in a more precise location of the QTL as predicted (Haley 1995, Schook and Alexander 1997).

Typing of additional markers at the proximal end of the SSC4p resulted in a flat line in the first 80 cM of SSC4, both in the half-sib and the line cross analyses for IMF. The suggestive QTL for IMF that was detected in the half-sib analysis of the previous study with a best position at 6 cM did not reach the suggestive significance level in this study. Probably, in the previous study a false positive was detected due to the relatively low coverage with markers in that region. On the q-arm of SSC4 the suggestive QTL for IMF in the line cross analysis is detected, located in the FABP4– S0073 interval. This QTL is in the same region as the QTL for mean fat depth detected by Andersson et al. 1994, and confirmed for several growth and fat traits in later studies (Andersson-Eklund et al. 1998, Knott et al. 1998, Walling et al. 1998, Marklund et al. 1999, Paszek et al. 1999). In none of these studies IMF was measured. In our Meishan x Large White cross both IMF and BFT were measured, and no effect for BFT was observed on SSC4. This is in agreement with Rohrer et al. (1998), who did not detect QTLs for several backfat traits on SSC4 in a Meishan x Large White cross.

In this study, QTL analyses revealed evidence for a QTL for BFT on SSC2 and for imprinting of this QTL. Recently this QTL has been confirmed using all 39 families in the pedigree (unpublished results). A paternally expressed QTL for muscle mass and fat deposition was recently mapped to the IGF2 locus at SSC2 (Jeon et al. 1999, Nezer et al.

1999). Although the marker within the IGF2 gene (SWC9 at 2 cM) typed in our cross is highly informative and polymorphic in both breeds, the maternally imprinted QTL found in our study has its best position at 59 cM. At the position of SWC9 the test statistic is far below the suggestive threshold and does not reach the genome wise significance level before the marker SW240 that is located 50 cM proximal (Figure 1c). No transmission ratio distortions were observed at the SWC9 locus or at the best position of the QTL. In addition in this study no line specific alleles at the SWC9 locus could be found. Therefore, the imprinted QTL observed in this study is expected to be different from the recently reported maternally imprinted QTL. However, it could not be excluded that the QTL alleles describes by Nezer et al (1999) are not segregating in our cross, and therefore no effect is seen at the SWC9 locus. These hypotheses can not be validated unless functional mutations for the assumed IGF2 variants will be identified.

The different QTL models indicate different best locations for the BFT QTL on SSC2. In the line cross analysis, the parental lines are assumed to be fixed for different QTL alleles. In the half-sib analysis, however, different QTL alleles allow for segregation in each family. This can explain the different graphs obtained with the two models. A possible explanation could be two neighboring QTLs; one additive QTL segregating in mainly three families and a paternally expressed QTL. With currently used QTL analysis models it is not possible to test this hypothesis. Furthermore, additional markers in the SW2443 - S0141 interval are necessary to furhter elucidate the observed effects in this region.

Conservation of synteny between the human and pig makes it possible to identify regions on the human map that are homologous to the QTL region in the pig. For the p arm of SSC2 the homologous human region is HSA11. However, comparative mapping is complicated by the fact that the QTL region for SSC2 is also partly crossing the breakpoint of two conserved synteny groups, HSA11 and HSA19. This breakpoint is likely to be located near the centromere of SSC2. The homologous regions for the p arm and the centromere of SSC2 in mice are located on chromosomes 7, 2 and 19.

It is known that several genes on HSA11p are imprinted. Besides the imprinted genes IGF2 en H19 on HSA11p15.5, several other human imprinted genes have been located in HSA11p15.5. In addition, imprinting has been reported for the Willms' tumor gene in human, located on HSA11p13 (Falls et al. 1999, Morison et al. 1998).

With the exception of the Y chromosome, in man putative QTLs affecting obesity related phenotypes are found on all chromosomes (Perusse et al. 1999), resulting in a total of almost 200 genes or markers linked to obesity phenotypes in humans. In addition, research on

obesity related traits in several other species have revealed a large number of QTLs in homologous regions of SSC2.

In mice a QTL for obesity, obq3, is located on chromosome 2 in a region that is homologous to SSC2p (Taylor and Philips 1997). In that study, several body composition traits (fat pad weights, gonadal fat percentage, inguinal fat percentage, 16-week body weight, BMI) were detected on the central part of mouse chromosome 2 in an AKR x C57l cross. A QTL for body weight was detected on mouse chromosome 2 with significance at the experiment-wide level with a best position at 56 cM (Brockmann et al. 1998). In addition, in a cross between mouse lines that were selected on fat content a QTL for fat percentage (Fob1) was detected on mouse chromosome 2 (Horvat et al 1999). Fob1 maps to the same region as obq3 (Taylor and Philips 1997). None of the above mentioned QTL studies report about imprinting mechanisms involved in the observed QTLs.

For SSC7, the typing of additional microsatellites in the QTL region has resulted in a smaller region and therefore has contributed to the fine mapping of the gene(s) responsible for the observed effect. For SSC2, imprinting analysis revealed a maternally imprinted QTL and interesting results for the line cross and half-sib analysis, suggesting the presence of two QTLs on this chromosome. In addition, a previously reported QTL for IMF on SSC4p was not supported in this study. Additional typing thus turns out to be beneficial to select QTLs for fine mapping and in that way avoids hunting a gene that is not present. Work is under way to create a higher density of genes on the porcine map to improve the comparative map between man, mouse and pig. This will facilitate fine mapping QTLs in pig and selecting candidate genes in homologous regions in man and mice.

This study indicates that genomic imprinting might be more common in livestock species then expected. Identification of imprinted genes for fat and growth traits can have several important implications in animal breeding. It is therefore important to include statistical testing for imprinting in further studies.

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Chapter 3

Genome-wide Scan for Body Composition in Pigs Reveals Important Role of Imprinting

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Abstract

The role of imprinting in body composition was investigated in an experimental cross between Chinese Meishan pigs and commercial Dutch pigs. A whole genome scan revealed significant evidence for five quantitative trait loci (QTL) affecting body composition, of which four were imprinted. Imprinting was tested using a novel statistical model that separated the expression of paternally and maternally inherited alleles. For backfat thickness, a paternally expressed QTL was found on SSC2 and a Mendelian expressed QTL on SSC7. In the same region of SSC7 a maternally expressed QTL affecting muscle depth was found. Chromosome 6 harbored a maternally expressed QTL on the short arm and a paternally expressed QTL on the long arm. The individual QTL explained 2 up to 10% of the phenotypic variance. The known homologies to human and mouse did not reveal positional candidates. This study demonstrates that testing for imprinting should become a standard procedure to unravel the genetic control of multifactorial traits.

Introduction

It is well established that quantitative trait loci (QTL) underlying the genetic variance of multifactorial traits can be mapped in experimental as well as outbred populations (1,2). Whole genome scans have revealed a number of genes contributing to genetic variation and have provided insight into the form of gene action. The genome scans can also be used to search for non-Mendelian forms of inheritance (3) but these opportunities have not been exploited systematically. Knowledge of form of inheritance of identified QTL is important for medical and agricultural applications.

Parental genomes undergo modifications during gametogenesis, resulting for some genes in parent-of-origin-specific expression in the offspring. This phenomenon of genomic imprinting, as a form of epigenetic gene regulation, has been shown to influence several sub-chromosomal areas in mammals (4). In human and mouse, most imprinted genes are arranged in chromosomal clusters[†] and their linked organization suggests coordinated mechanisms controlling imprinting and gene expression (5,6). It is generally viewed that imprinting is involved in fetal growth and brain development (7).

Different approaches have been used over time to identify imprinted areas in the genome. Both Robertsonian and reciprocal translocations resulting in mice with uni-parental disomy for portions of the genome have been used to identify imprinted regions on six

chromosomes (8). Furthermore chromosomal anomalies associated with imprinted diseases in humans helped to identify new imprinted genes and to narrow regions of interest (9,10). More recently, molecular genetic approaches taking advantage of e.g. methylation patterns observed for imprinted genes, have been used to isolate imprinted genes (11-14). The number of known genes is increasing rapidly, but imprinting has been reported only for about 30 (8). In livestock, evidence for imprinting was found for one specific chromosomal region in sheep and one in pigs (15-17). Imprinting effects, however, have not been systematically studied for multifactorial traits. We present results of a novel genome-wide approach to detect imprinted regions for multifactorial traits in an experimental cross of pigs.

Materials and methods

Experimental population. Boars from the Chinese Meishan pig breed were crossed with sows from commercial Dutch pig lines. From the resulting F_1, randomly selected boars and sows were mated to create the F_2 population (18). This experimental population facilitates the dissection of the genetics underlying phenotypic differences between these breeds for body composition traits. Meishan pigs are characterized by high fatness compared to Dutch pigs, which have been selected for lean growth for many generations. On 785 F_2 pigs we recorded three body composition traits after slaughter: backfat thickness and muscle depth measured between the third and fourth rib, and percentage of intramuscular fat inside the *Musculus longissimus* (18). The phenotypic mean (± standard deviation) of the F_2 population was 22.0 (± 5.7) mm for backfat thickness, 40.6 (± 6.7) mm for muscle depth and 1.84 (± 0.87) % for intramuscular fat content (18). Assuming Mendelian expression, analyses for backfat thickness and intramuscular fat content on part of this population revealed significant evidence for quantitative trait loci (QTL) on chromosome 2 and on chromosome 7 affecting backfat thickness (19).

Genotyping and statistical analyses. A whole-genome scan including a test for imprinting was used to map autosomal QTL on the F_2 population. Genotypes were obtained for 132 microsatellite markers, covering more than 90% of the porcine genome (19). Genotypes were obtained for the F_2 animals, their F_1 parents and the purebred Meishan grandparents.

The statistical analyses were based on the line cross concept (20), where original breeds are assumed homozygous for different QTL alleles. Extension of this model to test for imprinting has been suggested (3) and used in the analysis of the *IGF2* region in pigs (17).

This model, however, gives no indication whether there is paternal or maternal expression. The model for imprinting (3), therefore was re-parameterized to enable a direct test for the contribution of the paternally and maternally inherited effect. For every F_2 individual it is inferred what the probabilities are that it inherited two Meishan alleles (p_{11}), two Dutch alleles (p_{22}), or one from each line (p_{12} or p_{21}, different subscripts according to parental origin; first subscript is paternally inherited allele) at 1 centiMorgan (cM) intervals across the genome. Under the traditional line cross approach, an additive effect (a) and a dominance effect (d) are estimated using the regression of the phenotypes on $p_a=p_{11}-p_{22}$ and $p_d=p_{12}+p_{21}$. To separate the contribution of the parents, we introduced the probability that the individual inherited a Meishan allele from its father ($p_{pat}=[p_{11}+p_{12}]-[p_{22}+p_{21}]$) or from its mother ($p_{mat}=[p_{11}+p_{21}]-[p_{22}+p_{12}]$). In the saturated model, where the phenotypes were regressed on p_{pat}, p_{mat} and p_d the contribution of the individual components to the reduction in residual variance was evaluated by a partial F test. The genetic model for a putative QTL was inferred from the variance explained by components in the model.

Significance thresholds and confidence intervals. For the inferred genetic models the significance thresholds and the confidence intervals of the QTL position were determined empirically. The significance threshold was set at the 5% genome-wise risk level (21). This accounted for testing the entire genome but not for testing multiple traits. These thresholds were determined by permutation with at least 10,000 replicates (19).

Empirical confidence intervals for the QTL position were obtained by bootstrapping the data followed by analysis of the replicates under the inferred genetic model. From each of 10,000 bootstrap replicates, the best test statistic was stored. The 95% cut-off point of the sorted (in descending order) test statistics provided an empirical threshold to define the boundaries of the confidence interval. This is an alternative to other bootstrapping strategies where QTL positions of the replicates are sorted to determine an empirical confidence interval (3). The method used here allows for non-continuous confidence intervals and is closer to the traditional Lod drop-off methods.

Results

Our genome scan resulted in five significant QTL affecting body composition traits, of which four were imprinted. For backfat thickness, there was strong evidence for a paternally expressed QTL on porcine chromosome 2 (SSC2) (Table 1).

thickness on SSC7, both the paternal and maternal component were highly significant implying Mendelian expression for this QTL. For muscle depth, a highly significant QTL mapped to the same area as the QTL for backfat thickness on SSC7. In contrast to the QTL for backfat thickness, the QTL for muscle depth was maternally expressed (Table 1). From these results, it cannot be determined whether there are two linked loci or one locus with pleiotropic effects that shows imprinting during one stage of development and Mendelian expression during another.

With a model ignoring imprinting, suggestive evidence for a Mendelian QTL for intramuscular fat content was reported on the long arm of SSC6 (19). The present analysis, however, revealed that this was caused by a significant paternally expressed QTL (Table 1). In addition, a maternally expressed QTL affecting the same trait was found on the short arm of the same chromosome. The phenotypic variance explained by the individual QTL varied from 2% for the QTL affecting intramuscular fat content on SSC6 and 10% for the QTL affecting backfat thickness on SSC7.

Location	F ratio*			Inferred Genetic model	QTL effect§
	Paternal effect	Maternal effect	Dominance		
Backfat thickness (mm)					
SSC2, 36 cM	24.07†	2.85	0.51	Paternal expression	0.95 (0.20)
SSC7, 57 cM	30.27†	49.35†	0.04	Mendelian expression	−2.30 (0.25)
Muscle depth (mm)					
SSC7, 56 cM	4.74	50.33†	2.20	Maternal expression	-1.69 (0.24)
Intramuscular fat content (%)					
SSC6, 23 cM	0.07	14.53†	0.00	Maternal expression	0.14 (0.04)
SSC6, 117 cM	14.71†	1.34	0.31	Paternal expression	-0.13 (0.03)

Table 1. *Genetic model for QTL affecting three body composition traits. * Partial F ratio for the individual components of a model including a paternal, maternal and dominance component at the most likely position of the QTL. † $p < 0.0001$ ‡ Empirical confidence intervals obtained by bootstrapping for the relevant model. § Estimates of QTL effects for the inferred genetic model. The additive effect (Mendelian expression) and the paternal or maternal effect (imprinting) are expressed as the deviation of the Meishan allele. Standard errors of the estimates are in parentheses.*

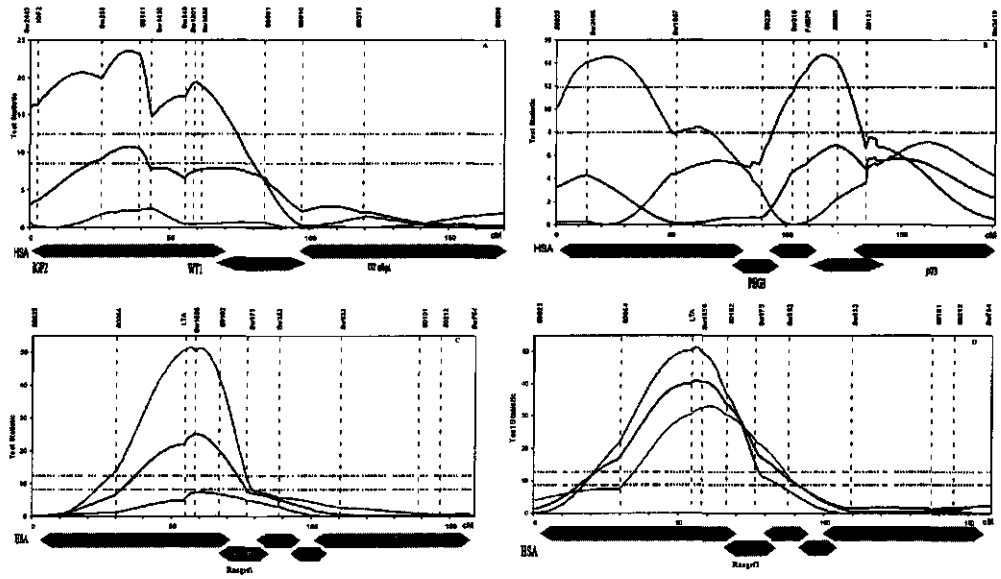

Figure 1. *Test statistic profiles for three porcine chromosomes that exhibit imprinting effects for one of the body composition traits: (A) SSC2 and backfat thickness, (B) SSC6 and intramuscular fat content, (C) SSC7 and muscle depth, and (D) SSC7 and backfat thickness. The black line represents the test statistic for a Mendelian QTL vs. no QTL. The blue line represents the test statistic for a paternally expressed QTL vs. no QTL. The red line represents the test statistic for a maternally expressed QTL vs. no QTL. The black horizontal line denotes the 5% genome-wise threshold for the Mendelian model, and the blue line indicates the same threshold for the imprinting model. (A) SSC2 and backfat thickness; (B) SSC6 and intramuscular fat content; (C) SSC7 and muscle depth; (D) SSC7 and backfat thickness. Homologous regions in humans are indicated as colored bars (22-25)‡. Imprinted genes located within these human chromosomal areas are underneath (5).*

A graphical comparison of results obtained under the imprinting and Mendelian models is in Fig 1. The imprinted QTL on SSC2 maps 35 cM from the *IGF2* region, for which an imprinted QTL for body composition has been reported (16, 17). Although the confidence interval does not exclude *IGF2* as a candidate gene, our results indicate that an additional imprinted QTL is present further proximal on this chromosome.

The general outline of the comparative map between pig and human for the regions of interest has been established‡ (22-25) and is in Fig. 1. Genes that have been mapped more precisely in pigs, by linkage analysis or on the radiation hybrid panel (25), facilitated

refinement of the comparative map. We realize that the comparative map presented here is not comprehensive, and that some genes originating from other chromosomes are reported but not represented in Fig. 1.

QTL affecting body composition traits in pigs can have implications for obesity research in humans (20). Although several obesity genes that are reported in humans and mice map to homologous regions of the imprinted QTL found in this study (26), imprinting has only been reported for the Prader-Willi Syndrome (HSA15q11.2-q12, refs. 9,10).

The QTL on SSC7 can be narrowed to a region homologous with HSA6p21.3-p22. This region contains the major histocompatibility complex, including *LTA*, and shows extensive conservation in gene order (27). Imprinted genes have not been reported for this region in humans or mice (5).

For the maternally expressed QTL affecting intramuscular fat content on SSC6p several genes that map to the area are located on HSA16q22-ter. No imprinted genes have been reported for this region in humans. For the paternally expressed QTL affecting intramuscular fat content on SSC6q, candidate genes *MC5R* (28), *FABP3* (29) and *UOX* (25) map between markers SW316 and S0003. These genes are located on human chromosomes 18p11.2, 1p33-p32, and 1p22, respectively and in humans imprinting has not been reported for these regions. However, the confidence interval of this QTL extends on both sides to homologous regions in humans, where imprinted genes have been reported: *p73* on HSA1p36 and *PEG3* on HSA19q13.4 (only imprinted in mice).

For SSC2, imprinting is reported for the *IGF2* area, but until now homology to other imprinting clusters could not be established clearly. Data on imprinting of the Wilms Tumor gene 1 (*WT1*) on HSA11p13 are contradictory (5).

Discussion

The progress of the genome projects, In particular the large number of polymorphisms that have been characterized in many species, has boosted the search for genes involved in multifactorial traits such as obesity, diabetes, and schizophrenia. Genomic imprinting, however, is regarded to be a rare phenomenon and consequently is ignored in most studies. Our results indicate that genomic imprinting might be a more common phenomenon than previously thought. We detected five QTL, of which four were subject to imprinting. For at least two of these regions, imprinting has not been reported in pigs, and the known homologies to humans and mice did not reveal obvious positional imprinted candidates. To

our knowledge, only one study for susceptibility loci for diabetes has considered imprinting in a genome-wide analysis, and these results indicated that uni-parental expression, both paternal and maternal, might indeed be involved in diabetes (30).

The statistical analysis presented here provides information on the mode of expression of genes. In addition, analysis under different modes of expression increases the power of finding genes. This is exemplified by the results for intramuscular fat content on SSC6, where significant evidence for QTL was found only under the imprinting model. The approach is implemented here for a cross between outbred lines but can be extended to other designs and methods of analysis, including mapping methods used in human genetic studies. For implementation, it is essential that parental origin of alleles can be derived for individuals with phenotypic observations. This requirement excludes studies based on crosses between inbred lines that are commonly used in mice and rats (3). Outbred crosses, such as used cross between two pig breeds in our study, are the ideal resource for detection of imprinted regions.

