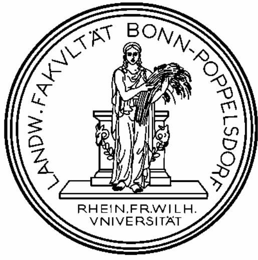

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Molecular genetic analysis of positional candidate genes for mammary gland characteristics in pigs

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Dedicated

with reverence

to

my beloved parents

Die molekular-genetischen Analysen von positionellen Kandidatengenen für Zitzenmerkmale beim Schwein

Stülpzitzen reduzieren die Anzahl funktionierender Zitzen in betroffenen Sauen und führen zum Rückgang der Produktivität der Ferkelerzeugung und des Ferkelwachstums während der Laktation. Stülpzitzen findet man in kommerziellen Schweinerassen mit Frequenzen von 8 bis 30 %. Da von Defekten betroffene Tiere selektiert werden, wird also auch eine wirtschaftliche Produktion in Zuchtprogrammen berücksichtigt.

Die vorliegende Arbeit zielt darauf ab, Punktmutationen (SNPs) in den positionellen Kandidatengenen für den Gendefekt Stülpzitze im Schwein zu finden und die physikalische Lokalisation der Gene TGFB1, RLN und PTHLH zu untersuchen bzw. den Genort über Kopplung zu bestimmen. Weiterhin wurde die Assoziation der Gene TGFB1, RLN und PTHLH zum Merkmal Stülpzitze errechnet. Das Transforming Growth Faktor Beta 1 Gen (TGFB1) und das Relaxin Gen (RLN) wurden mit dem positionellen Kandidatengenansatz, welcher die Kopplungsinformation für den Stülpzitzendefekt und die Kartierungsinformation eines Kandidatengens verknüpft, auf Assoziation getestet. Beide Gene, welche in den QTL Regionen für Stülpzitze kartieren, die in der Bonn-Berlin Ressourcen Familie gefunden wurden, sind an Proliferations- und Differenzierungsprozessen der Milchdrüsen beteiligt. Der Familien-basierte Assoziationstest (FBAT) wurde verwendet, um die allele Assoziation zwischen dem Defekt Stülpzitze und den Genotypen zu untersuchen. Die hohe Signifikanz belegt die Assoziation zwischen der Affektion des Defektes Stülpzitze und des Genotyps von RLN in der Bonn-Berlin Dumi Ressource Population. Es wurden keine signifikanten Effekte von TGFB1 auf den Defektstatus gefunden. Des weiteren wurde das Parathyroid Hormon Like Hormon Gen (PTHLH) als ein funktionelles Kandidatengen erwählt, das Epithel - Mesenchymale Interaktionen während der Bildung der Milchdrüsen reguliert. Es wurde ebenfalls eine hoch signifikante Assoziation mit dem Defektstatus gezeigt. Eine hoch signifikante Assoziation wurde ebenfalls zwischen den Genorten TGFB1, RLN und PTHLH und der Anzahl Zitzen und Anzahl Stülpzitzen gefunden.

The molecular genetic analyses of positional candidate genes for mammary gland characteristics in pigs

Inverted teat reduce the number of functional mammary complexes in affected sows leading for reduction of piglet productivity and litter growth during the lactation period. This detect occures in commercial pig breeds with frequencies between 8 to 30%. Due to selection of affected animals economic consequences in the breeding programm are considerate. The study aims to identify single nucleotide polymorphism (SNPs) in the positional candidate genes for the inverted teat defect in porcine, and to determine the linkage and physical location of TGFB1, RLN and PTHLH gene. Moreover the association of TGFB1, RLN and PTHLH gene on inverted teat trait were evaluated. The transforming growth factor beta 1 gene (TGFB1) and relaxin gene (RLN) were proposed to be tested for association in a positional candidate gene approach which combines linkage information for inverted teat defect and mapping information of a candidate gene. Both genes mapped in QTL regions for inverted teats discovered in the Bonn-Berlin resource family and involved in proliferation and differentiation processes of mammary gland. The family-based association test (FBAT) was used for allelic association between inverted teat defect and genotype. Highly significant evidence for association of RLN and the inverted teat defect affection in the Bonn-Berlin DUMI resource population was found. No significant effects of TGFB1 on affection status were detected. In addition, parathyroid hormone like hormone gene (PTHLH) was proposed as a functional candidate gene that regulates epithelial mesenchymal interactions during the formation of mammary gland. It could also been shown to be highly significantly associated with affection status. Highly significant association was also found between TGFB1, RLN and PTHLH loci and number of teats and number of inverted teats. Further confirmation of these results in independent samples of other populations will be conducted.