Genome wide screens for QTL often result in estimates of QTL position that lack precision, which complicates the identification of the responsible gene. Knowledge of the fact that the QTL is subject to imprinting will help in identifying the genes. Expression studies aimed at the identification of mono-allelic expression of positional candidates will further aid the identification of the gene(s) responsible for the observed QTL effect. Genotypes of the parents can be used to discriminate between random inactivation and parent-of-origin effects.

For the practice of animal breeding, identification of major imprinted loci affecting body composition has several implications. Our results call for a revision of methods for genetic evaluation that currently ignore non-Mendelian expression. The net result of gametic imprinting is a reduction of the expected phenotypic covariance between parents and offspring relative to that between sibs. Identification of imprinted loci opens new perspectives for crossbreeding, which is common practice in pig breeding. Imprinted genes could further accommodate differentiation between sow lines, which are required to have optimal body composition to support their reproductive performance, and boar lines, which ensure high-quality pork.

Although the mechanisms underlying imprinting are not totally unraveled (5), this study clearly demonstrates the important role of imprinting for body composition traits. We strongly urge, therefore, the inclusion of statistical testing for imprinting in human and animal genetic research, both in genome scans and in evaluating candidate genes.

Footnotes

†A World Wide Web Site is provided by C.V. Beechey, B. M. Cattanach, R. L. Selley, MRC Mammalian Genetics Unit, Harwell, Oxfordshire. http://www.mgu.har.mrc.ac.uk /imprinting/imptables.html

‡The comparative map of the pig can be viewed at http://www.toulouse.inra.fr /lgc/pig/cyto/cyto.htm. Alignment of the porcine cytogenetic and linkage map is adapted from http://sol.marc.usda.gov/genome/swine/htmls/chromosome_list.html

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Chapter 4

A high-resolution comparative RH map of porcine chromosome (SSC) 2

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Abstract

A high-resolution comparative map was constructed for porcine chromosome (SSC) 2, where a QTL for back fat thickness (BFT) is located. A radiation hybrid (RH) map containing 33 genes and 25 microsatellite markers was constructed for this chromosome using a 3000 rad porcine RH panel. In total, 16 genes from human chromosome (HSA) 11p, HSA19p, and HSA5q were newly assigned to SSC2. One linkage group was observed at LOD 3.0 and five linkage groups at LOD 4.0. Comparison of the porcine RH map with homologous human gene orders identified four conserved segments between SSC2 and HSA11, HSA19, and HSA5. Concerning HSA11, a rearrangement of gene order is observed. The segment HSA11p15.4-q13 is inverted on SSC2 when compared to the distal tip of SSC2p which is homologous to HSA11p15.5. The boundaries of the conserved segments between human and pig were defined more precisely. This high-resolution comparative map will be a valuable tool for further fine mapping of the QTL area.

Introduction

Total genome scans have been successfully applied to map loci affecting economically relevant traits in livestock. Recently, an imprinted QTL accounting for differences in back fat thickness (BFT) in a cross between Meishan and White breeds was detected on porcine chromosome (SSC) 2 (Nezer et al. 1999; Jeon et al. 1999; de Koning et al. 1999; Rattink et al. 2000). However, livestock genome maps lack the resolution required for further fine mapping of QTL regions to finally identify the underlying genes. The implementation of data from the much more detailed human and murine maps is useful to accelerate the improvement of livestock maps. Comparisons at the phenotypic level concerning obesity related traits indicated a number of QTLs to be located on HSA11 (Chagnon et al. 2000). However, these QTLs are not mapped precisely either. To facilitate the switch from candidate regions to candidate genes within small chromosome segments, the homologous regions need to be defined accurately (Schibler et al. 1998).

The general picture of the comparative map between pig and human is based on bi-directional chromosome painting (Rettenberger et al. 1995; Goureau et al.1996), somatic cell hybrid mapping and FISH (http:\\www.toulouse.inra.fr/lgc/pig/cyto/gene/chromo/SSC2.htm). Bi-directional chromosome painting indicated homology between SSC2pter and HAS 11p15-q13. The q-arm shows homology to HSA19pter (SSC2q12-q21) and HSA5q14-q35

(SSC2q22-q29). In addition, EST mapping (Fridolfsson et al.1997; Lahbib-Mansais et al. 1999) and the introduction of CATS primers (Lyons et al. 1997) improved the comparative map. The cytogenetic map of SSC2 (Yerle et al. 1996), encompasses 34 expressed sequences, 5 genes have been placed on the basic genetic map (Archibald et al. 1995; Rohrer et al. 1996), and 3 genes on the radiation hybrid (RH) map of Hawken et al. (1999). QTL studies on meat quality traits presented the genetic mapping of *MYOD1* and *IGF2* on SSC2, confirming homology between SSC2pter and HSA11p15-q13 (Jeon et al. 1999; Nezer et al. 1999). Nevertheless, considerable rearrangements within conserved blocks between mammalian species have been described (Carver and Stubbs 1997; Pinton et al. 2000). Therefore, a high-resolution comparative gene map is needed.

In this study, a detailed comparative RH map was constructed for SSC2. In addition to genes known or expected to be located on SSC2, microsatellite markers were mapped on the RH panel for a better link with the porcine genetic map. A gene-dense map of SSC2 is presented identifying precisely the borders to the human homologous segments.

Materials and Methods

Development of PCR primers. Existing primers for the genes known to map to SSC2 were selected from literature (Table 1). If a clear product could not be obtained with these primers, new primers were designed based on available sequence data in the database. In addition, the GDB sequence database (http://gdbwww.gdb.org/) was searched for homologous porcine sequences for all genes located on HSA11pter-q13 and HSA19q13. BLAST searches were performed with gene name and with the accession number. If possible, primers were developed using the Primer3 software (Rozen and Skaletsky 1998) in the 3'untranslated region (UTR) of the porcine cDNA sequence to reduce the chance of amplification from the rodent background in the cell lines. If no 3'UTR sequence was available, information on intron/exon boundaries was taken into account to design primers. Otherwise, primers were selected on the basis of the available sequence data. All PCR products resulting from previously unpublished primers were sequenced to insure that the right locus was amplified.

For microsatellite markers, primer sequences, and conditions have been described previously for SW1650, SWR1445, SW1450, SW1686, SW2167, SW1857, SWR1342, SW1883, SW2192, SWR2157, SW1564, SWR1338, and SW1695 (Alexander et al. 1996a), SW2443, SWR2516, SWC9, SW2442, and SW2513 (Alexander et al. 1996b), SW1201, SW240, SW1026, SW747, SWR783 and SW776 (Rohrer et al. 1994), S0141 (Jung et al.

1994), S0091 (Ellegren et al. 1993), S0378 (Robic et al. 1997), S0010 (Fredholm et al. 1993), S0036 (Brown et al. 1994).

Typing of the porcine RH panel and map construction. An RH panel was purchased from Research Genetics (Huntsville, USA). The RH panel was created by exposing the porcine cell line to 3,000 rad of x-rays and fusion with nonirradiated thymidine-deficient hamster recipient cells (A23). The panel consists of 101 clones, complemented by a pig and hamster genomic DNA as a control. For mapping, 94 cell lines were selected (Lopez Corrales, pers. comm.) to fit a 96-well format panel. The cell lines removed from the panel are Q7, Q10,T2, W4, X2, X7, and Y7.

Primers for 87 loci were first tested on the seven cell lines that were not included in the final RH mapping panel. If no clear PCR product could be produced, new primers were designed based on sequence data (Table 1). PCR reactions were performed in a total volume of 12 µl using 25 ng of RH cell line DNA. Amplification of PCR products was carried out using a standard PCR program with 5 min. denaturation at 95°C, 35 cycles for 30 s at 95°C, 45 s annealing, 90 s extension at 72°C, and a final extension for 10 min at 72°C. The annealing temperature was varied from 55° to 62° (Table 1). PCR products were separated on a 1.5 % agarose gel. Scoring of PCR products was carried out independently by two investigators.

The RHMAP 3.0 package (Lange et al. 1995) was used to construct RH maps. First, the RH2PT program was used for a two-point analysis to construct linkage groups with a LOD score of 3.0 and 4.0. To determine locus order, these linkage groups were reanalyzed using the RHMAXLIK program to construct a framework (ADDMIN = 2.0) and a comprehensive map. The ordering strategy used is the stepwise locus ordering, using the equal retention probability model. This model combines minimization of the obligate number of breaks required to explain the observed retention patterns with maximum-likelihood analysis. Loci that could not be placed in a linkage group on SSC2 with LOD 3.0 were typed on the somatic cell hybrid panel (Yerle et al. 1996) to obtain a regional assignment (http://www.toulouse.inra.fr/lgc/pig/pcr/pcr.htm).

Gene	Primer sequence		Product length	Temp	Reference for primers / Accession number
RNH	AGGCTCCTTTTCCCTGGAC	GTGCCGAAAGTTCTCTCACG	100	60	M58700
MUC5AC	CGGTCCAGGTGACTGAATCT	GGAGCAGACCCTTCTGTGAC	125	60	AF054584
IGF2	AATTCTGCGGTGCCACCATC	ATACAGACCAAGCCAATTGG	214	60	X56094
H19	AAGCTCCAGCGACACTGTTT	GAGCAGAATCTTCCAGAACT	326	55	Unpublished[1]
ADRBK1	GATCAGCTACGTGTACAAGAC	CCTCTCTGCAAGAAACTCACG	365	55	Unpublished[1]
GAL	ATCTTCGTCTCGGCGTTGT	TTTATTTTGCGGCTCAAACC	150	60	M13826
CFL1	TTTAACGACACCCCAGTTCC	CTGGTCCTGCTTCCATGAGT	193	55	M20866
FTH1	TAAGCTGGCCTCCCGGAGAC	GGTACACTAAGGAAAGAACT	108	55	D15071
TCN1	AAACTCAAACCCTCCTCCGT	TGTGCAAAGAGAGAAGCCAA	134	62	X52566
CNTF	ACAGTTCTCTGAGGCCTCAC	TCAGACGAGTCATCCAGAAC	263	60	U57644
CD59	GGAGAGTTTCTCCTCAACTTCC	ATTCGAATGGTTACCGCAAA	287	55	AF020302
CAT	TGCCTCTGAAACAAAACGTG	TTCAAAAGACCCCAAAGCAT	459	55	D89812
WT1	TTAACATTCCTCCTGGCTCG	GCCTTGCCCTCTGATTTATTT	414	60	AB010969
FSHB	CATTGCCATGAGCTATGGTG	TCCTTGACCTATCACGAGGC	318	62	Moran et al. 1993
BDNF	ATATCAGGTGCTCACAGTGC	GACTTAACTCTAGGAGTTCC	612	55	Unpublished[1]
MYOD1	GCTACGACGGCACCTATTAC	GGCTTAGTGTTTCATTCCCT	148	55	Soumillion et al.1997
LDHA	ACATGGCATTGTACACTATTCTG	GCCAGTGTTGCAGATGCT	226	58	Fridolfsson et al. 1997
RPS13	GAAAGCATCTTGAAAGGAACAGA	AGAGGCTGTGGATGACTCGT	146	62	C94688
PTH	ACCAGGAAGAGATCTGTGAGTG	TGCCCTATGCTGTCTAGAGC	305	60	Lyons et al. 1997
ADM	GTGAATGTCTCAGCGAGGTG	TTCTTCCCCAAGAGGCATC	100	55	Rohrer et al. 1996
INSL3	CCCTTGGCTTTGCAGAAATA	GGCTGTCAGGGGACTAGCTT	201	58	X73636
PRDX2	AGCTATGTCGCTCCAGGAAA	CCCTGTACTGACCCAGGAAA	140	60	Jorgensen et al. 1997
LDLR	GACGTGCTCCCAAGATGAGT	CAGGTGGAGCTGTTGCACT	159	55	AF118147
PDE4A	GAAGTGGACATCCCATCACC	CCTCTTGATCGGTCTTCACC	202	60	U97587
CNN1	GGAGCACTACGAGGTCCAAC	CATGCAGTTTGCTCCCACT	197	60	Z19538
DNASE2	CTCAGGGGCCAATTCAGACT	TTAGCAATCCTGAGGCAGGT	134	55	AF060221
CANX	TCCAATCCCTCTCCTCAATT	TGTCCATGCAGAAATAACAC	230	55	Fridolfsson et al. 1997
GPX4	AGCTCAACAAGTGTGTGCTGA	GCCAAAGGGACCTTCCTC	146	60	S80257
AMH	AGGCTGTGCGGCATGCC	AGCCATGCCTGCAGCTGCT	112	62	AF006570
NFIC	TATAAGAGGTCTGAGGGCGG	CCCTCTCAGTGCAGCTTCTT	105	62	X12764
C3	GGCAACCAAAGACGACCAT	AATCAGGCTCCGATGAAGTG	320	62	Fridolfsson et al. 1997
CSF2	CAGCATGTGGATGCCATC	GTACAGCTTCAGGCGAGTCT	935	60	Lyons et al. 1997
IL4	GATCCCAACCCTGGTCTGCT	GGCAGCAAAGACGTCGTCAC	430	62	Rettenberger et al. 1996
FOLR1	AGACGGTCCTTCTGCCTGT	TTGAGGAGGAGCCTATGGTTT	165	60	U89949

Table 1. *Description of genes typed on the RH panel. If available, previously published primers were used (Reference for primers), otherwise primers were designed from porcine sequence data (Accession number). Genes previously unmapped to SSC2 by other mapping methods are underlined.*
[1] *Primers derived from BAC containing the gene.*

Results

Construction of the RH map. From the 74 loci tested, 59 loci showed a single distinct PCR product on the RH cell lines. Thirteen loci amplified several products, and two showed the same product on the hamster background. These fifteen loci could therefore not be typed on the RH panel. The mean retention fraction was 25.9% and varied from 10.6 % (SWR2157) to 48.9% (SW1857). At a LOD score of 2.0 within the RH2PT program of RHMAP 3.0, one linkage group of 58 markers and genes was observed, covering the entire chromosome 2

except for one locus (FOLR) that could not be placed in the group. For LOD = 3.0, one large linkage group, and one small group of two loci (SW2442 and SWR1338) was observed. At this LOD score two additional loci could not be placed (SWR783, S0036). Figure 1 shows the RH map using a LOD score of 4.0. The genes and markers are grouped into 5 groups of respectively 15, 12, 9,15, and 7 loci.

To calculate the distances between the five linkage groups at LOD 4.0, a comprehensive map was calculated of combined neighboring groups (group I and II, group II and III, group III and IV, and group IV and V) with ordered framework markers from the linkage groups. The distance between linkage group IV and V could not be calculated since the order of the markers in the combined group changed. This results in a total map length for the first four groups of 2129.5 cR_{3000}. Two point linkage data between C3 and S0010 (Hawken et al. 99) were used to estimate the distance between group IV and V, resulting in a total length of the five linkage groups of 2470.7 cR_{3000}. According to the genetic map (Rattink et al. 2000), the genetic length between markers SW2443 and S0091 is 84 cM (using the Haldane map function) and the RH distance is 1514.7 cR_{3000}. Therefore, 1cM is equivalent to a distance of 18.0 cR_{3000} in this region. The total physical length of SSC2 is estimated at 168 Mbp (Rohrer et al 96). The marker interval SW2443 - S0091 covers around 48.8 % of SSC2 (82 Mbp), assuming a total genetic length of 172 cM (Rattink et al. 2000). For this region, 1 cR_{3000} is estimated to be equivalent to 54.1 Kbp.

Comparative map. In total 33 genes and 25 microsatellite markers were placed on the RH map of SSC2 (Figure 1). Nineteen genes from HSA11p-q13 were mapped to SSC2 in pigs (*RNH, MUC5AC, IGF2, H19, ADRBK1, GAL, CFL1, FTH, TCN1, CNTF, CD59, CAT, WT1, FSHB, BDNF, MYOD1, LDHA, PTH,* and *ADM).* However, one gene, *FOLR* (HSA11q13.3-q14.1), could not be linked to one of the linkage groups of the RH map on SSC2, although based on its human position, it was expected to be located on SSC2. *FOLR* was typed on the somatic cell hybrid panel (Yerle et al. 1996) and assigned to porcine Chr 9p21-p24. From HSA19p13, nine genes (*DNASE2, INSL3, PDE4A, CNN1, LDLR, C3, NFIC, AMH,* and *GPX4*) were placed together on the RH map of SSC2. Finally, three genes (*CANX, IL4,* and *CSF2*) map to HSA5q35 and 5q31.1. The gene *PRDX2* is reported to map to HSA13q12. Out of the 33 genes, 17 genes had been mapped to SSC2 previously by either FISH, linkage mapping, somatic cell hybrid mapping or RH mapping (Figure 1, http://www.toulouse.inra.fr/lgc/pig/cyto/gene/chromo/SSCG2.htm).

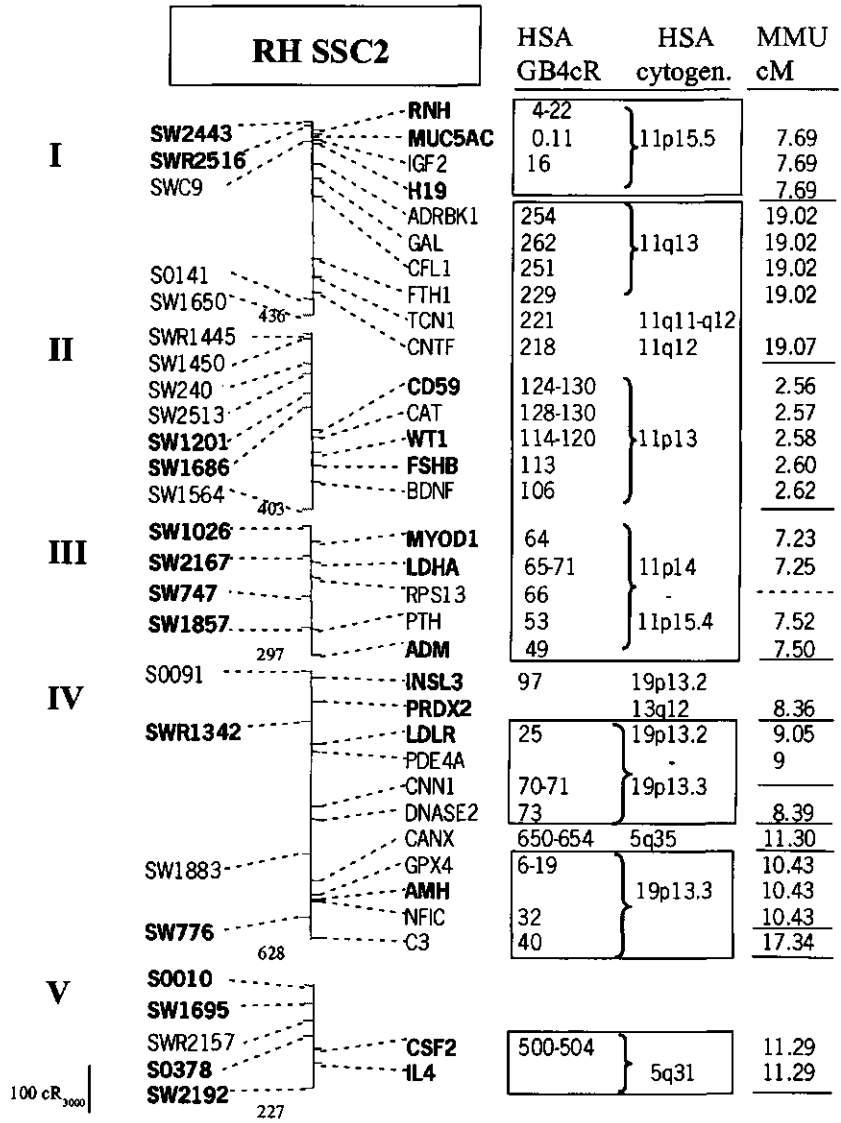

Figure 1. *A comprehensive radiation hybrid map for SSC2 with 58 loci. Framework markers are in bold, whose order is supported by linkage at LOD 4.0. The lengths of linkage groups are indicated in centirays (cR_{3000}) under the last marker of the linkage group. The distances between the linkage groups are, from group I to group V, 102.3, 124.8, 150.6, and 114.2 cR_{3000}. On the right of the RH map of SSC2 are the human radiation position (GB4: http://www.ncbi.nlm.nih.gov/genemap99/, in cR), human cytogenetic location, and murine linkage position (chromosome, cM) of the genes mapped on the RH panel.*

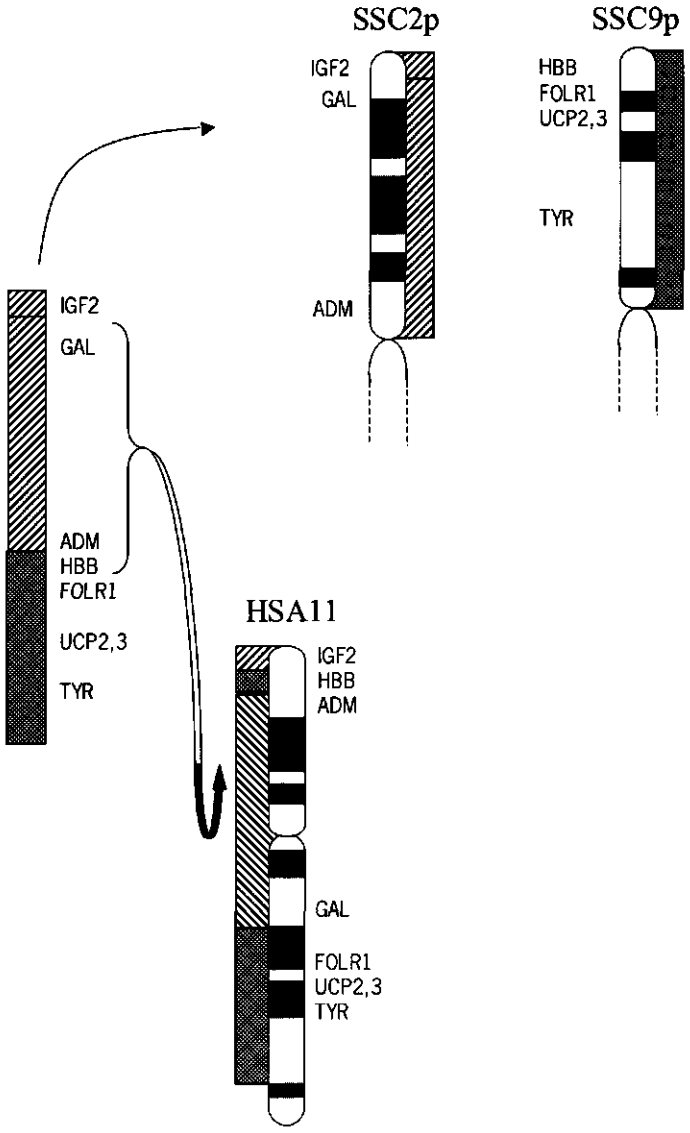

Figure 2. *A cytogenetic comparative map between SSC2p, SSC9p and HSA11 describing a model for the evolution from a common ancestor chromosome (left). Regions with conserved gene order are indicated with crosshatched bars. The arrows indicate evolution to Homo sapiens (bottom) and Sus scrofa (top). Gene names are illustrations to clarify translocations and inversions.*

Discussion

Construction of the RH map. This study describes the construction of a gene-dense comparative RH map of porcine Chr 2. One linkage group was observed at LOD 3.0 covering two thirds of SSC2. The two-point analysis resulted in five linkage groups at a LOD score of 4.0 (Figure 1). The length of the five linkage groups together is 2470.7 cR_{3000}. One cR_{3000} is estimated to be equivalent to 54 Kbp. Hawken et al. 1999 estimated 70 Kbp per cR for the INRA-UMN map. Although that estimate was based on the genome and our estimate of 54 Kbp is calculated for just one chromosome, it is in the range for the individual chromosomes (50-116 Kbp/cR) in the INRA-UMN map. However, based on the lower doses of radiation used to construct the present panel, the Kbp/cR_{3000} ratio was expected to be higher than the INRA-UMN ratios and it is expected to consist of larger linkage groups. Six linkage groups (with 5, 7, 9, 10, 12, and 14 loci) and one unlinked marker at LOD score 4.8 were observed in the present study. Hawken et al. (1999) observed ten linkage groups (with 2, 2, 2, 3, 3, 4, 5, 6, 9, and 11 loci) and one unlinked marker at the same LOD score.

There is general agreement between the map in this study and the INRA-UMN map. Nevertheless, the order of some loci in group II is switched (SWR1445 with SW1450, SW240 with SW2513), but these markers map very close together and could not be placed as framework markers with a LOD 2.0 support in the multipoint analysis. Linkage group IV joins the loci S0091, SWR1342, and SW776 that map to three separate small groups on the INRA-UMN map. The order of these linkage groups in the RH map given by Hawken et al. (1999) follows the genetic map of Rohrer et al. (1996) and is not in agreement with the map of this study. However, the genetic map of Archibald et al. (1995) is in agreement with our order and all three markers belong to the framework map giving strong support for the order presented here. On the distal part of SSC2q, the most telomeric marker S0036 on the genetic map could not be placed precisely on the panel. Nevertheless, with SW2192 as final marker, this RH map is expected to cover around 88% of the total genetic length of SSC2.

Mapping genes and comparative mapping. Bi-directional painting indicated one large conserved segment between SSC2 and HSA11, HSA19, and HSA5 (Goureau et al. 1996). From this study it can be seen that two conserved segments are present with preserved gene order between HSA11pter-q13 and SSC2 (Fig 1). The position of the 20 genes mapping to HSA11 shows a small segment on the distal tip of SSC2p17 homologous to HSA11p15.5 and one large inverted segment of HSA 11q13-11p15.4 covering the remaining part of SSC2p.

There is no indication of further rearrangements within this inverted segment, because the order of these genes is well in accordance with the human radiation hybrid map. For group II of the porcine RH map, the gene order of the framework loci CD59, WT1, and FSHB is exactly as expected according to the physical map in human (Gawin et al. 1999). Sample sequencing of porcine BAC clones from the area reveals homologies with additional genes and expressed sequences that bridge the gap between positions 130 and 218 cR on HSA11 (manuscript in preparation). Recently, Pinton et al. (2000) mapped additional anchor loci using gene-containing BACs from goats in heterologous FISH on porcine chromosomes. These results already indicated the inversion shown in this study at a higher resolution.

In the analysis for the RH map of SSC2 one gene (*FOLR*) was not linked with SSC2 loci in the two-point analysis. It has to be pointed out that a cluster of *FOLR*-genes is located at HSA11q13.3-q13.5 and our primers are designed in a region of the gene with high homology to the other FOLR genes. Therefore, we are not able to distinguish which FOLR-gene we have amplified, but based on its human position, the *FOLR* cluster was expected to map to SSC2. Typing of the somatic cell hybrid panel, however, assigned *FOLR* to SSC9p21-p24. The comparative map between pig and human shows (Figure 2) that a breakpoint between two conserved groups is located near the *FOLR* cluster. The closely linked gene *GAL* maps to the same position (262.72cR) on the human GB4 map as the *FOLR* cluster (GB4 position 262.5-263.4cR), *GAL*, however, does map to SSC2. The breakpoint of homology at HSA11q13 with SSC2 and SSC9 therefore could be narrowed down to this small interval between the *FOLR* cluster and *GAL*.

Lahbib-Mansais et al. (1999), describe discrepancies for SSC2p14-p17 between the bi-directional painting and the mapping of genes on the somatic cell hybrid panel. *CANX* was mapped to the SSC2p14-p17 while its human position is on HSA5q35. Based on the existing comparative map, this gene was expected to map on SSC2q. On the present RH map, *CANX* is located at a distance of 37.5 cR_{3000} from *AMH* (SSC2q21). Our results indicate that either the assignment of this gene to SSC2p14-p17 was incorrect or that the characterization of the cell lines for SSC2 in the somatic cell hybrid panel is inaccurate. We therefore conclude that up to now no internal rearrangements with segments from HSA5 have occurred on the short arm of SSC2.

Interestingly, the segment HSA11q13 – 11p15.4 is flanked on both sides by loci that map together on SSC9p. Besides *FOLR*, the genes for *UCP2/3*, and *TYR* on HSA11q13 (GB4 positions 271cR and 309cR, respectively) are also known to map to SSC9p (*UCP2/3*: Werner et al. 1999; Cepica et al. 1999; *TYR*: Chowdhary et al. 1994). On the distal tip of HSA11p,

HBB (36 cR) was also mapped to SSC9p24 (Pinton et al. 2000). This might indicate that the segment encompassing *HBB* and *GAL* was inverted in humans after the two species were separated from the common ancestor. Figure 2 describes a model for the evolution of HSA11 and SSC2p from a common ancestral chromosome. In the lineage leading to *Sus scrofa*, a chromosomal translocation might have occurred separating a part of the segment including *HBB* to SSC9p whereas in human a segment encompassing *GAL* and *HBB* was inverted later.

For SSC2q, the comparative picture showing the homology with HSA 19p13 and 5q3 could be extended. However, the conservation in gene order is less clear than for HSA11. Map construction was difficult for linkage group IV with a total of nine loci that could not be placed on the framework map. Combining groups IV and V to estimate the distance between the groups resulted in several rearrangements, e.g. moving *DNASE2* and *CNN1* to group V near *CSF2* and *IL4*. This indicates that the resolution of the panel is too low in this area. The estimated distance between C3 and S0010 was 114 cR_{3000}. The human map position of *PRDX2* on HSA 13q12 might define an additional area of homology with SSC2q. However, one STS from *PRDX2* was mapped to HSA19 on the GB4 map at 71-75 cR and another STS to HSA6 at 440 cR. Additionally, on the mouse map *PRDX2* is located near *DNASE2* on mouse chromosome (MMU) 8 (Fig. 1).

Microsatellite markers from BACs located on SSC2 are being developed and will be included in the QTL analysis. In total, this will considerably reduce the interval of the QTL for BFT located on SSC2.

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Chapter 5

Improving the comparative map of SSC2p-q13 by sample sequencing of BAC clones

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Abstract

To improve the comparative map for pig chromosome 2 and increase the gene density on this chromosome, a porcine BAC library was screened with 17 microsatellite markers and 18 genes previously assigned to pig chromosome 2. Fifty-one BAC clones located in the region of the maternally imprinted quantitative trait locus for backfat thickness were identified. From these BACs 372 kb was sample sequenced. The average read length of a subclone was 442 bp. Contig assembly analysis showed that every basepair was sequenced 1.28 times. Subsequently, sequences were compared with sequences in the nucleotide databases to identify homology with other mammalian sequences. Sequence identity was observed with sequences derived from 35 BACs. The average percentage identity with human sequences was 87.6%, with an average length of 143 bp. In total, sample sequencing of all BACs resulted in sequence identity with 29 human genes, thirteen human ESTs, seventeen human genomic clones, one rat gene, one porcine gene and nine porcine ESTs. Eightteen genes located on human chromosome 11 and 19, and seven genes from other human locations, one rat gene and one porcine gene were assigned to pig chromosome 2 for the first time. The new genes were added to the radiation hybrid map at the same position as the locus from which the BAC that was sequenced was derived from. In total 57 genes were placed on the radiation hybrid map of SSC2p-q13.

Introduction

A maternally imprinted quantitative trait locus (QTL) for backfat thickness (BFT) spanning a large region was detected on pig chromosome (SSC) 2 (Nezer *et al.* 1999, Jeon *et al.* 1999, De Koning *et al.* 2000, and Rattink *et al.* 2000). In addition, there are indications that more QTL for BFT are present on SSC2 (Rattink *et al.* 2000). In order to identify the genes responsible for the observed QTL effects on SSC2, fine mapping of this large region is necessary. The existing linkage maps for SSC2, however, contain very few genes and not many markers have been assigned physically (Archibald *et al.* 1995, Rohrer *et al.* 1996, the pig genome database http://www.thearkdb.org/pig). Conservation of genome organisation between species makes it possible to take advantage of genetically well-characterised species. Nevertheless, the extraction of relevant information from the human genomic sequencing effort is a challenge. Bi-directional chromosome painting revealed homology between porcine chromosome 2 and human chromosome 11 and 19 (Rettenberger *et al.* 1995,

Goureau *et al.* 1996). However, small chromosomal rearrangements and possible conservation in gene order between man and pig can not be observed with this method. A study to improve the comparative map of SSC2, based on mapping of genes and microsatellite markers on a radiation hybrid (RH) panel, has been described previously (Rattink *et al.* 2001). Based on this RH map, a more detailed comparative map with previously undetected rearrangements between SSC2, HSA11 and HSA19 was constructed. In order to increase gene density, porcine BAC clones from the QTL region were isolated and subjected to sample sequencing. The resulting porcine sequence data were compared with sequences in the public databases. In the present study, a total of 27 genes were added to the RH map of SSC2p-q13 at the location of the microsatellite or gene where the BAC sequencing originated from. Sample sequencing proved to be a very efficient way to rapidly improve the gene-density of medium density maps.

Material and methods

Sample sequencing of BACs. Screening of the porcine BAC library (Rogel-Gaillard *et al.* 1999) resulted in several BACs per locus for 18 genes and 17 microsatellite markers. Per locus, only one or two BACs were selected for sample sequencing. BAC DNA from a 1 ml overnight culture was digested with 10 U *Eco*RI or 10 U *Hin*dIII (Gibco) for 90 min., ligated into pTZ18R and transformed into competent *E.coli* (DH5α). For sample sequencing, DNA was isolated from 12 randomly selected subclones per BAC, per digestion (Qiaprep 96 miniprep kit; Qiagen). The cycle sequencing reactions were performed according to the manufacturer's instructions (Perkin Elmer, ABI), with 200 ng of plasmid DNA, 2 µl of Half Big Dye terminator (Genpak Ltd), 2 µl Big Dye Terminator Rtmix (Perkin-Elmer) and 1 µl of M13 forward or M13 reverse sequence primer (0.8 pmol/µl) in a total reaction volume of 10 µl. The excess dye terminator was removed by precipitation with 67% isopropanol. Sequencing products were run in a 96 well format for 7 hrs. on a 36 cm 4.75 % denaturing Long Ranger gel (FMC) on an automated sequencer (ABI377).

Database searches. All sequences were analysed using the PREGAP4 program of the STADEN software package (Bonfield *et al.* 1995, http:\\www.mrc-lmb.cam.ac.uk/pubseq). In PREGAP4, sequences were cleaned from vector and *E.coli* sequences as well as from sequences of low quality. In GAP4, contigs were constructed based on overlap between

sequences of subclones, with a minimal initial match of 20 bp, a maximum of 25 pads per read, and a maximum of 5% mismatches. Resulting sequences were subjected to BLAST database searches (nucleotide (GenBank, EMBL, DDBJ, and PDB sequences), EST and high throughput genomic sequences) in the Genbank database of the National Center for Biotechnology Information (NCBI), using the BLAST network client software (blastcl3, http://www.ncbi.nlm.nih. gov). To be considered a match, a minimal e-value of 1e-5 was necessary. Map locations of human genes were derived from Unigene (http://www.ncbi.nlm.nih.gov/Unigene) and mouse map locations from the Mouse Genome Database (http://www.jax.org). To obtain chromosomal location of ESTs, accession numbers were compared to the human genome working draft sequence database at NCBI. Details on the radiation hybrid panel and the construction of the radiation hybrid map were described previously (Rattink *et al.* 2001)

Results

Sample sequencing of BACs. A porcine BAC library (Rogel-Gaillard *et al.* 1999) was screened with primers from 18 genes and 17 microsatellite markers (35 loci in total) located on SSC2p encompassing the QTL region. In total, 51 BACs were identified, subcloned and sequenced. The total number of subclones sequenced was 1104, which were sequenced both in forward and reverse direction. All reads were processed into PREGAP4 for filtering and quality control, 1182 (53.5%) reads passed the quality measures of the program. Before contig construction, 522,684 bp were subjected to a BLAST database search. The average read length of the sequences that passed PREGAP4 was 442 bp (range 78 - 867).

In addition, all the good quality reads based on PREGAP4 analysis were used to construct contigs in the GAP4 program, taking into account possible overlap between both forward and reverse sequences of one subclone and overlap between subclones of one BAC. Out of the 1182 reads, 918 contigs with a total of 417,874 bp were assembled. The contig assembly analysis showed that in this experiment every basepair was sequenced 1.28 times.

BLAST database searches and sequence identity. The separate reads (1182) were submitted to a BLAST database search (nucleotide, EST and HTGS databases). Sequencing of BACs from 28 loci resulted in homologies with sequences in the databases (Table 1). In addition, BACs were identified for *MUC5AC, ADM, COMPII, R33609, ADRBK1, SW1564, and SWC9*. After sequencing of the BACs from these loci no sequence identities were detected in

the database searches. In total, for 68.6% of all BACs homologous sequences were found. No significant difference was observed between the number of hits through sample sequencing of subclones of BACs originating from a gene or from a microsatellite marker. In addition, from the 27 new genes that were detected in this study, 16 genes were detected from BACs originating from microsatellite markers and 11 genes from BACs originating from genes. The mean percentage identity with human sequences was 87.6% (range 72 - 100%), with a mean length of 143 bp (range 29 - 470 bp). Sequence identities with porcine ESTs ranged from 81% to 98%, with a mean of 89.3%. The mean length of observed sequence identity was 168 bp (range 40 - 355 bp).