Contents Page

Abstract		V
List of abbreviations		XII
List of tables		XV
List of figures		XVI
1	Introduction	1
2	Literature review	3
2.1	Experiments to address the roles of candidate genes in the mammary gland	3
2.2	Genetic background of teat number and inverted teat	5
2.3	The candidate gene approach	8
2.4	Single nucleotide polymorphism (SNP)	9
2.5	Linkage and association analysis in complex disease	10
2.6	Candidate genes for inverted teat defect	12
2.6.1	Transforming growth factor beta 1 gene (TGFB1)	12
2.6.1.1	Gene structure	12
2.6.1.2	Polymorphism in TGFB1 gene	13
2.6.1.3	Mapping	13
2.6.1.4	Association	13
2.6.2	Relaxin gene (RLN)	14
2.6.2.1	Gene structure	15
2.6.2.2	Polymorphism	15
2.6.2.3	Mapping	16
2.6.3	Parathyroid hormone like hormone gene (PTHLH)	16
2.6.3.1	Gene structure	16
2.6.3.2	Polymorphism	17
2.6.3.3	Association	18
2.6.3.4	Mapping	18

Contents (continued) Page

3	Material and Methods	20
3.1	Material	20
3.1.1	Experimental animals	20
3.1.1.1	Unrelated animals	20
3.1.1.2	Bonn-Berlin-DUMI resource population	20
3.1.1.3	Commercial Pigs	21
3.1.2	Chemicals and equipments	21
3.1.2.1	Chemicals	21
3.1.2.2	Reagents	23
3.1.2.3	Buffers	24
3.1.2.4	Competent Cells	25
3.1.2.5	Medium	25
3.1.2.6	Commercial Kits	26
3.1.2.7	Enzymes	27
3.1.2.8	Equipments	27
3.1.2.9	Used software	28
3.2	Methods	29
3.2.1	RNA isolation and cDNA synthesis	29
3.2.1.1	RNA isolation	29
3.2.1.2	cDNA synthesis	30
3.2.2	DNA isolation from agarose gel	30
3.2.3	Ligation	31
3.2.4	Transformation	31
3.2.5	Sequencing	32
3.3	Screening for polymorphism in genes	32
3.3.1	Transforming growth factor beta 1 gene (TGFB1) screening	32
3.3.1.1	PCR condition	33
3.3.1.2	PCR condition and Single strand conformation polymorphism (SSCP) analysis	33

Contents (continued) Page

3.3.1.3	Silver stain procedure	34
3.3.2	Relaxin gene screening	34
3.3.2.1	PCR condition	35
3.3.2.2	Genotyping the porcine RLN gene polymorphism	35
	PCR-SSCP	35
	Restriction fragment length polymorphism (RFLP)	36
3.3.3	Identification of parathyroid hormone like hormone gene (PTHLP)	36
3.3.3.1	Isolation of porcine PTHLP gene by RACE-PCR	36
3.3.3.2	Linkage mapping analysis by radiation hybrid panel	38
3.3.3.3	Genotyping method for PTHLH gene	39
	TaqMan allelic discrimination using real-time PCR	39
3.4	Linkage mapping	40
3.5	Association analysis between the markers and mammary gland trait	40
3.5.1	Family Based Association Tests (FBAT)	40
4	Results	41
4.1	Transforming growth factor beta 1 gene (TGFB1)	41
4.1.1	cDNA sequence of porcine TGFB1 and screening SNP in TGFB1 gene	41
4.1.2	Polymorphism in the porcine TGFB1	41
4.1.3	Development of methods for genotyping the allelic polymorphism	42
4.1.3.1	Genotyping method by using SSCP analysis	42
4.1.4	Genetic variation of the porcine TGFB1 in the DUMI resource population	42
4.1.5	Genetic variation of the porcine TGFB1 in the commercial breeds	45
4.1.6	Linkage mapping	45

Contents (continued) Page

4.2	Relaxin gene (RLN gene)	46
4.2.1	Polymorphism in the porcine RLN gene	46
4.2.2	Development of methods for genotyping the allelic polymorphism	47
4.2.2.1	Genotyping method by using SSCP analysis	47
4.2.2.2	Genotyping method by using RFLP analysis	47
4.2.3	Genetic variation of the porcine RLN gene in the DUMI resource population	48
4.2.4	Genetic variation of the RLN gene in the commercial breeds	50
4.2.5	Linkage mapping	51
4.3	Parathyroid hormone like hormone gene (PTHLH)	51
4.3.1	cDNA sequence of porcine PTHLH	51
4.3.2	SNP screening in PTHLH gene	52
4.3.3	Polymorphism in the porcine PTHLH gene	52
4.3.4	Development of method for genotyping the allelic polymorphism	55
4.3.4.1	Genotyping by using Taqman assay	55
4.3.5	Genetic variation of the porcine PTHLH gene in the DUMI resource population	56
4.3.6	Genetic variation of the porcine PTHLH gene in the commercial breeds	56
4.3.7	Linkage mapping	57
4.4	Family Based Association Tests (FBAT)	59
5	Discussion	63
5.1	Motives to address the inverted teat defect	63
5.2	Strategies to identify genes controlling economically important traits	64
5.3	Gene mapping approach	65