Locus[1]	BAC	Accession[2]	BLAST hit	Accession[3]	bp homology	% identity	HSA[4]	RH GB4[5]	MMU[6]
RNH	71G08	BH021458	HRC1	M91083	34	97	11p15.5	20-22	
	71G08	BH021459	Hs.299034	AI768677	59	88	11*		
GAL	364A05	BH021460	Porcine EST	AJ236925	343	97	11*		
SW2623	996G10	BH021461	Human clone	AL023803	70	84	20q11.22-12		
SW256	234B08	BH021462	EMK1	NM_004954	71	97	11q13	232	19 (3)
SWR783	1089G04	BH021463	INCENP	NM_020238	79	83	11q12-q13	229	19 (0)
	1089G04	BH021464	Human PAC	AC005190	220	76	Xq23		
	226A03	BH021465	Porcine EST	BE013369	171	85	UN		
FTH1	978F05	BH021466	HS14_10305	AC004828	470	85	14*		
CNTF	52G02	BH021467	LDPL	NM_004811	96	86	11p11-q12	219	
	52G02	BH021468	Human EST	AL045404	88	86	11	218	
SO141	322F10	BH021469	SSRP1	NM_003146	92	92	11q12	200-214	2 (54)
	322F10	BH021470	HS11_9424	AI907163	72	88	11*		
SW575	1031B05	BH021471	CUGBP1	NM_006560	155	97	11p11	167-169	2
	1031B05	BH021472	Hs11_9135	AW043965	349	95	11*		
SWR1445	160D10	BH021473	KIAA0197	D83781	107	94	11		
	160E10	BH021474	KIAA1014	AB023231	330	90	11	169	
	160D10	BH021475	Hs.158902	AI378640	68	72	11		
SW1450	254F09	BH021476	HS11_9113	AA135852	228	78	11*		
	254F09	BH021477	Human PAC	AC005137	29	93	11p14.3		
	473G08	BH021478	PTPRJ	D37781	215	84	11p11.2	210	2
	254F09	BH021479	HS11_9563	AI611820	166	81	11*		
	254F09	BH021480	HS11_9113	BE069275	147	84	11*		
SW1201	616A05	BH021481	Human PAC	Z82196	161	85	22q12-qter		
	616A11	BH021482	NCOR1	NM_006311	68	100	11p11.2/17p11.2		
	616A11	BH021483	Human clone	AC002553	277	94	17		
CD59	729B10	BH021484	G2	U10991	80	88	11p13	125-128	
	729B10	BH021485	CD59	W48705	100	93	11p13	124-130	2 (55)
	729B10	BH021484	Porcine EST	AW786233	355	98	11*		
	729B10	BH021486	Porcine EST	AW480509	143	88	UN		
	729B10	BH021486	Porcine EST	BE032376	136	91	UN		
	729B10	BH021487	FBX3	AF176702	41	92	11	130	
	729B10	BH021488	Hs.99766	AW992904	128	82	11	129	
CAT	1039B07	BH021489	ELF5	AF115403	97	90	11p13-p15		
	1039B07	BH021490	Human clone	AL035079	200	83	11p12-13		
	1039B07	BH021491	CAT	NM_001752	97	87	11p13	128-130	2 (57)
BDNF	484E11	BH021492	Human YAC	AJ011601	199	87	11p14		
MYOD1	411D05	BH021493	KCNC1	S56770	264	88	11p15.4		7 (23.5)
	420C05	BH021494	Human PAC	AC004736	265	88	11p14.3		
	420C05	BH021495	MYOD1	AF027148	121	88	11p15.4		7 (23.5)
	420C05	BH021496	Human PAC	AC004582	269	82	11p14.3		
SW2167	861B11	BH021497	LDHC	NM_002301	143	92	11p15.5-p15.3	70	7 (23.5)
LDHA	980A06	BH021498	KIAA0872	AB020679	63	88	16	453	
	980A06	BH021499	LDHA	X03083	289	89	11p15.1-p14.1		7 (25.5)
	980A06	BH021500	Porcine EST	BE030523	40	92	UN		
RPS13	752C03	BH021501	PIK3CA	U79143	124	91	3q26.3		3 (12)
	752C03	BH021502	KCNMB3L	AP000365	79	89	22q11.2		
	753A08	BH021503	COX7RP	NM_004718	135	90	2	134	12
	753A08	BH021503	Porcine EST	BE032079	53	84	12*		
	753A08	BH021504	HS2_5565	AC006038	100	84	2*		
SW747	237A10	BH021505	TCRA	M87864	30	83	14q11.2		14 (19)
	237A10	BH021506	Human clone	AC002418	79	87	X		
	237A10	BH021507	Defb1 porcine	AF132038	53	94	8p23.2-p23.1	53	8 (9)
	237A10	BH021508	Porcine EST	BE032564	173	81	15*		

Locus[1]	BAC	Accession[2]	BLAST hit	Accession[3]	bp homology	% identity	HSA[4]	RH GB4[5]	MMU[6]
SW1857	432A09	BH021509	Hs.288368	AI638460	86	88	11	55	
	432A09	BH021510	HS11_18010	AI041524	51	90	11*		
	432A09	BH021511	HS11_18010	AA376285	128	84	11*		
PTH	419C11	BH021512	Human PAC	AC004225	79	86	5		
	419C11	BH021513	Porcine EST	AU059923	217	83	UN		
	419C11	BH021513	Human EST	AW812031	136	84	UN		
	137A05	BH021514	SRP9	NM_003133	234	83	1		
SWR1338	1075F05	BH021515	KIAA1218	AI792220	55	87	7q22-q31		
	1075F05	BH021516	Human BAC	AC005099	372	82	7q22-q31		
S0091	684G10	BH021517	Unc13h1	U24070	202	86	9p11-p12		
	684G10	BH021518	Human PAC	AC002036	58	87	11q23		
SW776	698A04	BH021519	PIASY	AF077952	62	91	19	31	
CANX	612D12	BH021520	Human BAC	AC005023	144	85	Xq25-q26		
SW1466	394F03	BH021521	CALR	NM_004343	69	92	19p13.3-p13.2	71	8 (37)
	394F03	BH021522	FARSL	AF042347	147	87	19p13.2	71	
CAPN1	172C11	BH021523	Human PAC	AC004540	196	89	7q15		

***Table 1**: BAC sample-sequencing hits and accession numbers.[1]primers originating from this locus used to screen the BAC library. [2]Accession number of the sequenced porcine BAC subclone. [3]Accession number of the hit. [4]human cytogenetic position. [5]human RH GB4 location. [6]murine chromosome and position on chromosome in cM between brackets.* Human position derived from comparison of EST accession number with the human genome working draft sequence database at NCBI.*

Sample sequencing resulted in sequence identity with 29 human genes, one rat gene (*Munc13-1*) and one porcine gene (*Defb1*). Sequence identity was observed with 19 human genes located on HSA11, three genes from HSA19 and with sequences from seven genes located on different human chromosomes. Finally, in four cases (*CD59, CAT, LDHA,* and *MYOD1*) a part of the same gene that had been used to screen the BAC library was identified. In those cases where sequence identity was observed with both a human gene and its murine ortholog, only the human gene is listed in Table 1.

With thirteen human ESTs sequence identity was detected; four human ESTs map to HSA11, eight were assigned to HSA11 through a human genome working draft database search and one EST has an unknown human position. In addition sequence identity with nine porcine ESTs was found. Finally, considerable sequence identity was observed with 17 human genomic clones from which six clones are located on HSA11, eleven clones map to other chromosomes. In total, sequence identities with 70 human, murine and porcine sequences was observed with sequences originating from 28 different BACs, derived from 13 genes and 15 microsatellite markers mapped to SSC2.

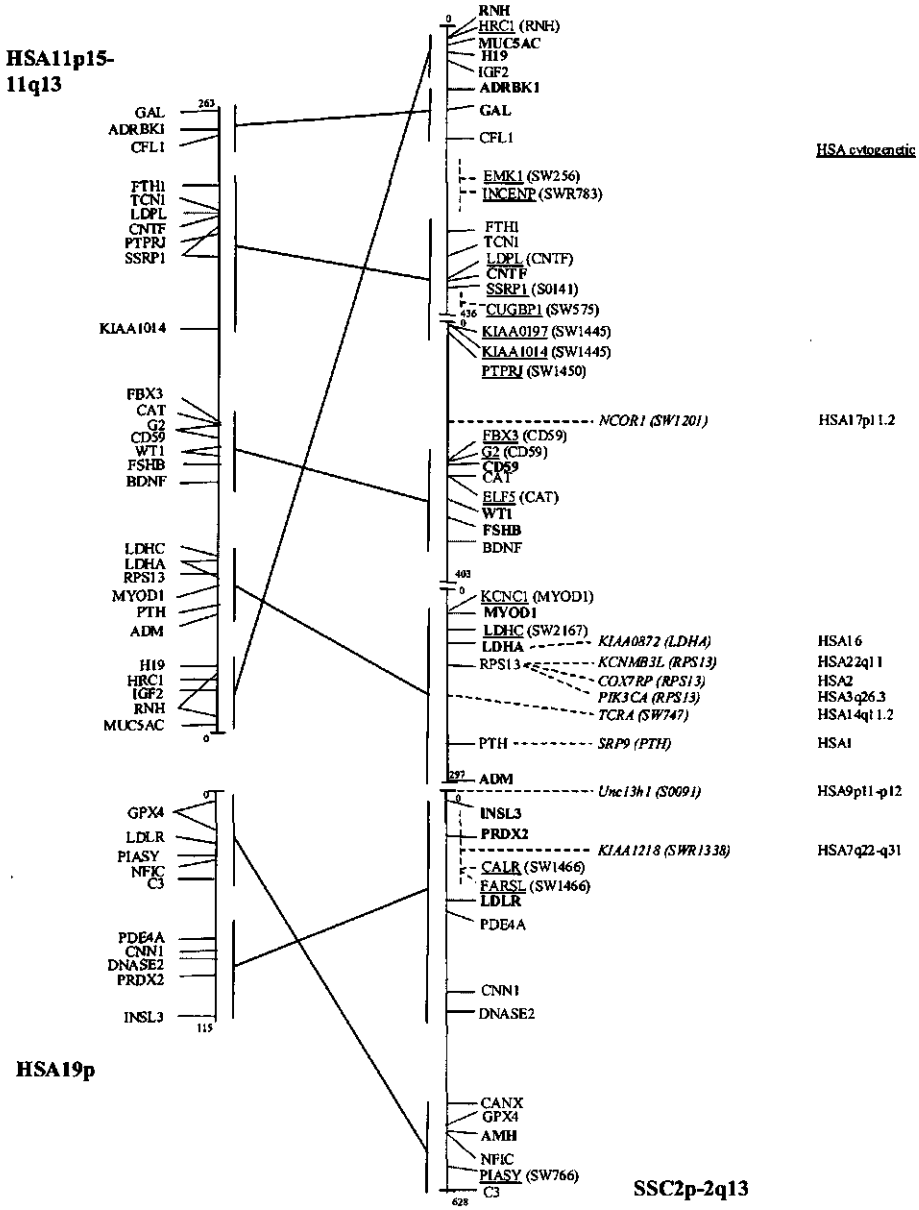

***Figure 1**: Comparative map showing gene order and rearrangements between HSA11, HSA19 and SSC2. The map of HSA11 is inverted (HSA11p15.5 is at the bottom, and HSA11q13 is at the top) to clarify the conservation in gene order between HSA11 and SSC2. Human RH positions were taken from the G4 RH map (http://www.ncbi.nlm.nih.gov/ genemap99/, in cR3000) and the porcine positions from the RH map for SSC2 (in cR3000) (Rattink et al. 2001). Loci indicated in bold represent framework markers on the RH map of SSC2. The new genes were added to the radiation*

hybrid map at the same position as the locus from which the BAC that was sequenced was derived from; underlined loci are newly assigned genes to SSC2 located on HSA11 or HSA19, that fit in the current comparative map. Loci in Italics refer to newly assigned genes from other human locations than expected from the current comparative map, with their cytogenetic position in man indicated on the right of the figure. Hits from loci that were not mapped on the porcine RH panel are indicated with a dotted bar and line at their most likely position based on existing linkage map (http://www.thearkdb.org/pig).

Previously, we (Rattink *et al.* 2001) presented a comparative map between SSC2, HSA11 and HSA19, based on genes mapped on the RH panel for SSC2. In Figure 1, the location of the 27 newly assigned genes identified by sample sequencing and the previously assigned genes are indicated. The new genes are added to the radiation hybrid map at the same position as the locus from which the BAC that was sequenced was derived from.

Discussion

Sample sequencing of BACs and BLAST database searches. In total, 51 BACs derived from 35 loci on SSC2p-q13 were analysed in this study. The aim of this study was to increase the number of mapped genes on SSC2 and not to intensively sequence a specific part of the chromosome. Therefore, only one or two BACs were selected for sample sequencing per locus. The average insert size of the clones in the BAC library was 135 kb (Rogel-Gaillard *et al.* 1999). Therefore, in a range of 4 to 6 Mbp of sequence from SSC2p-q13 was available for sequencing. From these BACs, almost 418 kb unique sequence was obtained. The length of pig chromosome 2 was estimated to be 168 Mbp (Rohrer *et al.* 1996), indicating in this study 0.25% of the entire chromosome and 0.75% of the region SSC2p-q13 was sequenced.

In this study, 1182 porcine sequences of good quality were compared to human, murine and porcine sequences in the databases. This resulted in 6.5% of the sequences detecting identity to a unique sequence (genes, ESTs and genomic clones) which is less than observed in mouse-human sequence comparison by Bouck *et al.* (2000). In that study, 4806 reads with on average 617 quality bases resulted in 440 regions of identity, which means that in 9.2% of the sequences a unique hit was observed. As expected, more hits were observed when the mouse sequences were compared to the database with unfinished genomic data. The average read of good quality sequence in that study is 621 bp (Bouck *et al.* 2000). The average read in our study (442 bp) and the total length sequenced (roughly 400,000 vs.

3,000,000) were lower. The amount of sequence identity observed, however, seems to be similar to the mouse-human comparison.

A number of different subclones showed sequence identity to several different parts of the same gene, further strengthening the significance of the observed sequence identity. For a BAC originating from *S0091*, sequence identity with the 191 bp of coding sequence of the rat gene *Unc13h1* was detected but not with the human ortholog of this gene. The proteins encoded by the rat and human genes have 94% similarity. The 191 bp region which is conserved between rat and pig, however, is lacking in the coding sequence of the human gene.

Predominantly, sequence identity is found between SSC2 and HSA11 and HSA19. In several cases, however, sequence identity with human genes located on other chromosomes was detected. This was observed for 12 BACs originating from microsatellites (6) and genes (6). The matches to human genes located on other chromosomes than HSA11 and HSA19 did not have shorter basepair homologies or percentage identity than the matches to genes on HSA11 and HSA19 (Table 1). However, based on the current comparative map for SSC2, these hits are not very likely. Alignment artifacts can be an explanation for the matches that are not consistent with the previous comparative data. Another explanation can be that homology is detected with a paralogous gene rather than the ortholog. Several hits are detected in this study to members of gene families. For example, *COX7RP* is part of the cytochrome-c oxidase family. At least one member is known to be located on HSA11q13 (*COX8*), but no sequence identity was observed with this family member. In addition, several members of this gene family have not yet been assigned and unidentified family members or pseudogenes may exist. Moreover, members that are present in man might have been lost during evolution in the pig lineage. Furthermore, the RH position in the pig or in man can be inaccurate, or previously undetected small rearrangements within large conserved segments might have occurred. Finally, we can not rule out the possibility that some of the BACs used in this study are chimeric. The BAC library used in this study contains around 4% chimeric clones (Rogel-Gaillard *et al.* 1999), indicating that in this study approximately two BACs are expected to contain chimeric inserts.

Comparative mapping between SSC2, HSA11 and HSA19. Studies have observed different gene orders within conserved synteny groups across species (Schibler *et al.* 1998, Johansson *et al.* 1995). In general, the comparative map between HSA11p and SSC2p-q13 shows a highly conserved gene order with exclusion of the small conserved segment between

HSA11p15.5 (*RNH, MUC5AC, IGF2,* and *H19*) at the tip of SSC2p (Figure 1). For HSA19p, several small rearrangements have occurred in comparison to SSC2. *PRDX2* is mapped to SSC2 on the RH panel, but has been physically assigned to HSA13q12 by FISH and on the RH panel to HSA19 (Rattink *et al.* 2001). In Figure 1, the position on HSA19 is indicated. In addition, *AMH* is physically assigned to HSA19p13.3-p13.2 but a location on the GB4 RH map is not available. As seen in Figure 1, a fair number of small intra- and interchromosomal rearrangements have occurred between SSC2 and HSA19p, assuming the SSC2 RH map is indeed correct. According to Carver and Stubbs (1997) small intrachromosomal rearrangements may be the rule rather than the exception. In addition, rodents have undergone more sequence changes than other mammals, and mammals more than other vertebrates (Andersson *et al.* 1996). However, the order of genes between HSA19p13.3 and mouse chromosome 10 is well conserved, only one inversion is observed in which the gene order is conserved as well (Puttagunta *et al.* 2000).

In total, 27 genes have been assigned to SSC2 by sample sequencing of BACs in this study. Sample sequencing resulted in refinement of the RH map of SSC2 and in improvement of the comparative map. In total 57 genes and 25 microsatellite markers are placed on the radiation hybrid map of SSC2p-q13. Further defining the position of the breakpoints of the conserved segments is underway. In addition, new microsatellite (Faivre *et al.* 2001) and SNP markers have been developed from SSC2p. The described detailed comparative map will improve the characterization of candidate genes for the backfat thickness QTL located on this chromosome (Rattink *et al.* 2000).

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Chapter 6

Refinement of the SSC2 – Human comparative map by assignment of eight porcine genes

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Abstract

Comparison of the porcine and human genome reveals homology between a large region of human chromosome 11 (HSA11p-q13) and the p-arm of pig chromosome 2 (SSC2p). Genes that are expected to map near the breakpoint between these conserved segments were mapped in pigs to determine the breakpoints more precisely. For the investigation of the borders of the conserved segments human cDNA sequences of genes located on HSA11 were used to identify porcine EST sequences that are expected to map on the pig near the breakpoints of the conserved segments between HSA11 and SSC2. Primers were designed and six genes were assigned to SSC2 on the somatic cell hybrid panel and SSC2 radiation hybrid map. Moreover, two genes were assigned to SSC9. In addition, human IMAGE clones were selected from these eight genes, and were hybridised to porcine BAC library filters to obtain porcine BAC clones. The BAC clones were subcloned and sample sequenced to identify additional homologies to human sequences, which resulted in the mapping of an additional three genes to SSC2. The mapping of eight genes resulted in refinement of the borders of the conserved segments to regions less than 4 Mb and in the detection of a new rearrangement in the comparative map between HSA11 with the porcine genome.

Introduction

Comparative mapping between human, mouse and domestic animal species improves the low-density map of animal species and in addition could lead to better understanding of the human genome. Several methods to improve the comparative maps have been applied to livestock species. Chromosome painting revealed conservation of synteny between man and pig, but without details on orientation, small rearrangements and precise breakpoints between the conserved segments (Rettenberger et al., 1995). With this method it is only possible to identify a region of a chromosome on which a gene of interest can be located. However, to be able to select candidate genes from the human genome based on comparative mapping information, the gene density in pig has to be improved. This will allow the comparison of gene orders within conserved segments and a more accurate identification of the borders of the conserved segments (Johansson et al., 1995, Schibler et al., 1998). To increase the number of genes mapped in the pig, several strategies can be followed. First, sequences of human genes can be compared with the available porcine sequence data to identify porcine genes that can be mapped in the pig. Second, BAC clones with known map location can be sample sequenced which subsequently

can be compared with human sequence data to reveal new homologies and to identify previously unassigned genes in the pig. Third, sequencing of cDNA clones will result in ESTs that can be assigned to the pig genome by various mapping techniques, such as radiation hybrid mapping, somatic cell hybrid mapping, cytogenetic mapping and linkage mapping (Fridolfsson et al., 1997, Lahbib-Mansais et al., 1999, Yerle et al., 1996, Hawken et al., 1999).

Comparison of the porcine and human genome reveals homology between a large region of human chromosome 11 (HSA11p-q13) and the p-arm of pig chromosome 2 (SSC2) (Rettenberger et al., 1995; Goureau et al., 1996). Genes that based on the human map are expected to map in pig near the breakpoint between these conserved segments can determine the breakpoints more precisely. Previously, the screening of a porcine BAC library with microsatellite markers and genes located on SSC2 already resulted in the improvement of the comparative map of this chromosome (Rattink et al., 2001b). However, the number of described microsatellite markers and genes on SSC2 is limited. To further investigate the borders of the conserved segments eight genes were assigned to SSC2. The genes were selected based on the location of the orthologous genes in man on HSA11. In addition, screening of a porcine BAC library with human IMAGE clones for those eight genes identified BAC clones that were subsequently sample sequenced.

Materials and Methods

Mapping of genes on the SCH and RH panel. From HSA11q13 eight genes were selected; *INPPL1, DHCR7, PC, ACTN3, MEN1, and PLCB3. APBB1*, and *AMPD3* were selected from HSA11p15 (Table 1). Development of primers on porcine sequences homologous to these selected human genes, characteristics of the somatic cell hybrid (SCH) panel and the radiation hybrid (RH) panel and the construction of the map of SSC2 have been described previously (Yerle et al. 1996, Rattink et al. 2001a). Briefly, PCR results were analysed using the website http://www.toulouse.inra.fr/lgc/pig/pcr/pcr.htm to obtain a regional assignment of the BACs. BACs that based on the SCH panel mapped to SSC2 were subsequently typed on the RH panel (Research Genetics, Huntsville, Ala., USA) and integrated in the existing RH map of SSC2 (Rattink et al. 2001a, Figure 1). PCR products were sequenced to verify the inserts. In addition, primers were designed on selected subclones of all the BACs to map them on the porcine RH panel and SCH panel.

Hybridisation of BAC filters with human IMAGE clones. Clones containing the human genes *INPPL1, DHCR7, GSTP1, PC, ACTN3, MEN1, PLCB3, FKBP2, CARS, APBB1*, and *AMPD3* were obtained from the IMAGE consortium (Lennon et al., 1996). Clones were plated and grown overnight at 37^0C on LB-agar plates containing ampicillin (conc 100 µg/ml). Single colonies were grown overnight in a 4 ml LB medium with freezing medium and ampicillin. DNA was isolated from the clones according to the IMAGE consortium DNA isolation procedure. The DNA was digested with the appropriate restriction enzymes for three hours at 37^0C in a total volume of 10 µl, containing 350 ng DNA, 1 µl of the appropriate buffer and 10 units of each restriction enzyme. Insert size was verified on a 0.8% agarose gel (110 V, 3.5 hours). In case a different insert size than expected was observed, the clone was plated again and the procedure was repeated. Hybridisation conditions were tested using Southern blots with human and pig DNA (Meishan and Dutch lines).

The BAC library filters were obtained from the HGMP Resource Centre, Hinxton, United Kingdom. The total set consists of six filters representing a 4.7 fold genome coverage. The probes were radioactively labelled with P^{32} by random priming (RediprimeII, Amersham Pharmacia Biotech, Little Chalfont, England) and hybridised to the BAC library filters. An additional precipitation step was performed before the probes were added to the blots. The filters were pre-hybridised in Church buffer for one hour at 60^0C, and hybridised overnight at 60^0C in Church buffer. The blots were washed three times for 20 minutes with Wash buffer I (1XSSC, 0.1%SDS) at 60^0C, three times with Wash buffer II (0.1XSSC, 0.1%SDS) at 60^0C for 20 minutes and subsequently subjected to autoradiography. All BACs are spotted twice on the BAC filters and only those that displayed hybridisation to both spots were indicated as positive.

Sample sequencing of BACs. Sample sequencing of positive BACs has been described in detail previously (Rattink et al 2001b). In short, BAC DNA was digested with *Eco*RI or *Hin*dIII, ligated into pTZ18R and transformed into competent *E.coli*. For sample sequencing, DNA was isolated from 12 randomly selected subclones. Sequences were subjected to BLAST nucleotide, EST and high throughput genomic database searches, using the BLAST network client software (blastcl3, http://www.ncbi.nlm.nih. gov).

Results

Mapping of genes on SSC2. Primers were designed on porcine EST sequences that had a high sequence identity with the selected genes on HSA11 (Table 1). All genes were typed on the SCH panel. Six genes (*ACTN3*, *AMPB3*, *DHCR7*, *MEN1*, *PC* and *PLCB3*) were assigned to SSC2 and subsequently typed on the RH panel (Figure 1). Typing of *APBB1* and *INPPL1* on the SCH panel resulted in assignment to SSC9p21-p24.

Gene	Primer sequence		Product length	Temp (°C)	Accession number
ACTN3	CAAGAACTACATCACTGCTGAGG	CGTAGAGGGCACTGGAGAAG	146	65	AJ301019
AMPB3	CATTGTTGGAGCCAGGATCT	AGCCAATCAGAGGCTGAAAC	134	60	BG835121
APBB1	GTGAGCAGTGGACACCGAGT	ATGATGAACGCAAAGGTGTG	151	60	BE235360
DHCR7	GAATCGGGAAGTGGTTTGAC	TGCAGGATGTTGACCAAGAC	148	65	AW786254
INPPL1	GCAGTGCCTCTCTGCCTATT	GCGGCTCTAAAAGGTGAGG	102	60	BG384897
MEN1	GACCTCTCATCCGACCCTTT	TCATCTTCCTCGCAACTGAA	152	62	BG732325
PC	TCGGATGTGTACGAGAACGA	AGGGTGTCACCTTGATGAGG	154	62	BI339495
PLCB3	TGAGTCGGTCAACTCCATCC	GCTGCTCCTGACACTCCTG	149	62	BG835750

Table 1. *Description of genes typed on the SCH and RH panel. Primers were designed from porcine sequence (Accession number)*

Hybridisation of BAC filters with human IMAGE clones. IMAGE clones from the eight genes were obtained from the IMAGE consortium. Four of these IMAGE clones were rejected because of a wrong insert size (*AMPD3* and *DHCR7*) or poor hybridisation to the test Southern blots (*PLCB3* and *INPPL1*). The remaining four IMAGE clones were hybridised to the pig BAC library filters. To verify the presence of the gene on the BACs, a PCR reaction was performed with the primers of the porcine sequence homologous to the genes. Six BACs for the four genes containing the porcine ortholog of the IMAGE clone were identified; *APBB1* (PigE BAC 108J06), *ACTN3* (PigE BAC 047P07 and 086L20), *MEN1* (PigE BAC 178M07 and 227E11), and *PC* (PigE BAC 245J15) (Table 1). In addition, to verify the location of the BACs, they were typed on the SCH panel and subsequently on the SSC2 RH panel. All BACs for *ACTN3, MEN1* and *PC* were assigned to SSC2 very close to the location of the porcine genes on the RH map.

Locus[1]	IMAGE clone	BAC	Subclone	Accession[2]	BLAST hit	Accession[3]	bp homology	% identity	bp	HSA[4]	RH GB4[5]
ACTN3	628357	PigE BAC 086L20	P013H08R	BH608541	Porcine EST	AW359546	80	83	524	UN	
		PigE BAC 047P07	P013E04Q	BH608542	Porcine EST	AW483450	113	94	120	UN	
		PigE BAC 047P07	P013B05R	BH608543	Porcine clone	AC087180	177	86	356	UN	
APBB1	2141200	PigE BAC 108J06	P013B10R	BH608544	Human BAC	AC084337	39	89	404	HSA11p15.5	
MEN1	1992296	PigE BAC 178H07	P014F03Q	BH608545	MEN1	U93237	183	93	603	HSA11q13	247.35
		PigE BAC 178H07	P014E03Q	BH608546	RASGRP2	XM_006536	287	91	625	HSA11q13	238.35
		PigE BAC 227E11	P015A01Q	BH608547	Porcine EST	BE030713	337	93	173	HSA11q13*	
		PigE BAC 227E11	P015A01Q	BH608547	MEN1	U93237	90	87	173	HSA11q13	247.35
		PigE BAC 227E11	P015B04R	BH608548	Human EST	BE145576	35	94	506	UN	
		PigE BAC 227E11	P015C02Q	BH608549	Human EST	AW505360	377	86	598	HSA11q13*	
		PigE BAC 227E11	P015C05R	BH608550	Murine BAC	AC006956	529	86	698	UN	
		PigE BAC 227E11	P015D02Q	BH608551	ZNF162	XM_006534	157	85	607	HSA11q13	240.19
		PigE BAC 227E11	P015G01Q	BH608552	Porcine EST	BF192278	106	96	561	HSA11q13*	
		PigE BAC 227E11	P015G01Q	BH608552	PYGM	U94775	132	88	561	HSA11q12-q13.2	238-247
PC	2209982	PigE BAC 245J15	P014D09Q	BH608553	Porcine EST	AW480283	127	85	554	UN	
		PigE BAC 245J15	P014G08Q	BH608554	Porcine EST	BE231963	300	92	621	UN	
		PigE BAC 245J15	P014B09Q	BH608555	Human BAC	AP001319	146	88	625	HSA11q13	

Table 2. *Sample sequencing hits from obtained through BLAST database search. [1]human IMAGE clone originating from this locus was used to screen the BAC library. [2]Accession number of the sequenced porcine BAC subclone. [3]Accession number of the hit. [4]human cytogenetic position. [5]human RH GB4 location. [6]murine chromosome and position on chromosome in cM between brackets. * Human position derived from comparison of EST accession number with the human genome working draft sequence database at NCBI.*

Sample sequencing. To increase the number of identified genes on SSC2 and further improve the comparative map, the five BACs located on SSC2 and the SSC9-derived BAC positive for *APBB1* were sample sequenced and BLAST database searches were performed. Homology with human and murine sequences was obtained for all BACs (Table 2). Sample sequencing of a BAC for *MEN1* (PigE BAC 227E11) resulted in homology with three genes located on HSA11 (*RASGRP2, ZNF162,* and *PYGM*). Three genes were added to the RH map of SSC2 at the position of the locus from which the sequenced BAC was derived (Figure 1). On the sequence map of HSA11, all four genes are mapped within a 100 kb region. Homology to the human gene *MEN1* was detected for both BACs positive for the human IMAGE clone of this gene.

In total, homology with three genes, one human EST and two BACs located on HSA11 was observed. In addition, homology with two other human ESTs, one murine BAC, six porcine ESTs and one porcine clone was detected for which no location in the genome was available. However, for one human EST and for two porcine ESTs a human location on HSA11 could be derived. A BLAST database search with the ESTs in the high through put genome sequence (HTGS) database resulted in high sequence identities of the ESTs with sequences located on HSA11.

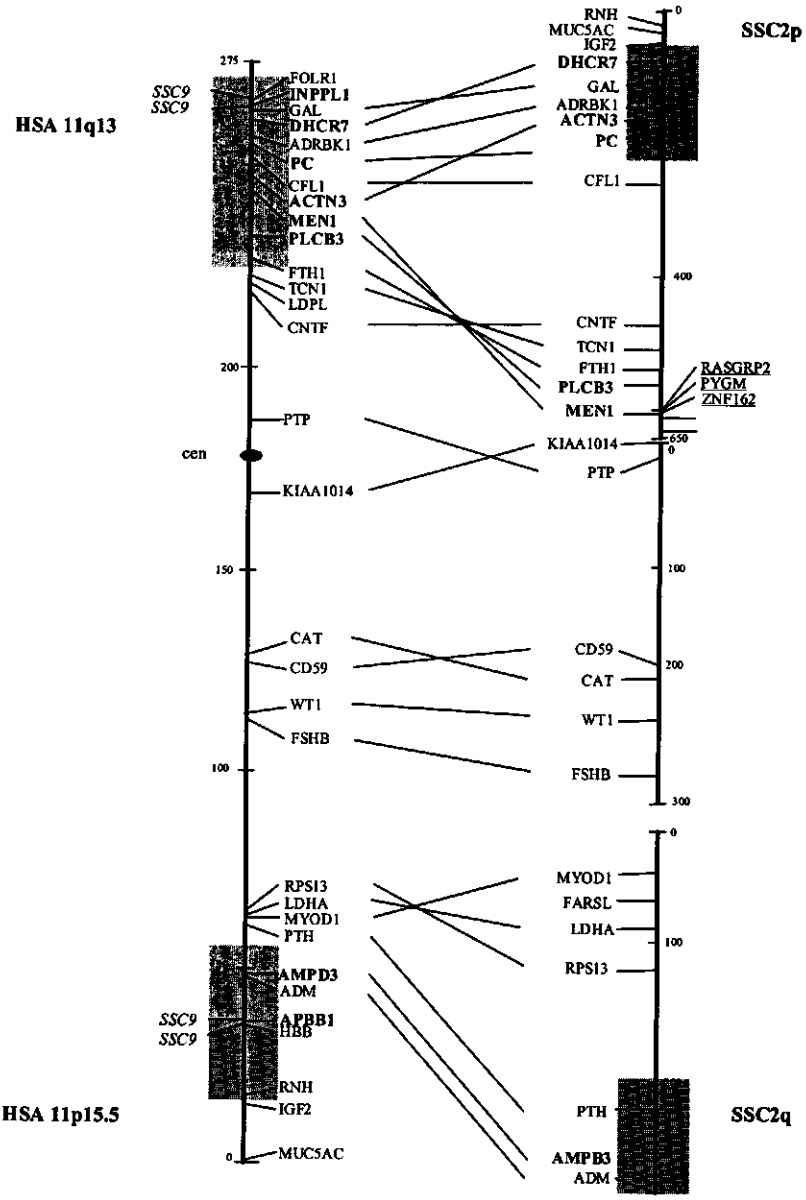

Figure 1. *Selected genes and IMAGE clones from HSA11 and their position on the GB4 radiation panel and porcine RH panel. The map of HSA11 is inverted (HSA11p15.5 is at the bottom, and HSA11q13 is at the top) to clarify the conservation in gene order between HSA11 and SSC2. Human RH map positions were taken from the G4 RH map (http://www.ncbi.nlm.nih.gov/ genemap99/, in cR3000) and the porcine positions were taken from the RH map for SSC2 (in cR3000) (Rattink et al. 2001a). Loci indicated in bold represent genes mapped in this study on the RH map of SSC2. Underlined loci are newly assigned genes to SSC2 located on HSA11. They are placed at the same position as MEN1.*

Discussion

Boundaries of conserved segments. For the investigation of the borders of the conserved segments between HSA11 and SSC2 human cDNA sequences of genes on HSA11 were used to identify porcine EST sequences that are expected to map on the pig near the breakpoints of the conserved segments. Six genes were assigned to SSC2, which resulted in a further improvement of the comparative map between SSC2 and HSA11p-q13.

On the RH map of SSC2 *GAL* and *IGF2* are positioned 34.2 cR apart. In man, however, *GAL* (GB4 RH 262.72 cR_{3000}) and *IGF2* (GB4 RH 16.4 cR_{3000}) are 246 cR apart. Based on the current comparative map between HSA11p-q13 and SSC2p the borders between the conserved segments are expected to be located in man between *FOLR1* and *GAL* on one site and *RNH* and *ADM* on the other site (Rattink et al., 2001, Figure 1). In pigs, *GAL* maps to SSC2 (radiation hybrid position) whereas *FOLR1* is located on SSC9 (Yerle et al., 1996). In this study *INPPL1* was also mapped to SSC9. This indicates that the breakpoint is located is between *INPPL1* and *GAL*. INPPL1 is positioned at 72.9 Mb on the human sequence map. *GAL* is not on the HSA11 sequence map, but *DHCR7*, the next gene in this comparative map is located at 72.2 Mb. The breakpoint therefore should be located within this region between these genes, which spans less than 1 Mb.

In the chromosomal region of *RNH* and *ADM* during evolution, another breakpoint occurred compared with SSC2. In addition, a rearrangement of a small fragment containing at least two genes between man and pig is detected in this region. *APBB1* was assigned to SSC9p21-p24 in this study. Previously, *HBB* was mapped to SSC9p24 (Pinton et al., 2000). On one side the breakpoint has occurred between *APBB1* and *ADM*, which are approximately 4 Mb apart. On the other side the breakpoint is within a block of 3 Mb between *HBB* and *IGF2*.

Hybridisation of BAC filters with human IMAGE clones. Hybridisation of the porcine BAC library resulted in six BACs for four genes (*APBB1, ACTN3, MEN1* and *PC*). However, several other BACs were identified after hybridisation that could not be verified for the porcine genes homologous to the original human IMAGE clones. To see whether hybridisation with gene family members with high sequence identity occurred in stead of hybridisation with the expected gene, these BACs were mapped on the SCH panel to obtain a porcine chromosomal location (Data not shown). Based on the current comparative map a syntenic region in man was derived for these BACs. For some BACs it became clear that hybridisation to gene family members with high sequence identity occurred, but for others no described family members

were detected. For example, four BACs identified with the human IMAGE clone for *APBB1* were mapped to porcine regions which are syntenic to regions on HSA22, HSA1q42.1-q43, HSA6, HSA16 and HSA14. In these regions, no known members of the amyloid beta A4 precursor protein binding family are reported. On the other hand, one BAC that hybridised to the human IMAGE clone of *ACTN3* was mapped to SSC7q12-q23 or SSC7q26. The homologous regions in man are HSA14 and HSA15, respectively. On HSA14, the gene *ACTN1* is located, which has 83% sequence identity with *ACTN3* and also belongs to the alpha actinins belong to the spectrin gene superfamily which represents a diverse group of cytoskeletal proteins. Most likely, the BAC contains *ACTN1* in stead of *ACTN3*.

The technique using human probes to screen a porcine BAC library is not described in detail in literature. For all four genes positive BACs were obtained that were verified for the presence of the orthologous porcine gene and for their location on SSC2. However, in some cases hybridisation of the IMAGE clone with a BAC harbouring a porcine gene family member with high sequence identity to the IMAGE clone could explain the mapping of the BACs to other chromosomes than SSC2. However, not in all cases such an explanation could be found. This indicates that cross-species hybridisation of BAC libraries as described in this experiment is not very efficient.

Sample sequencing of subclones of the BACs of *MEN1* resulted in the assignment of three new genes to SSC2: *RASGRP1, ZNF162*, and *PYGM*. Three genes were added to the RH map of SSC2 at the position of the locus from which the sequenced BAC was derived. The BACs for *APBB1*, *MEN1* and *PC* showed sequence identity with genes, BACs, and ESTs positioned at HSA11, that all fit in the present comparative map of SSC2 with HSA11p-q13.

Concluding, nine genes and five BACs were placed on the RH map of SSC2 for further improvement of the comparative map of SSC2 and HSA11p-q13 and two genes were assigned to SSC9. This resulted in a more accurate identification of the borders of the conserved segments. Furthermore, a new rearrangement was detected between HSA11 and the porcine genome.

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Chapter 7

General Discussion

This chapter describes the contribution of the main findings of this thesis to the field of animal genetics and the consequences of this work for animal breeding. The chapter consists of four sections. In the first section, the results of the QTL study presented in chapter 2 and 3 are discussed and compared with published results obtained from studies with other pig mapping experiments. In addition the approaches to detect genes that influence important production traits in livestock are discussed. The second section describes the imprinting effects in the pig as described in chapter 3 in relation to the increasing knowledge of imprinting in man. In the third section, the construction of the high resolution map of SSC2 (Chapter 4, 5, and 6) and the comparative mapping efforts with the human and mouse genomes to identify candidate genes based on location and biological function are discussed. In addition, background information on the genetics of human obesity in general and on HSA11 and HSA19 in particular are provided in this section. In the last section, the main conclusions of this thesis and directions for further research are presented.

Chapter 7.1 QTL analyses

Several studies to identify genes involved in fatness and growth traits in pigs have been performed (reviewed by Rothschild, 1998). In the regions on SSC2, SSC4 and SSC7 where QTL for fatness traits were detected (Chapter 2 and 3) several other groups also found QTLs for growth or fatness traits. For SSC7 QTLs for fatness and growth traits with a cryptic allele have been reported (Chapter 2). On the q-arm of SSC4 the suggestive QTL for intramuscular fat detected in this thesis is located in the same region as the QTL for mean fat depth detected by Andersson et al. (1994) which was confirmed for several growth and fatness traits in later studies (Chapter 2). No effect for backfat thickness was observed on SSC4 in the experimental cross presented in this thesis. For SSC2, a paternally expressed QTL for muscle mass and fat deposition was mapped to the region containing the *IGF2* locus at SSC2 (Jeon et al., 1999; Nezer et al., 1999). They found that *IGF2* is imprinted and expressed exclusively from the paternal allele in several tissues in pigs. In addition, a suggestive QTL for fatness traits was detected on SSC2. It is located at the extremity of the p arm close to the *IGF2* locus. In a cross of Large White and Meishan pigs this QTL explained 1 to 2% of the phenotypic variance (Bidanel et al., 2001). In another large QTL experiment (Malek et al., 2001a), twenty significant QTLs at the 5% chromosome-wise level for the different traits associated with back fat thickness were detected, but none of these are located on SSC2. For SSC2, however, in that

study only three markers for the first 71.3 cM of the chromosome were typed. A QTL for tenderness at the distal part of the SSC2 approached the 5% genome-wise significance level (Malek et al., 2001b). For none of these studies a functional mutation in a gene explaining the observed differences in the QTL studies or even a strong candidate gene is reported.

Several candidate genes in the pig with expected function in the fat metabolism were screened for association with fat related traits. Leptin is a hormone that influences the quantity of food consumed relative to the amount of energy expended and mutations in the leptin gene are associated with an increased fat deposition in humans (Friedmann and Halaas, 1998). If leptin biology is similar for livestock species and human and rodent species, it is clear that it may have a major impact on the performance and well-being of livestock species (Houseknecht et al., 1998). Only suggestions of a possible association between a polymorphism at position 3649 of the leptin gene and fatness in pigs was detected by Jiang and Gibson (1999) but the evidence is not conclusive. Linkage disequilibrium with another mutation seems a more likely explanation for the association, because the mutation is not in the translated region of the gene and thus does not affect the protein structure. *MC4R* is a signalling molecule involved in the regulation of energy homeostasis that responds to leptin signalling. Several mutations in *MC4R*, including frameshift and nonsense mutations, are associated with inherited obesity in humans. In pigs, a missense mutation was identified in the *MC4R* gene on SSC1, and a significant association with backfat, growth rate and feed intake was detected (Kim et al., 2000). Fatty acid binding proteins (FABP) are intracellular proteins that transport fatty acids from the cell membrane to sites of fatty acid oxidation or phospholipid or triacylglycerol synthesis. *A-FABP*, located on SSC4, showed no linkage with a QTL for intramuscular fat. Suggestive and chromosome wise significance with a QTL affecting intramuscular fat on SSC6 was detected for *H-FABP*, but not for backfat thickness (Gerbens et al., 2000). This QTL on SSC6 was found to be highly significant under the multiple QTL analyses by De Koning (2001), with *H-FABP* located within the confidence interval. For all other candidate genes, no connection with in literature described QTLs on the chromosomes where the candidate genes are located, could be made.