Contents (continued) Page

5.4	Single nucleotide polymorphism (SNP) and genotyping method	66
5.5	Quantitative trait loci for inverted teat defect (and number of teats)	68
5.6	Candidate gene analysis	72
5.6.1	Transforming growth factor beta 1 gene (TGFB1)	72
5.6.1.1	Experimental and functional evidence for an aetiological role in the inverted teat defect	72
5.6.1.2	Polymorphism	73
5.6.1.3	Candidate gene for disease in other species	74
5.6.1.4	Gene mapping	75
5.6.2	Relaxin gene (RLN)	77
5.6.2.1	Experimental and functional evidence for an aetiological role in the inverted teat defect	77
5.6.2.2	Polymorphism	78
5.6.2.3	Gene mapping	78
5.6.3	Parathyroid hormone like hormone gene (PTHLH)	80
5.6.3.1	Cloning and characterization of cDNA for porcine PTHLH	80
5.6.3.2	Experimental and functional evidence for an aetiological role in the inverted teat defect	81
5.6.3.3	Polymorphism	82
5.6.3.4	Gene mapping	83
5.7	Association and statistic analysis	84
5.8	Future prospects for investigation of candidate genes for inverted teat defect in pigs	87
6	Summary	89
7	Zusammenfassung	92
8	References	96

List of abbreviations

A	: Adenine
A260	: Absorbance at 260 nm wavelength (UV light)
APS	: Ammonium persulfate
ATP	: Adenosine triphosphate
bp	: Base pairs
BSA	: Bovine serum albumin
C	: Cytosine
cM	: Centimorgan
ddH_2O	: Distilled & deionized water
ddNTP	: Dideoxyribonucleoside triphosphate
DGGE	: Denaturing gradient gel electrophoresis
dH_2O	: Deionized or distilled water
DMSO	: Dimethyl sulfoxide
DNA	: Deoxynucleic acid
dNTP	: deoxyribonucleoside triphosphate
	(usually one of dATP, dTTP, dCTP and dGTP)
DTT	: Dithiothreitol
E.coli	: *Escherichai coli*
EDTA	: Ethylenediaminetetraacetic acid (powder is a disodium salt)
EtBr	: Ethidium bromide
EtOH	: Ethanol
FBAT	: Family based association tests
G	: Guanine
g	: Gram
h	: Hour
HGM	: Human gene mapping
Ile	: Isoleucine
IPTG	: Isopropylthio-ß-D-galactoside
kb	: Kilobases
l	: Litre

Leu	: Leucine
LOD	: Logarithm of odds
mA	: Milliamperes
mg	: Miligrams
min	: Minute
$MgCl_2$	: Magnesium chloride
ml	: Milliliters
mRNA	: Messenger RNA
mmole	: Milimole
MW	: Molecular weight
NaCl	: Sodium chloride
ng	: Nanograms
nm	: Nanometers
NPL	: Non Parametric Lod Score
OD260	: Optical density at 260 nm wavelength (UVlight); = A260
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate buffered saline
Pro	: Proline
PCR	: Polymerase chain reaction
PFGE	: Pulsed field gel electrophoresis
PIC	: Polymorphism information content index
pmol	: Picomolar
PTHLH	: Parathyroid hormone like hormone gene
QTL	: Quantitative trait loci
RACE	: Rapid amplification of cDNA end
RFLP	: Restriction fragment length polymorphism
RLN	: Relaxin gene
RNA	: Ribonucleic acid
rpm	: Revolutions per minute
SDS	: Sodium dodecyl sulfate
Ser	: Serine
SMART^{TM}	: Switching mechanism at 5'end of RNA transcript

SNP	: Single nucleotide polymorphism
SSCP	: Single-strand conformation polymorphism
T	: Thymine
TAE	: Tris-acetate buffer
TBE	: Tris- borate buffer
TDT	: Transmission disequilibrium
TE	: Tris- EDTA buffer
TEMED	: N,N,N',N'-tetramethylethelenediamine
Tet	: Tetracycline
TGFB1	: Transforming growth factor beta 1 gene
Thr	: Threonine
tRNA	: Transfer RNA
U	: Units
UTR	: Untranslated region
UV	: Ultra-violet light
V	: Volts
VNTR	: Variable number of tandem repeats
v/v	: Volume per volume
W	: Watts
w/v	: Weight per volume
X-gal	: 5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