Candidate gene approach versus positional cloning

Two approaches can be followed for the identification of genes involved in fatness traits in the pig: QTL analysis as described in this thesis and the candidate gene approach. For QTL analysis, no prior biochemical or physiological understanding of the trait is needed. A detailed marker map is mandatory for this exercise (Bouchard et al., 1998). Many genes that each have a

small effect determine the polygenic trait fatness. It is difficult to reach the significance levels for genes with small effects in a single QTL study, thus mainly genes with large effects will be detected (Barsh et al., 2000). In the total genome scan approach a risk is the detection of an individual locus that may be linked to the trait only in that specific family or cross (Warden and Fisler, 1998). Once a trait has been located in a chromosomal region through QTL mapping, identifying the underlying gene remains a significant problem. Up to now, only a few studies did indeed identify a mutation in a gene explaining the observed QTL effects (Riquet et al., 1999; Milan et al., 2000).

The candidate gene approach makes use of the investigation of functional or non-functional mutations or polymorphisms in genes with an expected impact on a certain trait (Bouchard et al., 1998). In the candidate gene approach it is very hard to determine whether a candidate gene has indeed the functional alleles that explain the differences in phenotype, or whether nearby loci have the true functional effects and the candidate gene is a marker (Warden and Fisler, 1998).

In practice a combination of the two described approaches is applied. This co-called positional candidate approach relies on a combination of mapping to a chromosomal subregion followed by a survey of the interval to see if attractive candidates reside here (Wolff, 1997). Based on a chromosomal region underlying a trait detected in a QTL study a positional candidate gene can be selected. This is an expressed gene present in the chromosomal region that shows some preliminary characteristics consistent with a role in the trait examined (Warden and Fisler, 1998).

QTL analyses have revealed several interesting chromosomal regions explaining the differences in production traits in livestock. In addition, studies applying the candidate gene approach for the identification of genes involved in complex traits have resulted in a number of associations of genes with these traits. But most of the candidate genes identified based on biological function are not located in the described QTL regions. This is referred to as the 'polygenic paradox' whereby evidence for an important role of a protein in regulation of a phenotype does not necessarily implicate the underlying gene as a QTL (Pomp, 2001). It has to be noted though that the significance thresholds that are used in both approaches are not the same. For a candidate gene, association is only determined by testing at a specific locus. In QTL analysis across the entire genome significance is tested, making this multiple testing more accurate than association testing at one locus. Even though differences in approaches of detecting genes is quite different and is providing conflicting evidence, it does not implicate that the biology and physiology of the traits is different, but more that the statistics applied are

different. In addition, several other factors can cause this paradox. Publication of only significant QTLs can cause a publication bias. Moreover, a QTL can also influence loci on other places in the genome than the QTL region. In general, QTLs indicate large effects on the trait. Candidate genes, however, are usually representing small effects based on current knowledge of the trait.

Chapter 7.2 Imprinting

For the QTL detected on SSC2 an imprinting analysis was performed (Chapter 2 and 3). A genome wise significant paternally expressed QTL on SSC2 with the best position at 63 cM was described. A maternally imprinted QTL on SSC2 with a major effect on muscle mass and fat deposition (Nezer et al., 1999 and Jeon et al., 2001), and a paternally expressed QTL for teat number are also located on SSC2 (Hirooka et al., 2001). Besides these QTLs and the imprinted QTLs described in Chapter 2 and 3 of this thesis, no imprinted QTLs have been described in pigs. The *IGF2* locus is imprinted in pigs (Nezer et al., 1999), however for other genes it is not known. In man, much more research is performed on imprinted genes and imprinting mechanisms. Based on comparative mapping the QTL region for backfat thickness on SSC2 is homologous to HSA11p-q13 and HSA19p (Chapter 2, 4, and 5). Comparison of imprinted regions in several mammalian species will provide more insight in the evolution of arrangements and clusters of imprinted genes. This can lead to identification of porcine candidate genes through human homologs with location that fits in the comparative map between human and pig and imprinting features (Reik and Walter, 2001). On HSA11 several imprinted genes are located, but on HSA19p as yet no imprinted genes were reported.

Imprinting on human chromosome 11

On HSA 11 one large imprinted cluster of genes is located. In addition other regions on this chromosome are subjected to imprinting. The cluster on HSA11p15.5 contains at least 10 imprinted genes. The paternally imprinted genes in this cluster are *IPL/TDAG51, IMPT1, CDKN1C, KVLQT1, TAPA1/CD81, H19* and *ASCL2/HASH2*. The genes *IGF2*, *INS2* and *IGF2AS* are maternally imprinted. In addition, the gene *2G3.8* gives rise to multiple transcripts that mostly show biallelic expression, but at least one transcript is imprinted. The two most investigated imprinted genes are *H19* and *IGF2*. *H19* is a developmentally regulated gene with putative tumour suppressor activity. Expression of *H19* results in a non-protein-coding fetal RNA. The gene is expressed in a number of organs during fetal development and in adult

skeletal and cardiac muscle (Bartolomei et al., 1991). The maternally imprinted gene *IGF2* encodes insulin-like growth factor II. The gene is inactive on the chromosome inherited from the mother in most normal tissues. Loss of this imprinting results in the production of a double dosage of active *IGF2* gene product. This overexpression contributes to overgrowth of many somatic tissues, which can result in tumours. Although the neighbouring genes *H19* and *IGF2* share an enhancer, *H19* is expressed only from the maternal allele and *IGF2* only from the paternal allele. The region of paternal-specific methylation upstream of *H19* appears to be the site of an epigenetic mark that is required for the imprinting of both *H19* and *IGF2*. There are more than 80 identified disease genes mapped to HSA11. Among these, for only one disease it is confirmed that imprinted genes play a role. *CDKN1C* and *IGF2*, involved in the Beckwith-Wiedemann Syndrome (BWS), are sometimes mutated or show loss of imprinting.

Imprinting and fat

Besides the *IGF2* locus on HSA11, there is only one locus in the genome with known role in the fat metabolism with imprinting effects: the Prader-Willi syndrome (PWS) locus on HSA15q11.2-q12. PWS is defined by upper-body obesity, short stature, mental retardation and hypogonadism (Kopelman, 2000). It is caused by a deletion of the parental chromosomal segment. The differential expression of the disease depends on whether the deletion is inherited from the mother or from the father. A deletion on the maternal chromosome results in another syndrome, the Angelman syndrom. This disease includes most of the same features as PWS, but not obesity (Bouchard et al., 1988). This indicates that apparent candidate loci that might explain the imprinting effects observed for the QTL for backfat thickness on SSC2 are not yet described in literature.

Chapter 7.3 A high-resolution map of SSC2 and comparative mapping

In this section the improvement of the genetic map of SSC2 is described. Followed by a discussion on the advantages and limitations of comparative mapping between SSC2, and the homologous regions in man and mouse. This comparative map provides a starting point for the search for genes involved in fatness in pigs. Therefore, an overview of human obesity research is given and subsequently the loci involved in fatness located on HSA11 and HSA19 are described. The relevance of these findings for the QTLs for fatness in pigs is discussed.

Bi-directional chromosome painting revealed homology between SSC2 and HSA11, HSA19, and HSA5 (Rettenberger et al., 1995, Goureau et al., 1996). As described in Chapter 4, 5, and 6, several small rearrangements have been detected comparing HSA11, HSA19 and SSC2. In addition, conservation in gene order was reported (Chapter 4 and 5) which could not be detected by chromosomal painting. By using Radiation Hybrid (RH) mapping, the comparative map can be studied at a much higher resolution. RH mapping makes use of a panel of somatic cell hybrids, with each cell line containing a random set of fragments of irradiated porcine genomic DNA in a hamster background. Markers are typed on each cell line of the panel. The results are recorded as a vector indicating the presence or absence of the markers in each of the cell lines. Linkage between markers is then calculated based on the degree of resemblance of the vectors. In the RH map of SSC2, Type I markers are included in addition to the species specific Type II markers (Chapter 4). Through the use of Type I markers, the RH map can be used to compare gene order within conserved segments between species. QTLs are often located by linkage mapping on species-specific maps. By using RH maps that include both Type I and II markers, homologous chromosomal locations for QTLs of specific traits can be identified in other species. This conservation of genome organisation between pig, man and mouse makes it possible to take advantage of genetically well-characterised species for the selection of candidate genes for, for example, the imprinted QTL on SSC2.

Comparative mapping between SSC2 and homologous regions in human and mouse

The radiation hybrid map of HSA11 consists of more than 30,000 unique gene-based markers typed in either the GB4 or GB3 RH panel. The panels contain human DNA fragments of average size of ~10 Mb or ~3Mb respectively, allowing markers to be ordered to approximately 1 Mb or 0.5 Mb (Gaudray et al., 1999). HSA11 is estimated to contain roughly 1700 genes. The major gene rich regions on the chromosome are 11p15, 11p13, 11q13, and 11q23. The comparative map between HSA11 and the mouse genome is based on approximately 450 genes whose homologues have been mapped in the mouse, on mainly four chromosomes: MMU2, MMU7, MMU9 and MMU19 (Gaudray et al., 1999). The mouse chromosomal region homologous to the entire human 11p15 imprinted domain is contained in a single syntenic block on mouse chromosome 7 (Onyango et al., 2000). Even between species that are very distantly related, like man and Pufferfish (Fugu), conserved synteny is observed. Miles and co-workers (1998) demonstrated conserved synteny between Fugu and 1.5Mb of HSA11q13.

The estimated number of conserved segments between mouse and man is 529 +/- 84. The mean length of a conserved segment is 2.8 cM (Kumar et al., 2001). Since the pig and man diverted later in evolution from each other than mouse and man, larger conserved segments can be expected between man and pig. The comparative map between SSC2, HSA11 and HSA19 described in Chapter 4 and 5, indicates large conserved segments with conservation in gene orders. However, a more intensive study of the borders of these segments already indicated that in these regions more small rearrangements and differences in gene order are present (Chapter 6). If the assumption is made that the genes are evenly distributed across the chromosome, the region of HSA11 that is homologous to SSC2 would roughly contain 1100 genes. Most of these genes will also be present in pig, both species being vertebrates. In this thesis, the mapping of approximately 75 genes is described, and in literature additional 50 genes are reported to be located on SSC2. This means that the current comparative map presented in this thesis encompasses 10% of the actual genes located on SSC2. Thus apart from what is observed so far for SSC2, it is likely that more detailed research of the large conserved segments of SSC2 will reveal a substantial number of small rearrangements not described in this thesis. These can be intrachromosomal rearrangements resulting in loss of gene order, or interchromosomal rearrangements, resulting in homology to other human chromosomes than HSA11 and HSA19. This indicates that selection of candidate genes from other species based on the current comparative map can be difficult and should be interpreted with caution.

Human obesity research

Selection of candidate genes based on chromosomal location or function in another species requires a well-determined definition of the trait of interest. The fatness traits described in this thesis are difficult to compare with similar traits in other domestic animals, since physiological differences in fat and meat metabolism and differences in selection pressure between species are observed. The traits in humans most comparable to fatness in pigs are obesity-related traits. In man, besides the physiological differences, fatness traits are measured in an another way than in pigs. Moreover, obesity is defined as excess weight, whereas the traits backfat thickness and intramuscular fat in pigs are based on variance in normal levels of fat. In addition, obesity in man is measured as a body mass index, including the weight and length of a person. This includes all fat depots in the body. Therefore it would be more comparable to porcine backfat thickness than to intramuscular fat. If we want to use the physiological and genetic data based on obesity research in humans for understanding the porcine fat metabolism and the selection of candidate genes for QTLs affecting fatness in pigs, we have to keep in mind

these differences in both species. Therefore, human data can not directly be translated to the pig. But at present, since the human genome project and the human obesity research field are much more developed than in pig, it is useful to explore these databases and extract relevant information to the porcine genome.

In man, obesity is determined by an interaction between genetic, environmental and psychosocial factors, acting through the physiological mediators of energy intake and expenditure (Kopelman, 2000). If the food intake and energy expenditure balance is dysregulated, extra food intake does not activate the feed-back mechanism to elevate whole body metabolism and additional fat is accumulated. Obese patients have an increased relative risk for diabetes mellitus. Ninety percent of the patients that have the form of diabetes called mellitus noninsulin-dependent diabetes mellitus (NIDDM) are obese (Albu and Pi-Sunyer, 1998). Obesity is an example of a disease in which relevant genes mediate susceptibility to the disease in a specific environment. Thus the environment remains a critical factor in any effort to elucidate the genetic bases for susceptibility to obesity and in determining what genes will be identified (Wolff, 1997). Heritability for obesity in humans varies between 10% and 80% (Warden and Fisler, 1998). Besides obesity research in man, several rodent models are available for the study of obesity. Rodent models have provided insight in physiology, interaction between genetic factors and the role of environment in the development of obesity.

Human and rodent obesity genes are detected in several studies: Mendelian disorders, single-gene rodent models, QTL from cross-breeding experiments, and association and linkage studies (Bouchard et al., 1998). Mendelian disorders show that single genes with large effects are important in the development of obesity, but only in a very small fraction of the obese population. Single-gene rodent models identified several genes that cause obesity in rodents (Bouchard et al., 1998). The genes best characterised are *ob*, *bd*, *fat*, *tub*, and *agouti*. Obesity of the *ob/ob* mouse begins to develop soon after birth and is associated with a decrease in brown adipose tissue thermogenesis and an enhanced insulin activity. The gene regulating this process is uniquely expressed in white adipose tissue and produces leptin (York and Hansen, 1998). Leptin is a hormone that is primarily made and secreted by mature adipocytes and binds to its receptor in the hypothalamus (Gregoire et al., 1998). During starvation, circulating leptin levels fall, thus activating a behavioural, hormonal and metabolic response that is adaptive when food in unavailable. Weight gain increases plasma leptin concentration and elicits a different respons, leading to a state of negative energy balance. Leptin deficiency, seen in *ob/ob* mice, is associated with an increase in fat deposition. (Friedman and Halaas, 1998). The diabetes mouse

(*bd/bd*) inherits its obesity as an autosomal recessive trait from a mutation in the leptin receptor gene (York and Hansen, 1998). The *fat* gene in mice has been shown to code for carboxypeptidase E (CPE). A single mutation reduces the activity in both the pancreatic islet and pituitary. CPE is required for cleavage of two arginine residues from the b chain of insulin during its processing from proinsulin. A mutation in the *CPE* gene results in very high levels of proinsulin rather than insulin in the circulation (York and Hansen, 1998). The *tub/tub* mutation is associated with distinct sexual dimorphism in blood glucose, serum insulin, all changes being more pronounced in male mice (York and Hansen, 1998). The *agouti* mouse shows dominant inheritance of obesity and is characterised by moderate obesity and high incidence of tumor growth. Increase of intracellular calcium levels may promote insulin resistance. The search for mutations in humans in the orthologs of *ob*, *db*, *fat*, *tub*, *agouti* and others has resulted in the identification of mutations in these genes in man, but only a small proportion of the genetic variance of obesity can be explained by these genes (Chagnon and Bouchard, 1999).

Most QTLs for obesity related traits are detected in animal models. Advantages are the possibility to keep environmental factors and the genetic background constant and the very dense gene maps and high homology between the human and rodent genomes. However, the question remains whether the genes detected in these animal studies have orthologs in man and whether they have the same location and function. Moreover, the confidence interval of the QTLs are often very broad which makes is difficult to identify the human locations (Barsh et al, 2000).

Linkage analysis can be performed with candidate gene markers or with anonymous polymorphic markers. It refers to the co-segregation of a marker and a trait locus within families (Bouchard et al., 1998). The Pima Indians of central Arizona have the highest reported rates of obesity in the United States and are frequently used in linkage studies to detect major genes involved in obesity (Krosnick, 2000). A large number of genes linked to obesity have been reported, but only for a few genes strong evidence for linkage was presented. Moreover, in most cases linkage with these genes could not be confirmed in other populations.

Association studies showed evidence for associations between candidate genes and obesity related phenotypes. In this type of studies a correlation between a genetic polymorphism with a phenotype is investigated (Bouchard et al., 1998). This can be performed in samples of unrelated individuals. In addition, gene-gene and gene-environment interactions have been reported. In several studies interactions between genes influencing obesity is described. For example, in a cohort study interactions between two polymorphisms in two genes, *ADRA2A* and *ADRB3*, were reported to influence abdominal and subcutaneous fat (Ukkola et al., 2000). In

addition, several studies reported the lack of association between candidate genes and obesity related traits (Perusse et al., 2001). This indicates that even though many genes associated with obesity have been detected, the effects of these genes are relative small. The selection of genes to test for association is based on current knowledge on obesity. Many more genes with small effects will be present, but to detect major loci, a total genome scan will be more efficient.

Summarising, in four years time the number of genes, markers, and chromosomal regions that have been associated or linked with obesity phenotypes in man and animal models increased from 74 in 1996 to more than 250 in the year 2000 on all chromosomes, except the Y chromosome (Perusse et al., 1997 and Perusse et al., 2001). Reported Mendelian disorders with obesity as one of their clinical manifestations that have been mapped increased from 10 to 24. Single gene mutations increased from 5 genes (*db, fat, ob, tub, a^y*) to 6 genes (*mg*). In total, 115 different QTLs from animal models were reported in 2000 compared to 24 in 1996. The map positions of obesity loci identified by quantitative studies do not correspond to classical obesity mutations such as *ob, tub* or *fat* (Barsh et al, 2000). Only thirteen studies reported positive associations with candidate genes in 1996. This increased dramatically to 130 positive associations with 48 candidate genes in 2000. Genome scans and other linkage studies resulted in 59 loci linked to obesity indicators in 2001, whereas only twelve linkage studies were reported in 1996.

Based on the number of loci associated with human obesity it is obvious that it is a very complex trait. As mentioned before in this chapter, the use of this very intensively investigated human obesity field, raises the question what relevance it can have for understanding fat metabolism in the pig and more specifically, for identifying the genes explaining the QTL effects for backfat thickness on SSC2. Human obesity research can help to determine the direction and give clues for further porcine research, but emphasis should be on better understanding of the fat traits in pigs themselves and improvement of the genetic map of the pig. Even in man, only a few genes explaining the observed differences in the QTL studies have been detected so far. It remains very difficult to narrow down the QTL region and identify the functional mutations underlying the QTL effects. Positional candidate genes can be identified in the QTL region and screened for functional mutations explaining the observed QTL effects, but with the current large QTL region, this is a daunting task. This will be an important challenge for the coming years in obesity related research, in addition to gene-gene and gene-environment interactions.

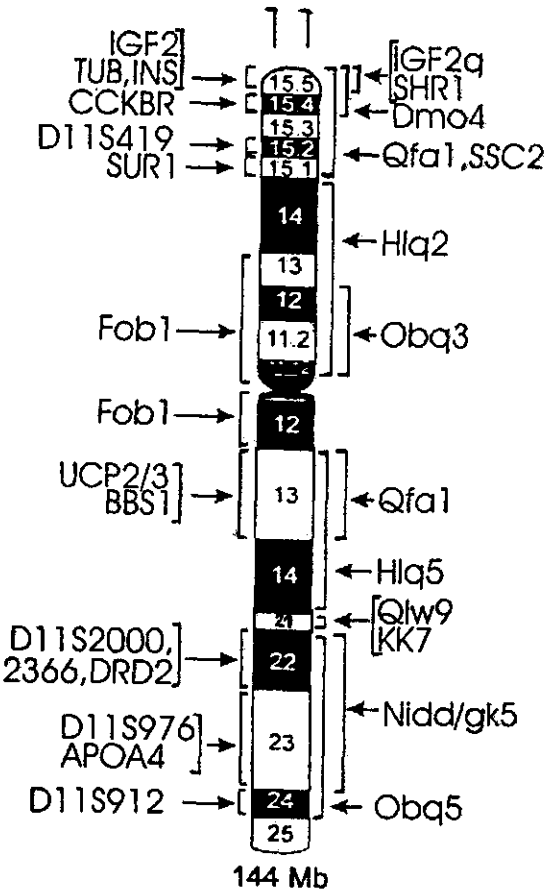

Figure 1. The obesity gene map of human chromosome 11 incorporating all putative obesity related loci from single-gene mutation rodent models of obesity, human obesity cases due to single-gene mutations, QTLs from crossbreeding experiments and genome-wide scans, all relevant Mendelian disorders and genes and markers that are associated or linked with an obesity phenotype. (Source: Perusse et al., 2001)

Fat related loci on HSA11p-q13

All loci on HSA11 related to obesity traits are indicated in Figure 1. One single-gene mutation (*TUB*) associated with obesity has been reported to be located on HSA11. The mouse ortholog of this gene has been investigated in depth. The *tub/tub* mouse is an autosomal mutation 2.4 cM distal to the *hbb* gene (York and Hansen, 1998). The mutation causes maturity-onset obesity. The human gene is located on HSA11p15.5. The *tub* gene is mainly expressed in the brain. It is suggested that the function of the *tub* gene has similarity with the *PCSK1* gene, which is the site of a mutation in the *fat/fat* mouse (Sahly et al., 1998).