List of Tables Page

Table 1:	Heritability of teat number	5
Table 2:	Heritability of inverted teat	7
Table 3:	Primer sequence for SNP identification in TGFB1 gene	33
Table 4:	Primer sequence for SNP identification in RLN gene	35
Table 5:	The primer and method of genotyping for RLN gene	36
Table 6:	List of primers employed for RT-PCR and 5'- and 3'-RACE-PCR	37
Table 7:	PTHLH primers for intron isolation	38
Table 8:	Primer and probe sequences used for SNP genotyping of PTHLH gene	39
Table 9:	Frequencies of alleles of the TGB1 gene	45
Table 10:	Two point link for TGFB1	46
Table 11:	Frequencies of alleles of the RLN gene	49
Table 12:	Allele frequencies of the RLN gene in the commercial breeds	51
Table 13:	Frequencies of alleles of the PTHLH gene	56
Table 14:	Result of the FBAT done considering of inverted teat as the affection trait	61
Table 15:	Association of each marker in DUMI resource population by FBAT analysis	62
Table 16:	QTL region for affection of inverted teat within DUMI resource population	70
Table 17:	QTL-Region for inverted teat affection with in commercial breeds German Landrace, Large white and F1 reciprocal cross German Landrace and Large white	70

List of figures Page

Figure 1:	Ultrastructure and scheme of normal teat and inverted teat	7
Figure 2:	Bonn- Berlin DUMI resource population	20
Figure 3:	Sequence analysis of porcine TGFB1 gene revealed single nucleotide polymorphism in exon 5 at position 797 of the coding sequence	41
Figure 4:	Sequence of intron 4 of TGFB1 gene	42
Figure 5:	Nucleotide sequence of TGFB1 gene	43
Figure 6:	Mendelian inheritance of porcine TGFB1 at exon 5 position 797 using SSCP analysis	44
Figure 7:	Sequence analysis of porcine RLN gene revealed single nucleotide polymorphism	47
Figure 8:	Mendelian inheritance of RLN gene in intron 1 position 9 using RFLP analysis	49
Figure 9:	Mendelian inheritance of relaxin gene SNP in exon 1 position 22 using SSCP analysis	50
Figure 10:	Sequence analysis of porcine PTHLH gene revealed single nucleotide polymorphism at position 375 of coding sequencing	52
Figure 11:	Nucleotide sequence and amino acid (AA) sequences of porcine PTHLH gene	53
Figure 12:	Sequence of intron 2A of the PTHLH gene	54
Figure 13:	Comparative amino acid sequence in different species	54
Figure 14:	Scheme showing genotypes in porcine PTHLH gene in DUMI resource population	55
Figure 15:	Physical mapping of PTHLH gene using radiation hybrid panel (IMpRH)	58
Figure 16:	Overview of SNP assay using TaqMan probe	68
Figure 17:	A comparative map of TGFB1 and the human genome	76
Figure 18:	A comparative map of RLN and the human genome	79
Figure 19:	A comparative map of PTHLH and the human genome	84

1. Introduction

The inverted teat defect in pigs occurs in many breeds and herds. This disorder results in non-functional teats with canals that are shortened. Inverted teats cannot be suckled by the piglets. As a consequence the number of piglets raised is reduced for sows suffering from inverted teats. This disorder has a considerable negative impact in pig production. It occurs in different commercial pig breeds with frequencies between 8 to 30 % (Blendl et al. 1981, Mayer and Pirchner 1995). The mode of inheritance is not fully understood. This disorder has a genetic cause, but the number of genes involved is unknown. It is most likely that a major gene and a few modifying minor genes are involved. There are heritability estimates ranging from 0.2 to 0.5 (Brevern et al. 1994, Mayer 1994). Because of the uncertainty of the genetics of this trait, culling of parents and littermates is not recommended. Individuals with a high proportion of inverted teats should not be placed in the breeding herd.

In order to efficiently select against this disorder the availability of a DNA-test would be of great advantage since this disorder is in contrast to many other disorders difficult to diagnose and is only visible late in life. There are new techniques in molecular genetics are being developed, aimed at the isolation and identification of DNA markers linked to genes or representing genes for disease resistance, disorders and economically important production traits. When available, these markers will provide animal breeders with an objective test system to identify, at birth or even earlier, animals carrying desirable genes.

The search for this trait centres on two major techniques, linkage mapping and the candidate gene approach. The candidate gene approach is based on knowledge of physiology, biochemistry or pathology which clearly indicates the mechanism of the trait (Rothschild and Soller 1997). They may be structural genes or genes in regulatory or biochemical pathways affecting trait expression as functional candidate genes. Some genes may be excellent candidate genes based on similar phenotypes seen in other species. To date, several major genes affecting quantitative traits have been found with the candidate gene approach. Following to this strategy, Parathyroid hormone like hormone gene (PTHLH) is proposed as a candidate gene for inverted teat defect.

The positional candidate gene approach (Ballabio 1993) combines linkage information for a particular trait and mapping information of a candidate gene, i.e. if, for a particular trait, the genetic linkage data implicate a specific region of a chromosome, the genes located in this region are therefore potentially candidates for the phenotype, if additional facts support the hypothesis that the gene is the QTL. Supporting data are often those about the pattern of expression of the gene. Additional information may also be obtained from comparative mapping data, if a candidate has been mapped in another species in a region of conserved synteny which corresponds to the QTL region in the interested species as positional comparative candidate gene. A combination of linkage mapping and a candidate gene approach has been the most successful method of identifying disease genes to date. This approach has certain advantages over traditional linkage mapping or positional cloning approaches. According to this strategy are proposed here transforming growth factor beta 1 gene (TGFB1) and Relaxin gene (RLN) as candidate genes for inverted teat defect.

The association studies are now widely accepted as an important complement to linkage analysis. Single nucleotide polymorphisms (SNPs) are thought to be ideally suited as genetic markers for establishing genetic linkage and as indication of genetic disease. In some case a single SNP is responsible for a genetic disease.

The objective of this study was (1) to identify single nucleotide polymorphism (SNPs) in the positional and functional candidate genes for the inverted teat defect in porcine, (2) to determine the linkage and physical location of TGFB1, RLN and PTHLH gene, (3) to evaluate the association of TGFB1, RLN and PTHLH gene on inverted teat trait, and (4) to improve the comparative pig gene map. Ultimately, the goal will be to identify genes or DNA markers, which can be used to select against the inverted teat defect.

2. Literature review

2.1 Experiments to address the roles of candidate genes in the mammary gland

The endocrine system coordinates development of the mammary gland with reproductive development and the demand of offspring for milk. Three categories of hormones are involved, reproductive hormones, metabolic hormones and mammary hormone (Neville et al. 2002).

Plath et al. (1997) have shown expression of transforming growth factor beta 1 (TGFB1) in the bovine mammary gland during mammogenesis, lactogenesis, galactopoiesis and involution. Both specific receptor binding and expression of TGFB1 mRNA was higher during the prepubertal and pubertal periods than during lactation. These data support a role of TGFB1 in regulation of pubertal mammary growth and development. TGFB1 had a striking biphasic effect: whereas relatively high concentrations of this cytokine inhibited colony formation, lower concentrations stimulated extensive elongation and branching of epithelial cords. Taken together, these studies indicate that HGF/SF is a stromal-derived paracrine mediator of mammary ductal morphogenesis, and that when present at low concentrations, TGFB1 can contribute to this process (Soriano et al. 1998). The TGFB is at least in part responsible for restricting the formation and growth the lateral buds, and functions normally to maintain the open pattern of branching that is required for alveolar development during pregnancy. TGFB does not, on the other hand, restrict the development of alveolar structures (Daniel et al. 1989). The effects of overexpression of TGFB1 in the mammary epithelium have been evaluated more extensively. TGFB1 has been targeted to the mammary epithelium from different transcriptional promoters in two transgenic mouse models (Smith 1996).

During gestation, estrogens are known to be required for mammary development and relaxin is also needed to stimulate total mammary gland growth (Farmer and Sørensen 2000). Relaxin acts synergistically with estrogens and progesterone to develop the mammary apparatus and at the same time to suppress lactation. Removing the inhibitory effects of relaxin at parturition may be an important prelude to lactogenesis (Harness and Anderson 1975). Relaxin play a major role during pregnancy in promoting

connective tissue remodelling including cervical ripening and relaxation of pubic ligament. Min and Sherwood (1996) have been reported that the relaxin promotes growth and softening of cervix and development of mammary gland in pregnant pig.

In mice relaxin is required for development of the mammary gland and the nipple during pregnancy. Second, relaxin is required for relaxation of the pubic ligament during the second half of pregnancy; third relaxin is involved in Posmol (basal plasma osmolality) regulation in pregnancy. Finally, relaxin is not necessary for maintaining pregnancy and does not appear to affect the length of gestation, but does seem to affect normal delivery in some animals (Zhao et al. 1999).

In rodent, it was confirmed that the relaxin promotes growth and differentiation of the mammary parenchyma (epithelial and myoepithelial cells) and the mammary stroma (fibroblatas, adipocytes and collagen) (Bani et al. 1985, Bianchi et al. 1986). Moreover, relaxin was demonstrated to be essential for development of normal mammary nipples and nipple function in pregnant rats (Kuenzi and Sherwood 1992, Kuenzi et al. 1995).

Relaxin knockout mice have been developed and are fully fertile. Zhao et al. (1999) used gene targeting to generate Rln -/- mice. These mice were fertile but had deficient mammary development such that pups were unable to suckle and died within 24 hrs unless cross-fostered with a wildtype foster mother.