Tub could also be a neuropeptide involved in neurophysiologic and endocrine functions regulating feeding behaviour (Sahly et al., 1998). Santagata and co workers (2001) demonstrated that tubby functions in signal transduction from G protein-coupled receptors and the regulation of gene expression.

The Bardet-Biedl syndrome 1 (BBS1) located on HSA11q13 is a Mendelian disorder characterized by mental retardation, pigmentary retinopathy, polydactyly, obesity and hypogentitalism. BBS1 has certain features, like obesity and retinal degradation, in common with the tubby phenotype, but probably another unidentified gene located near *TUB* is causing this syndrome.

Several QTL studies in different species identified loci homologous to loci on HSA11 in relation to obesity and diabetes related traits. The mouse chromosome 2 QTL (Obq10) for adiposity index (the sum of the weight of the three major fat pads divided by body weight) has a peak near the obesity QTL Obq3 previously identified in the AKR/C57L intercross (Taylor and Phillips, 1997). These QTLs are similar in that gonadal fat percentage is most strongly affected, and males are more affected than females. Obq3 exhibited a broad peak suggesting the possibility of linked loci, while Obq10 shows a relatively sharp peak, consistent with a single locus (Taylor et al., 2001). The syntenic region in man is HSA11p12-p11. In a polygenic obesity mouse model resulting from a long-term selection experiment a QTL on mouse chromosome 2 (Fob1) for percentage fat at 14 weeks maps to the region of Obq3 (Taylor and Phillips 1997). The inheritance of both these QTLs were found to be additive, raising the possibility that a common locus might be involved (Horvat et al., 2000). A QTL (Hlq2) influencing heat loss was identified on mouse chromosome 2 (Moody et al., 1999). The homologous region in man is HSA11p14-p11. Heat loss is an indicator of energy expenditure and maintenance energy requirements (Moody et al., 1999). In a rat strain that was used to identify the genes involved in susceptibility to NIDDM in relation to obesity, a QTL for Body Mass Index (BMI) in females was detected on rat chromosome 1, which is homologous to HSA11p15. This QTL Qfa1 is homologous to the human region were candidate genes such as *TUB*, and *IGF2* are located (Chung et al., 1997). In a backcross of a rat strain that is a model for obese NIDDM, a highly significant QTL (Dmo4) for fat weight and adipose index was detected on rat chromosome 1 (Watanabe et al., 1999). This region of rat chromosome 1 is homologous to HSA11p15.5-p15.4. In a backcross between diabetic and spontaneously hypertensive rats SHR1 a QTL for body weight was detected on rat chromosome 1 (syntenic region in man is HSA11p15.5), in the region flanked by *Igf2*. A QTL affecting adiposity and weight at 10 weeks, Adi3, on mouse chromosome 7 has significant linkage at the genome wide level and only

affects males. There are many candidate genes in this region that may be responsible for observed differences in adiposity, including *TUB, FIT1, PTH,* and *UCP2* and *UCP3* (Cheverud et al. 2001).

For 5 genes located on HSA11 an association with obesity is described (Perusse et al., 2001). *SUR1, IGF2,* and *INS* map to SSC2 and are candidates for the QTL for backfat thickness described in this thesis (Chapter 2 and 3) and are therefore described here briefly. *UCP2* and *UCP3* are also located on HSA11, but map in pig to SSC9 and therefore can be excluded as candidate genes for the QTL.

The sulfonylurea receptor (SUR1) is a member of the ATP binding cassette superfamily which senses changes in ATP and ADP concentration, affect K channel activity and thereby modulate insulin release (Aguilar-Bryan et al., 1995). In morbidly obese families a mutation in the *SUR1* gene was associated with morbid obesity and Type II diabetes. Obesity and NIDDM share some metabolic abnormalities and are often associated, suggesting that shared susceptibility genes might exist (Hani et al., 1997). The *SUR1* gene is located on HSA11p15.1. A polymorphism in the *IGF2* gene is associated with BMI in middle-aged man (O'Dell et al., 1997). The gene is expressed during prenatal development and is imprinted. The imprinting features of this gene have been described previously in this chapter. A deletion in the imprinting control region of *IGF2/H19* leads to a biallelic decrease in expression of *Igf2*, but not *H19*, and increased fat deposition and obesity of transgenic mice. This suggests that *Igf2* may affect fat metabolism (Jones et al., 2001). Insulin (INS) is synthesised by the beta cells of the islets of Langerhans and consists of two polypeptide chains. Both chains are derived from the proinsulin locus, located at HSA11p15.5. Proinsulin is converted to insulin by enzymatic removal of a specific segment. Mutations in the *INS* gene are linked with NIDDM (Olansky et al., 1992). Evidence for linkage between *INS* and obesity related traits such as BMI (O'Dell et al., 1999), Birth weight (Dunger et al., 1998) and Waist to hip ratio (WHR) in obese women (Weaver et al., 1992) was also found.

Fat related loci on HSA19p

One Mendelian disorder is described to be located on HSA19p13.3-p13.2. The insulin resistance syndromes (IRS) consists of a cluster of disorders and biochemical abnormalities and has been given the name Syndrome X or the deadly quartet. The characteristics of this syndrome are amongst other things NIDDM, central obesity, hypertension (Cefalu, 2001)..

Four QTLs were detected in model organisms in regions syntenic to HSA19p. Obq1, a QTL affecting adiposity index in mouse was mapped to the central region of mouse

chromosome 7 (Taylor and Phillips, 1996). The homologous region in human is HSA19q13.2-q13.3. Bw6f, a QTL for 6-week weight in mouse located on mouse chromosome 7 (homologous to HSA19q13), explains 9% of the variance for body weight in two lines divergently selected for body weight from a base population of a cross between two inbred strains (Keightley et al., 1996). In a mouse F2 intercross a QTL for late weight gain, Olw9, was detected on chromosome 9 (Cheverud et al., 1996). The homologous region in man is HSA19p13. Dmo5, a QTL involved in fat weight and adipose index detected on rat chromosome 3 (HSA19p13.2-q13.3) was described by Watanabe et al. (1999) in a backcross in a strain that is a model for obese NIDDM.

Association between two genes on HSA19p and obesity is reported. Firstly, an association between the insuline receptor gene (*INSR*) in hypertensive men with obesity was described (Zee et al., 1994). *INSR* is located on HSA19p13.3-p13.2. Several transcriptional initiation sites were identified in the gene, resulting in several transcripts. Mutations in either the structural gene or some of the processing steps may lead to insuline resistance. It is suggested by Taira et al. (1989) that mutations of the *INSR* gene that cause slightly decreased affinity of the receptor for insulin in combination with obesity might cause diabetes. Secondly, in several studies an association between the low-density lipoprotein receptor (*LDLR*) and BMI in hypertensives and obesity was detected, indicating that the *LDLR* gene has influence over adipose tissue deposition (Zee et al., 1996). *LDLR* is a transmembrane glycoprotein that brings ligands from the extracellular environment into the cell for degradation. It plays an important role in cholesterol homeostasis. The gene is mapped to HSA19p13.1-p13.3.

Relevance to porcine fat QTL

In man, on every autosomal chromosome loci associated with obesity are described. In particular on HSA11 and HSA19 multiple candidate genes are located that could explain the observed QTL effects on SSC2 in the experimental pig cross. Some candidate genes are more obvious to be involved in the fat metabolism in pigs than others, but all have to be taken into account until the QTL region on SSC2 can be narrowed down to a much smaller region. *IGF2* is a strong candidate gene, since a paternally expressed QTL affecting muscle mass and fat deposition and a paternally expressed QTL affecting skeletal and cardiac muscle mass were mapped to the *IGF2* locus (Jeon et al., 1999; Nezer et al., 1999). It can not be excluded that *IGF2* alleles described by Nezer and coworkers (1999) are not segregating in our cross. But based on the location of the imprinted QTL for backfat thickness on SSC2 (Chapter 2 and 3),

and the lack of transmission distortion at the microsatellite marker within the IGF2 gene in the analysis, it is likely that a different locus on SSC2 explains the QTL effects in our studies.

The radiation hybrid map and the comparative map of SSC2 presented in this thesis are helpful for the first rough estimate of the syntenic regions in man. However, since the comparative map between man and pig in general and for SSC2 in specific is still of a low resolution and the number of genes mapped on SSC2 is too low. It is difficult to select candidate genes at this point. The comparative map for selecting candidate genes from other species should be improved and more emphasis should be on improvement of the knowledge of the porcine genome itself. Large EST sequencing projects and ultimately the sequencing of the entire porcine genome will lead to more understanding of fat related processes in the pig.

Besides the contribution of human obesity research to the understanding of the QTLs for fat in livestock species that has been discussed in this chapter, crossbreeding experiments of pig, cattle and chicken can be of value for the study of human obesity. There are many advantages to the use of animal models. First, the phenotype can be controlled and more easily defined by inbreeding and ability to control the environment. Second, endpoints can be measured directly and more precise. Third, obesity can be induced in animals and young preobese animals can be studied. Finally, the availability of genetic models and the use of transgenics are providing insights into gene products that cause or reverse obesity and into the mechanisms of these effects. However, just like the concern of relevance of human obesity research to animals, it is also not clear-cut what the relevance of animals to human obesity can be (York and Hansen, 1998). For example, it is difficult to compare the phenotypes backfat thickness and intramuscular fat with obesity. Obesity is defined as excessive fat deposition and weight, whereas the porcine fat traits are variations in normal levels.

Chapter 7.4 Conclusions and directions for further research

The typing of additional markers as described in Chapter 2 and 3 did narrow down the regions of the QTLs, but not as sufficiently as needed for identification of the underlying gene(s). In the experimental cross that was used to identify the QTLs, the number of informative meiosis is fixed and additional markers will not greatly improve the fine mapping of the QTL region. To indeed narrow down the region more radically, more offspring need to be generated by making more generations in which the blocks of DNA that will be passed on to their offspring will be smaller and the animals carrying the smaller QTL region can be identified. At one of the breeding companies descendants of the Meishan X Large White F2

animals are still present. If these animals can be traced and the pedigree can be followed, these animals might be used for Identity By Descent (IBD) analysis to see whether some of these animals still carry the QTL and that might enable us to further narrow down the regions containing the identified QTLs.

QTL analyses

Besides the descendants of the cross, the haplotype of the animals can be investigated to identify the combination of alleles of the markers in a small segment of the QTL region that the animals carrying the favourable QTL allele have in common. In the animals carrying the allele for the QTL, the phase of the microsatellite markers can be investigated to see whether a common set of alleles is present, which can be looked for in the descendants. This haplotype analysis can only be used in a relative small segment of the QTL region. The question is, however, whether the QTL is still present in the animals descending from the cross, since these animals have undergone selection in the breeding company for the last eight years. If however all this is present, the animals can be typed for several markers within the QTL region. Either a new cross can be made with these animals or IBD analysis can be performed to obtain a smaller QTL region. Hopefully, this region can be as small as one or a few cM. This region can be screened for candidate genes based on possible function in the fat pathways and in addition a search for not yet detected genes can be made. Candidate genes can be screened for functional mutation that can explain the differences detected in the QTL analysis and verify these mutations in unrelated populations. Intensive investigation of the region with BAC contigs and intensive sequencing in combination with comparative mapping can lead to a full understanding of the genes located in this region and to the discovery of new genes.

In a project related to the work described in this thesis, the maternally imprinted QTL region on SSC2 is being saturated with single nucleotide polymorphism (SNP) markers. The SNPs were developed by sequencing PCR products from genes located on SSC2. Currently, these SNP markers are being used for detailed haplotyping of animals in the QTL cross. This can lead to identification of a small haplotype that the animals carrying the favourable QTL allele have in common. In addition, flow-sorting of SSC2 DNA from Meishan and Large White animals will result in the development of SSC2-specific SNPs that have different alleles in both breeds.

Microarrays

With the availability of large amounts of sequences of genomes it is possible to begin to understand how genes influence the downstream processing of RNA and proteins and thus the physiology of a species, and how the genes in the genome interact with each other. To study large sets of genes simultaneously microarrays are very helpful. A microarray is made by mechanically depositing double stranded PCR products from cloned cDNA libraries onto coated glass microscope slides. The sample spot sizes in microarray are typically less than 200 microns in diameter and these arrays usually contain thousands of spots on one slide. Two different samples, e.g. disease tissue and normal tissue, are hybridised to the same array and a ratio of the expression level between these two samples is calculated.

It has become clear from literature that array experiments can improve the knowledge on the role of genes in adipocyte tissue during prenatal and postnatal development. Identification of novel candidate genes involved in the pathophysiology of obesity will result from studies with DNA arrays containing clones from fat cDNA libraries. In a recent study by Gabrielsson and co-workers (2000), genes previously not reported to be expressed in adipose tissue were detected. Another study that used DNA microarrays to identity differences in gene expression in adipose tissue from lean, obese, and obese-diabetic mice was performed by Nadler et al. (2000). They found that the expression of genes normally associated with adipocyte differentiation were down-regulated in obese mice and large increases in gene expression were observed in genes coding for cytoskeletal and extracellular matrix proteins.

For pigs, a starting point to identify the genes involved in adipocyte tissue that can lead to the identification of candidates for the QTLs for fatness described in this thesis, can be a study of the expression of genes in fat and muscle tissue in postnatal piglets. However, porcine fat or muscle tissue microarrays are not yet available. Even though these arrays are not obtainable, a first experiment can be hybridisation of porcine RNA from fat tissue to human arrays. This will give a first indication of which genes are upregulated or downregulated in fat tissue in piglets. These genes can be candidates for the QTL detected on SSC2 based on their expression profile indicating involvement in fat development, and based on their chromosomal location.

Comparative map

To create a high resolution comparative map of SSC2 for the selection of candidate genes and to make pig-specific microarrays, many more Type I markers need to be mapped in the pig. Also species specific markers in QTL regions need to be developed for fine mapping of

the large QTLs. Human ESTs can be used to obtain a high-resolution comparative map by RH mapping. In addition, porcine EST assignments are important for a better characterisation of how the pig and human genomes are related. Not until many more porcine ESTs are mapped comparative positional candidates can be selected from the wealth of information on human genomic data. In the US, several groups are producing numerous ESTs for various tissues and are developing DNA array technology in pigs.

Bioinformatics

In general, production of data by large sequencing projects and mapping experiments as indicated above must be managed in an accessible way. Data acquisition and data integration in bioinformatics need to be improved. It has to be insured that existing databases can communicate with databases of other species to accelerate the comparative gene mapping effort. In addition, databases should contain the physiological functions of genes, mutations, common description of traits, orthologous genes and other information that could be used by researchers of other livestock species.

Definition of traits

In human obesity research a large number of different traits are related to obesity. However, the definition of obesity is not clear-cut. To use the human obesity research efforts for selecting candidate genes for the QTLs for fatness traits in pigs described in this thesis, it needs to be clear what human traits can be best compared to intramuscular fat and/or backfat thickness in the pig. As mentioned previously in this chapter, if this is possible and candidate genes are selected based on comparative mapping, these genes can have a different biological function in pigs. At least, to compare the traits better descriptions on how the traits are measured should be included.

One of the aims of this thesis was to identify genetic markers linked to separate genes controlling backfat thickness and intramuscular fat, enabling animal breeders to select separately for the two traits. In the QTL analyses presented in Chapter 2 and 3 on SSC2 a chromosomal region with effects for backfat thickness is detected and on SSC4 a QTL effecting intramuscular fat but not backfat thickness is described. In the same experimental cross on the X chromosome one genomic region is detected that has an effect on both intramuscular fat and backfat thickness (Harlizius et al., 2000). This indicates that in the different pig breeds different genes that influence intramuscular fat and backfat thickness are segregating in several

combinations. It is important to further investigate the genes involved in both intramuscular fat and backfat thickness and the genes involved in the separate traits. This can lead to the development of genetic tests to evaluate the genetic potential of individual animals, which can be included in breeding programs. In addition, the biological functions of these genes in the fat metabolism and the biological factors that are important in both types of fat production and in the separate fat types can be determined.

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Summary

In recent years, genome research in livestock animals such as cattle, pig and chicken has increased enormously. The discovery of highly polymorphic microsatellite markers in the livestock genomes and the completion of the sequencing of the human genome have a high impact on animal genome research. This has led to the construction of genetic linkage maps in farm animals and subsequently in a large number of linkage studies, with the aim to localise genes involved in all sorts of production traits of these species. This thesis deals with the identification of genes controlling fatness in the pig and in particular genes controlling intramuscular fat and backfat thickness. Markers linked to Quantitative Trait Loci (QTL) controlling intramuscular fat and backfat thickness in the cross between Meishan x Large White breeds will lead to the identification of the underlying genetic causes.

QTL for fatness traits were reported in an experimental Meishan x Large White and Landrace F2 cross. In **Chapter 2**, a set of 25 additional markers from these regions were typed on 800 animals (619 F2 animals, their F1 parents and F0 grandfathers) to further investigate the regions on SSC2, SSC4 and SSC7. Compared to the published maps, a modified order of markers was observed for SSC4 and SSC7. QTL analyses were performed both within the half-sib families as well as across families (line cross). Furthermore, a QTL model accounting for imprinting effects was tested. Information content could be increased considerably on all three chromosomes. Evidence for the backfat thickness QTL on SSC7 was increased and the location could be reduced to a 33 cM confidence interval. The QTL for intramuscular fat on SSC4 could not be detected in this half sib analysis, whereas under the line cross model a suggestive QTL on a different position on SSC4 was detected. For SSC2, in the half-sib analysis a suggestive QTL for backfat thickness was detected with the best position at 26 cM. Imprinting analysis, however, revealed a genome wise significant paternally expressed QTL on SSC2 with the best position at 63 cM. The results suggest that this QTL is different from the previously reported paternally expressed QTL for muscle mass and fat deposition on the distal tip of SSC2p.

The role of imprinting in body composition was investigated in the experimental cross. The whole-genome scan described in **Chapter 3** revealed significant evidence for five QTL saffecting body composition, of which four were imprinted. Imprinting was tested with a statistical model that separated the expression of paternally and maternally inherited alleles. For back fat thickness, a paternally expressed QTL was found on SSC2, and a Mendelian-expressed QTL was found on SSC7. In the same region of SSC7, a maternally expressed QTL affecting

muscle depth was found. Chromosome 6 harboured a maternally expressed QTL on the short arm and a paternally expressed QTL on the long arm, both affecting intramuscular fat content. The individual QTL explained from 2% up to 10% of the phenotypic variance. The known homologies to human and mouse did not reveal positional candidate genes. This study demonstrates that testing for imprinting should become a standard procedure to unravel the genetic control of multifactorial traits.

In **Chapter 4**, the construction of a high-resolution comparative map is described for SSC 2. A radiation hybrid (RH) map containing 33 genes and 25 microsatellite markers was constructed for this chromosome using a 3000 rad porcine RH panel. In total, 16 genes from human chromosome (HSA) 11p, HSA19p, and HSA5q were newly assigned to SSC2. One linkage group was observed at LOD 3.0 and five linkage groups at LOD 4.0. Comparison of the porcine RH map with homologous human gene orders identified four conserved segments between SSC2 and HSA11, HSA19, and HSA5. Concerning HSA11, a rearrangement of gene order is observed. The segment HSA11p15.4-q13 is inverted on SSC2 when compared to the distal tip of SSC2p which is homologous to HSA11p15.5. The boundaries of the conserved segments between human and pig were defined more precisely. This high-resolution comparative map will be a valuable tool for further fine mapping of the QTL area.

To improve the existing comparative map for pig chromosome 2 and increase the gene density on this chromosome, a porcine BAC library was screened with 17 microsatellite markers and 18 genes previously assigned to pig chromosome 2. The results of this study are reported in **Chapter 5**. Fifty-one BAC clones located in the region of the maternally imprinted quantitative trait locus for backfat thickness were identified. From these BACs 372 kb was sample sequenced. The average read length of a subclone was 442 bp. Contig assembly analysis showed that every basepair was sequenced 1.28 times. Subsequently, sequences were compared with sequences in the nucleotide databases to identify homology with other mammalian sequences. Homology was observed with sequences derived from 34 BACs. The average percentage identity with human sequences was 88.7%, with an average length of 155 bp. In total, sample sequencing of all BACs resulted in homology with 30 human genes, thirteen human ESTs, 20 human genomic clones, one rat gene, one porcine gene and nine porcine ESTs. Eighteen genes located on human chromosome 11 and 19, and eight genes from other human locations were assigned to pig chromosome 2 for the first time. Through radiation hybrid

mapping, a detailed comparative map was constructed. In total 58 genes were mapped on the radiation hybrid map of SSC2p-q13.

In **Chapter 6**, genes that are expected to map near the breakpoint of the conserved segments between HSA11p-q13 and SSC2 were mapped in pigs to determine the breakpoints more precisely. For the investigation of the borders of the conserved segments human cDNA sequences of genes located on HSA11 were used to identify porcine EST sequences that are expected to map on the pig near the breakpoints of the conserved segments between HSA11 and SSC2. Primers were designed and six genes were assigned to SSC2 on the somatic cell hybrid panel and SSC2 radiation hybrid map. Moreover, two genes were assigned to SSC9. In addition, human IMAGE clones were selected from these eight genes, and were hybridised to porcine BAC library filters to obtain porcine BAC clones. The BAC clones were subcloned and sample sequenced to identify additional homologies to human sequences, which resulted in the mapping of an additional three genes to SSC2. The mapping of eight genes resulted in refinement of the borders of the conserved segments to regions less than 4 Mb and in the detection of a new rearrangement in the comparative map between HSA11 with the porcine genome.

Chapter 7 describes the contribution of the main findings described in Chapters 2 through 6 to the field of animal genetics and the consequences of this work for animal breeding. In the regions on SSC2, SSC4 and SSC7 where QTL for fatness traits were detected, several other groups also reported QTL for growth or fatness traits. In addition, studies applying the candidate gene approach for the identification of genes involved in complex traits have resulted in a number of associations of genes with these traits. However, most of the candidate genes identified based on biological function are not located in the described QTL regions. Through the use of radiation hybrid maps that include both Type I and II markers, homologous chromosomal locations for QTL of specific traits can be identified in other species. The conservation of genome organisation between pig, man and mouse makes it possible to take advantage of genetically well-characterised species for the selection of candidate genes for the imprinted QTL for backfat thickness on SSC2. This QTL region on SSC2 is homologous to HSA11p-q13 and HSA19p. On HSA 11 one large imprinted cluster of genes is located, but apparent candidate loci that might explain the imprinting effects observed for the QTL for backfat thickness on SSC2 are not yet described in literature. The traits in humans most comparable to fatness in pigs are obesity-related traits. The number of genes, markers, and

chromosomal regions that have been associated or linked with obesity phenotypes in man and animal models is more than 250 dispersed on all chromosomes. Human obesity research can help to determine the direction and give clues for further porcine genetic research, but emphasis should be on better understanding of the fat traits in pigs themselves and improvement of the genetic map of the pig.

One of the aims of this thesis was to identify genetic markers linked to separate genes controlling backfat thickness and intramuscular fat, enabling animal breeders to select separately for the two traits. QTL for these traits and the molecular characterisation of the QTL region for backfat thickness on SSC2 is described in this thesis. Identification of the genes actually involved in intramuscular fat and backfat thickness can lead to the development of genetic tests to evaluate the genetic potential of individual animals, which can be included in breeding programs. In addition, the biological functions of these genes in the fat metabolism and the biological factors that are important in both types of fat production and in the separate fat types can be determined.

Samenvatting

In de afgelopen jaren is het onderzoek aan de genomen van landbouwhuisdieren, zoals koeien, varkens en kippen, enorm toegenomen. De ontdekking van zeer polymorfe microsatelliet merkers in deze diersoorten en het voltooien van de ontrafeling van het menselijke genoom hebben een grote impact op de kennis van de genetica van landbouwhuisdieren. Dit heeft geleid tot de constructie van genetische koppelingskaarten van landbouwhuisdieren en vervolgens tot een groot aantal koppelingsstudies; met de bedoeling om genen betrokken bij belangrijke, vaak kwantitatieve, productie kenmerken te identificeren. Dit proefschrift behandelt de identificatie van genen betrokken bij de vetopbouw in het varken en in het bijzonder de identificatie van genen betrokken bij de aanzet van intramusculair vet en rugspek. Quantitative trait loci (QTL) zijn plekken op het genoom waar vermoedelijk genen zich bevinden die te maken hebben met een bepaald kenmerk. Merkers die zijn gekoppeld aan een QTL voor de aanzet van intramusculair vet en rugspek gevonden in een experimentele kruising tussen het Chinese varkensras Meishan en Nederlandse commerciële varkenslijnen kunnen leiden tot de identificatie van genen die de verschillen in vet percentages tussen de twee rassen kunnen verklaren.

Voorafgaande aan het promotie onderzoek beschreven in dit proefschrift zijn QTL voor aan vet gerelateerde kenmerken gevonden in een experimentele kruising tussen Meishan en Nederlandse commerciële varkenslijnen. Om deze QTLs op varkens-chromosoom 2, 4 en 7 beter in kaart te brengen werden 25 extra merkers in de regio's getypeerd op 800 dieren (619 F2 dieren, de F1 ouders en F0 grootouders) van de experimentele kruising. De resultaten hiervan worden beschreven in **Hoofdstuk 2**. Vergeleken met de reeds gepubliceerde koppelingskaarten van deze chromosomen werden verschillen in volgorde van de merkers geconstateerd op varkens-chromosoom 4 en 7. QTL analyses werden uitgevoerd zowel binnen de families (familie-model) als over families heen (lijnkruisings-model). Naast deze analyses werd ook gekeken naar inprenting effecten. Genetische inprenting effecten zijn processen die de activiteit en expressie van genen of stukken van chromosomen reguleren door veranderingen op post-DNA- transcriptieniveau op basis van de ouderlijke herkomst van de genen en chromosomen. Dit betekent dat in een individu de allelen afkomstig van de vader een ander effect kunnen hebben dan de allelen afkomstig van de moeder.
De informatiedichtheid van de markers op de chromosomen is aanzienlijk verhoogd op alle drie de chromosomen door de 25 merkers die extra getypeerd zijn. De aanwezigheid van een QTL voor rugspekdikte op varkens-chromosoom 7 werd bevestigd en de locatie van dit QTL werd nauwkeuriger vastgesteld tot een regio met een betrouwbaarheidsinterval van 33

centiMorgan (cM). Het QTL voor intramusculair vet dat eerder was geobserveerd op varkens-chromosoom 4 kon in deze studie niet worden teruggevonden in het familie-model. Het lijnkruisingsmodel leverde op dit chromosoom echter wel een suggestief QTL voor intramusculair vet op, echter op een andere plek op het chromosoom dan de beschreven QTL in het familie-model. Op varkens-chromosoom 2 werd met het familie-model een suggestief QTL voor rugspekdikte gevonden waarvan de beste positie op 26 cM ligt. Met toepassing van het inprenting model werd echter een zeer significant QTL op varkens-chromosoom 2 voor dit kenmerk gevonden. De meest waarschijnlijke locatie is op 63 cM en dit QTL komt tot expressie door het allel dat overgeërfd is van de vader (paternale expressie). Deze resultaten geven aan dat het ingeprente QTL dat gevonden werd in deze studie anders is dan de reeds eerder gepubliceerde QTL voor spierdikte en vetaanzet, dat is gesitueerd op varkens-chromosoom 2 aan het distale uiteinde van de p-arm van het chromosoom.

De inprentings-effecten die eerder werden gevonden werden in detail bekeken in de experimentele kruising en worden beschreven in **Hoofdstuk 3**. Bewijs voor vijf significante QTLs die karkassamenstelling en de vleeskwaliteit beïnvloeden werden gevonden, waarvan er vier QTLs waren ingeprent. Inprenting werd onderzocht met behulp van een statistisch model dat onderscheid maakt tussen allelen in een individu die afkomstig zijn van de vader en die afkomstig zijn van de moeder. Op varkens-chromosoom 2 werd een QTL voor rugspekdikte gevonden waarbij het allel afkomstig van de vader het effect veroorzaakt. Op varkens-chromosoom 7 werden twee QTLs ontdekt; een QTL voor rugspekdikte en in dezelfde regio een QTL voor spierdikte waarbij het allel afkomstig van de moeder het effect veroorzaakt (maternale expressie). Twee QTLs voor intramusculair vet werden gevonden op varkens-chromosoom 6. Op de korte arm van dit chromosoom werd een QTL met maternale expressie gevonden en op de lange arm een QTL met paternale expressie. De individuele QTLs verklaarden 2 tot 10% van de totale fenotypische variatie. De chromosoom gebieden in de mens en de muis die homoloog zijn aan de in dit hoofstuk beschreven varkenschromosomen bevatten geen genen met reeds bekende functies die logischerwijs in het varken dezelfde functie zouden hebben en dus de waargenomen effecten kunnen verklaren. De resultaten beschreven in dit hoofdstuk zijn een indicatie dat inprenting een algemener fenomeen is dan tot dus verre werd aangenomen. Testen voor inprenting zouden daarom een standaard procedure moeten zijn bij de ontrafeling van genetische factoren die aan de basis liggen van kwantitatieve kenmerken.

Om de QTL regio voor rugspekdikte op varkens-chromosoom 2 beter in kaart te brengen werd een vergelijkende genetische kaart met hoge resolutie tussen varkens-chromosoom 2 en de homologe humane chromosomen geconstrueerd (**Hoofdstuk 4**). In dit hoofdstuk wordt de radiation hybrid (RH) kaart van varkens-chromosoom 2 bestaande uit 33 genen en 25 microsatelliet merkers gepresenteerd. De RH panel werd gemaakt door het fuseren van de chromosomen van somatische cellijnen afkomstig van een hamster met een set van aselecte fragmenten van varkens DNA, die zijn verkregen door bestralingen van de varkens-chromosomen. Uit het vaststellen van de frequentie van merkers en genen die samen voorkomen na de door bestraling geïnduceerde DNA fragmentatie kunnen de volgorde en de afstanden tussen de merkers en genen worden bepaald en kan een genetische kaart worden gemaakt. Van de 33 genen getypeerd op de RH panel, waren er 16 afkomstig van humaan chromosomen 5, 11 en 19 die niet eerder op het varkens genoom waren geplaatst. Eén koppelingsgroep met een LOD score van 3.0 and vijf koppelingsgroepen met een LOD score van 4.0 worden beschreven. Vergelijking van de RH kaart van varkens-chromosoom 2 met overeenkomstige gebieden op het menselijke genoom lieten zien dat er vier geconserveerde segmenten zijn tussen varkens-chromosoom 2 en humaan chromosoom 5, 11 en 19. Op humaan chromosoom 11 werd een andere volgorde van de genen zichtbaar, ten opzichte van varkens-chromosoom 2. De banden p15.4 tot q13 van humaan chromosoom 11 zijn omgekeerd aanwezig op varkens-chromosoom 2, maar het uiterste stukje van varkens-chromosoom 2 is homoloog aan het uiterste stukje van humaan chromosoom 11. De grenzen van de geconserveerde segmenten tussen de humane en varkens-chromosomen werden in deze studie veel nauwkeuriger in kaart gebracht. De vergelijkende kaart met hoge resolutie van varkens-chromosoom 2 is een waardevol hulpmiddel voor verder in kaart brengen van de QTL regio.

Om de huidige vergelijkende kaart tussen varkens-chromosoom 2 te verbeteren en bovendien het aantal genen dat op dit chromosoom is geplaatst te verhogen, werd een varkens Bacterial artificial chromosome (BAC) bank gescreend op de aanwezigheid van BACs die positief zijn voor 17 microsatelliet merkers en 18 genen die gelokaliseerd zijn op varkens-chromosoom 2. De resultaten van deze studie worden beschreven in **Hoofstuk 5**. Een BAC is een plasmide waaraan een stuk varkens-DNA is toegevoegd dat in een bacteriecel wordt opgenomen en zal worden vermenigvuldigd tijdens delingen van de bacteriecel. In de regio op chromosoom 2 met het QTL voor rugspekdikte met paternale expressie werden 51 BAC clonen geïdentificeerd. Van deze BACs werden 372 kilobasen gesequenced. Gemiddeld werden 442

baseparen per subclone gesequenced. De constructie van overlappende sequenties wees uit dat elk basepaar gemiddeld 1.28 keer gesequenced werd. Deze varkenssequenties werden vergeleken met sequenties in de nucleotide databases om homologie met andere zoogdiersoorten te identificeren. Voor sequenties afkomstig van 34 verschillende BACs werd homologie met andere zoogdieren gevonden. Het gemiddelde percentage overeenkomstige sequentie met humane sequenties was 88.7%, met een gemiddelde lengte van 155 basenparen. Het sample sequencing van alle 51 BACs resulteerde in homologie met 30 humane genen, dertien humane expressed sequence tags (ESTs), 20 humane genomische clonen, één rat-gen, één varkens-gen en negen varkens-ESTs. Achttien genen gelocaliseerd op humaan chromosoom 11 en 19 en 8 genen afkomstig van andere chromosomen werden geplaatst op varkens-chromosoom 2. Deze genen werden geplaatst op de RH kaart dat resulteerde in een gedetailleerde vergelijkende kaart voor varkens-chromosoom 2. In totaal zijn 58 genen geplaatst op de RH kaart van varkens-chromosoom 2.

In **Hoofdstuk 6** werden de breekpunten tussen de geconserveerde segmenten tussen humaan chromosoomsegment 11p-11q13 en varkenschromosoom 2 in meer detail bekeken. Genen waarvan verwacht werd, op basis van hun humane locatie, dat ze gelokaliseerd zijn dicht bij de breekpunten op varkens-chromosoom 2 werden geplaatst in het varkensgenoom. Humane cDNA sequenties van genen gelokaliseerd op humaan chromosoom 11 werden gebruikt om varkens-EST-sequenties op te sporen. Primers werden ontworpen voor deze genen en op de somatische cell hybrid en RH panel konden zes genen op varkens-chromosoom 2 worden geplaatst. Bovendien werden twee genen geplaatst op varkens-chromosoom 9. Naast deze genen werden er humane IMAGE clones geselecteerd voor deze acht genen die werden gehybridiseerd met een varkens BAC-bank. Positieve BAC-clonen werden vervolgens gesubcloneerd en gesequenced. Sequenties werden vergeleken met humane sequenties in de databases. Dit resulteerde in het plaatsen van nog eens 3 genen op varkens-chromosoom 2. Het positioneren van de acht genen in deze studie leidde tot een verfijning van de grenzen van de geconserveerde chromosoomsegmenten tot minder dan 4 Mb en tot de ontdekking van een nieuwe verschil in gen-volgorde in de vergelijkende kaart tussen humaan chromosoom 11 en het varkens genoom.

De bijdrage van de in Hoofdstuk 2 tot en met Hoofstuk 6 beschreven resultaten aan het vakgebied van de toegepaste landbouwhuisdieren genetica en de consequenties voor de fokkerij worden beschreven in **Hoofdstuk** 7. In de chromosoom regio's op varkens-

chromosoom 2, 4 en 7 waar QTLs voor vet-kenmerken zijn gedetecteerd, hebben verscheidende andere onderzoeksgroepen ook QTLs voor groei- en vet-kenmerken beschreven. Bovendien, studies waarin de kandidaatsgen benadering werd toegepast voor het identificeren van genen betrokken bij complexe kenmerken, hebben geleid tot een aantal associaties van genen met deze kenmerken. Maar de meeste van deze kandidaatsgenen die geïdentificeerd zijn op basis van hun biologische functie, liggen niet in de genoemde regio's op varkens-chromosoom 2, 4, en 7. Homologe chromosoomgebieden voor QTL voor specifieke kenmerken in andere diersoorten kunnen worden geïdentificeerd door middel van het plaatsen van zowel Type *I* merkers (genen) als Type *II* merkers (b.v. microsatelliet merkers). De geconserveerde genoomorganisatie tussen varken, mens en muis maakt het mogelijk om gebruik te maken van genetisch zeer goed in kaart gebrachte soorten organismen voor de selectie van kandidaatsgenen voor het ingeprente QTL voor rugspekdikte op varkens-chromosoom 2. Deze QTL regio op varkens-chromosoom 2 is homoloog met humaan chromosoom 11p-q13 en humaan chromosoom 19p. Op humaan chromosoom 11 is een groot cluster met ingeprente genen gelokaliseerd, maar kandidaatsgenen die de gevonden inprentings effecten voor het QTL voor rugspekdikte op varkens-chromosoom 2 kunnen verklaren zijn nog niet beschreven in de literatuur. De kenmerken die in de mens zijn onderzocht en het meest vergelijkbaar zijn met de kenmerken rugspekdikte en intramusculair vet in het varken zijn vetzucht gerelateerde kenmerken. Het aantal genen, merkers en chromosomale regio's waarmee associatie of koppeling met vetzucht gerelateerde fenotypes wordt beschreven is meer dan 250, verspreid over alle humane chromosomen. Humaan vetzucht onderzoek kan bijdragen aan het onderzoek aan vet kenmerken in varkens, maar de nadruk zal in de toekomst toch moeten liggen op het beter begrijpen van het vet metabolisme in het varken en het verbeteren van de genetische kaart van het varken.

Een van de doelen van dit promotieonderzoek was het identificeren van genetische merkers die gekoppeld zijn aan genen die rugspekdikte en intramuscular vet beïnvloeden zodat fokkers apart kunnen selecteren voor deze kenmerken in varkens. QTL voor deze kenmerken en de moleculaire karakterisering van de QTL regio voor rugspekdikte op varkens-chromosoom 2 is beschreven in dit proefschrift. De identificatie van de genen betrokken bij rugspekdikte en intramusculair vet kan leiden tot het ontwikkelen van genetische tests om het genetische potentieel van individuele dieren te evalueren, die kunnen worden toegepast in fokprogramma's. Bovendien kunnen de biologische functies van de genen in het vet metabolisme en de biologische factoren die belangrijk zijn in beide typen van vet productie en in de afzondelijke vet types worden bepaald.

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Annemieke

Curriculum Vitae

Annemieke Paula Rattink werd geboren op 28 april 1974 in Nieuwkoop. In 1992 behaalde zij het VWO diploma aan de Scholengemeenschap Albanianae te Alphen aan de Rijn. In hetzelfde jaar begon ze aan de studie Biologie aan de toenmalige Landbouwuniversiteit te Wageningen. Haar eerste afstudeervak heeft zij gedaan voor de vakgroep Humane Epidemiologie en Gezondheidsleer uitgevoerd bij TNO – Preventie en Gezondheid in Leiden, waar gekeken werd naar de associatie tussen polymorfismen in Factor VII en HRG en de ernst van arteriosclerose en diep veneuze trombose. Haar tweede afstudeervak betrof een studie naar het MHC DRB3 locus in het met uitsterven bedreigde Arabisch hert (*Oryx leucoryx*) aan Arizona State University, Tempe, USA. Hierna volgde een stage bij het Nederlands Kanker Instituut in Amsterdam waar gewerkt werd aan de positionele klonering van het colonkanker gevoeligheidsgen SCC1. In september 1997 studeerde zij af met als specialisatie Organisme, waarna ze in november 1997 bij de Leerstoelgroep Fokkerij en Genetica begon als Onderzoeker In Opleiding (OIO) aan het promotieonderzoek beschreven in dit proefschrift.

Sinds 1 februari 2002 is zij werkzaam als moleculair geneticus bij het Breeding Research Centre van Nutreco te Boxmeer.

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