Parathyroid hormone like hormone (PTHLH) is produced in the mammary gland during late pregnancy and lactation and is postulated to have multiple effects in the mammary gland as well as the neonate, including regulation of calcium transport, relaxation of smooth muscle, and regulation of cell growth and differentiation (Ratcliffe 1992, Thiede 1994). Therefore, PTHLH produced by the lactating mammary gland very likely modulates the activity of mammary epithelial and myoepithelial cells as well as local vascular tissues (Thiede 1994). Recent experiments have demonstrated that PTHLH and the PTHR1 (parathyroid hormone receptor 1) comprise one are important signal pathway involved in this exchange. Both are necessary for mammary development, and in their absence, although the mammary buds initially form, they fail to undergo the expected androgen-mediated destruction in males or the initiation of ductal branching morphogenesis in females (Wysolmerski et al. 1998, Dunbar et al. 1999). Instead, the

mammary epithelial cells disappear and the nipple sheath fails to form, leaving neonates without mammary glands or nipples. During the early stages of mammary bud formation, PTHLH is expressed within mammary epithelial cells and the PTHR1 is expressed in the condensed mammary mesenchyme as well as in the presumptive dermis (Wysolmerski et al. 1998, Dunbar et al. 1999). It appears that PTHLH from the epithelial cells acts on the mesenchyme in close proximity to the mammary mesenchyme and involved in supporting the initiation of branching morphogenesis (Wysolmerski et al. 1998).

Table 1: Heritability of teat number

Breed	No. of animals	No. of teats	Heritability	Reference
Iberian	26,913	11	0.33-0.42	Bejar et al. 1993
Lacombe, York.	27,000	14	0.27-0.47	Mckay and Rahnefeld 1990
Iberain	30,271	11	0.35-0.46	Toro et al. 1986
Landrace	1,370	14.27	0.20	Smith et al. 1986
L.White	456	14.44	0.20	Smith et al. 1986
L.White	2,148	14	0.10-0.20	Clayton et al. 1981
Brit. Landrace	3,200	13.70	0.20-0.30	Clayton et al. 1981

2.2 Genetic background of teat number and inverted teat

The teat number is an important trait regard to the mothering ability of sows, the pig industry has traditionally applied selection pressure to teat number (Pumfrey et al. 1980). In particular, teat number plays an important role when the number is less than the litter size. Nevertheless, information about the inheritance of teat number is limited in comparison to other reproductive traits of pigs (Hirooka et al. 2001).

The factors that affect the number of teats in pigs are of interest for both biological and practical reasons. Previous work indicates that there is a genetic component, principally from the dam. The proportion of males in a litter appears to be related to the anogenital distance of the gilts in the litter, possibly as a result of the intrauterine position effect. Drickamer et al. (1999) indicated that the teat number in female pigs is related to the proportion of males in the litter.

The number of glands which develop on a pig will be mostly affected by genetics of the animal, but this occurs within a range of teat numbers; in the case of the pig this is a fairly wide range compared to most species. Heritability of teat number is low to medium as shown in table 1. Also, there is little relationship between teat number and maternal performance. Nevertheless, gilts are still chosen for breeding based partially on the number of teats. Most breed associations require 12 functional teats for pure bred registry. Fernandez et al. (2004) studied to evaluate heterosis and recombination effects in a crossing experiment involving two distinct European and Asian breeds (Iberian and Jiaxing). Teat number was recorded in the right and left sides of piglets and analysed by fitting a mixed linear model including the Dickerson's crossbreeding parameters. The result confirms that the rupture by recombination of coadapted genomes decreases developmental stability in domestic pigs.

The inverted teat is characterised by failure of nipples to protrude from the udder surface. The teat canal is held inward, forming a small crater so that normal milk flow is prevented (Figure 1). This abnormality has a genetic cause, but the number of pairs of genes involved is unknown. The heritability is estimated to be 0.1-0.6 (Table 2). The wide distribution of teat abnormalities is shown by investigation of slaughter pigs: 17% of boars and 6% of sows were affected by inverted teats. 75% of the inverted teats were seen periumbilical (Beilage et al. 1996). An interesting observation from the study by Labroue et al. (2001) was that almost all of the inverted teats become functional and give milk at the time of lactation. Therefore, both normal and inverted teats should be considered as functional teats. The small, supernumerary and blind teats should be considered as non-functional and should be excluded from the counts.

Table 2: Heritability of inverted teat

Reference	Heritability
Molenat and Thibault 1977	0.30-0.65
Clayton et al. 1981	0.21
Hittel 1984	0.32
Mayer 1994	0.19
Brevern et al. 1994	0.50

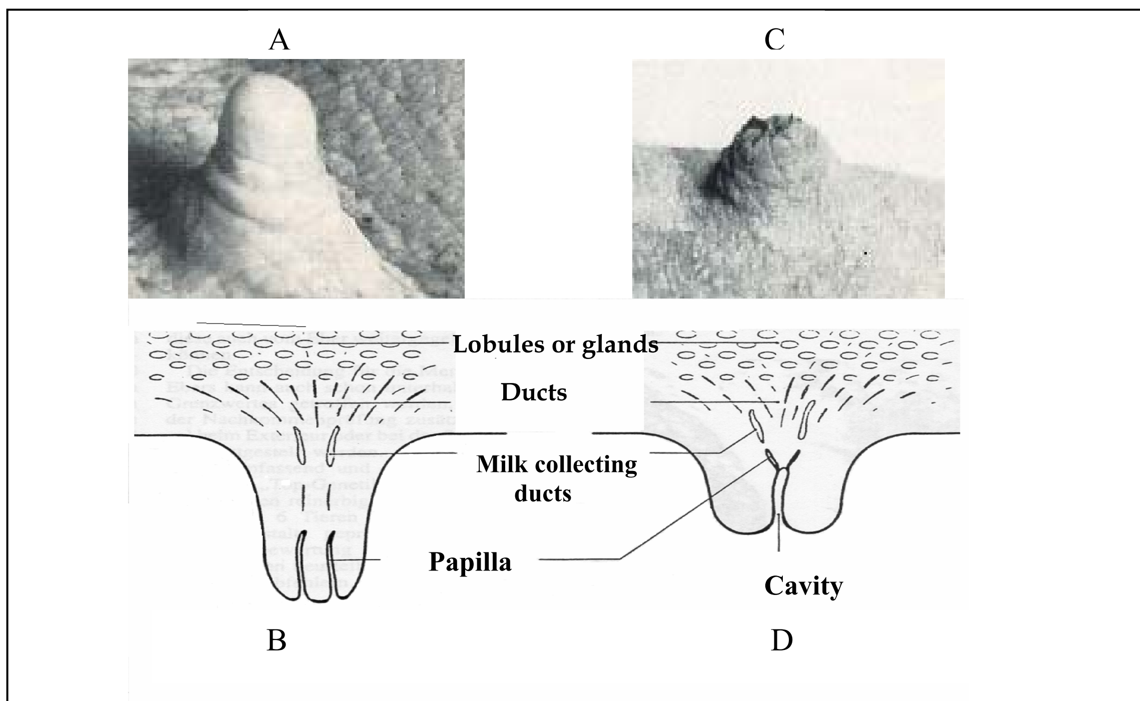

Figure 1: Ultrastructure and scheme of normal teat (A, B) and inverted teat (C, D) (Steffens 1993, Beilage et al. 1996)

2.3 The candidate gene approach

The candidate gene approach is based on knowledge of physiology, biochemistry or pathology which clearly indicates the mechanism of the trait (Rothschild and Soller 1997). They may be structural genes or genes in regulatory or biochemical pathways affecting trait expression as functional candidate genes. Some genes may be excellent candidate genes based on similar phenotypes seen in other species.

The most general approach is that of "positional cloning" (Collins 1992) or QTL mapping. It is based on the search of regions containing QTL through linkage mapping to anonymous markers approaching the unknown gene more and more closely, fine mapping the QTL region and finally cloning the QTL.

A number of QTL for different traits have been identified in different resource populations that were subjected to genome scans (Andersson et al. 1994, Rothschild et al. 1995, Bumstead 1998, Walling et al. 1998, Wang et al. 1998, Yonash et al. 1999, Marklund et al. 1999, Harlizius et al. 2000, de Koning et al. 2001, Malek et al. 2001a). Unfortunately, genome-wide scans cannot resolve the location of a QTL more precisely than 10-30 cM (Darvasi and Soller 1997). As this is equivalent to a region containing 5-15 MB of deoxyribonucleic acid (DNA) and 300-900 genes, on average, it is a major task to fine map the QTL region in order to finally positional clone the gene.

The “positional candidate” gene approach (Ballabio 1993) combines linkage information for a particular trait and mapping information of a candidate gene, which may be identified because of its specific expression pattern (functional candidate); i.e. if, for a particular trait, the genetic linkage data implicate a specific region of a chromosome, the genes located in this region are therefore potentially candidates for the phenotype, if additional facts support the hypothesis that the gene is the QTL. Supporting data are often those about the pattern of expression of the gene. Additional information may also be obtained from comparative mapping data, if a candidate has been mapped in another species in a region of conserved synteny which corresponds to the QTL region in the interested species as positional comparative candidate gene.

The functional candidate approach benefits from the fact that it only deals with cDNA, devoid of intronic and intergenic sequences, which represent only a few percent of the

total genome (about 3% in mammals). Differential expression screening approaches are therefore more closely associated to gene function. Since it is logistically near impossible to sequence the 100,000 different genes in a pig, the next best approach is to sequence only parts of the genes called express sequence tags (ESTs).

A combination of linkage mapping and a candidate gene approach has been the most successful method of identifying disease genes to date. The candidate gene approach is useful for quickly determining the association of a genetic variant with a disorder and for identifying genes of modest effect. This approach has certain advantages over traditional linkage mapping or positional cloning approaches.

2.4 Single nucleotide polymorphism (SNP)

SNPs are major contributors to genetic variation, comprising some 80% of all known polymorphisms, and their density in the human genome is estimated to be on average 1 per 1,000 base pairs. SNPs are mostly biallelic, more frequent, mutationally and more stable, making them suitable for association studies in which linkage disequilibrium (LD) between markers and an unknown variant is used to map disease-causing mutations. In addition, because SNPs have only two alleles, they can be genotyped by a simple plus/minus assay rather than a length measurement, making them more amenable to automation.

SNPs promise considerable advantages over microsatellite markers: (1) lower mutation rates, (2) more robust in laboratory handling and data interpretation (Krawczak 1999), (3) suitability for standardized representation of genotyping results as a digital DNA signature (Fries and Durstewitz 2001) and (4) suitability for various genotyping techniques and high potential for automation (Kruglyak 1997). One disadvantage is that any SNP has lower information content, compared with a highly polymorphic microsatellite. But this disadvantage can be compensated for by a higher number of markers.

Individual SNPs are less informative than other marker systems, especially as the widely used microsatellites, but they are more abundant and have a great potential for automation (Wang et al. 1998, Cronin et al. 1996). Markers used for genome scans

should allow the polymorphisms to be typed quickly, accurately and inexpensively. SNP markers possess the properties that fulfil these requirements. First, DNA sequence variations, based on point mutations are estimated to occur once every 500 to 1000 bp when any two chromosomes are compared (Harding et al. 1997). Second, SNPs are diallelic in populations, and their allele frequencies can be estimated easily in any population through a variety of techniques (Kwok et al. 1994). Third, the mutation rate per generation of SNPs is low compared to tandem repeat markers where the high mutation rates can confound genetic analysis in population (Hastbacka et al. 1992). Fourth, many of the mutations of the trait result from single nucleotide changes in genes; it is likely that a subset of SNPs is functionally important in complex traits. Last, promising high-throughput genotyping methods are now available for efficient genotyping of SNPs (Wang et al. 1998).

The methods for genotyping of single nucleotide polymorphisms include gel-based and non-gel-based approaches. The gel-based genotyping approaches are for example DNA sequencing, RFLP and single-strand conformation polymorphism (SSCP) analysis (Orita et al. 1989). All non-gel-based genotyping approaches achieve allelic discrimination by one of four mechanisms: allele-specific hybridization, allele-specific primer extension, allele-specific oligonucleotide ligation and allele-specific cleavage of a flap probe. By combining one of these allelic discrimination mechanisms with either a homogeneous or solid-phase reaction format or a detection method such as fluorescence intensity, fluorescence polarization or mass spectrometry fast, reliable, automated and large-scale genotyping is warranted (Kwok 2000, Griffin et al. 1999, Li et al. 1999).

2.5 Linkage and association analysis in complex disease

Porcine genome maps have been constructed based mainly on three methods: genetic linkage mapping, RH panal mapping and cytogenetic mapping. The current numbers of markers place on the porcine genome are over 1800 in linkage map from (Archibald et al. 1995, Marklund et al. 1996, Rohrer et al. 1996), 900 in RH map form (Hawken et al. 1999), and 700 in cytogentic map form (Yerle et al. 1995, 1997).

Genetic linkage techniques are powerful tools for analyzing complex disease-related genes because they detect genes that have a major influence on the development of a disease (Greenberg 1993). However, linkage studies are less sensitive than association studies because they do not detect less influential genes (Greenberg 1993). A linkage study, therefore, may be negative in the absence of major genes contributing to disease susceptibility. The principle of linkage analysis is based on the fact that if two genes or markers are close together on a chromosome, they will cosegregate because the likelihood that a recombination will occur between them during meiosis is low. Therefore, if a tested marker is close to a disease susceptibility gene, its alleles will cosegregate with the disease in families. The logarithm of odds (LOD) score is the measure of the likelihood of linkage between a disease and a genetic marker (Ott 1996). The LOD score is the base-10 logarithm of the odds ratio in favour of linkage. In Mendelian disorders, a LOD score of greater than 3 (*i.e.*, odds ratio greater than 1000) is considered strong evidence for linkage (Ott 1996).

Association studies are more sensitive than linkage studies and therefore are better for fine-mapping of linked genetic regions, because the association signal increases as the markers get closer to the susceptibility gene. Association analysis is highly sensitive and may detect genes contributing less than 5% of the total genetic contribution to a disease (Risch and Merikangas 1996).

Association analyses are performed by comparing the frequency of the allele studied in family. This is usually performed by typing each individual of each for the tested marker, but recently, methods for DNA pooling have been developed, which could simplify large-scale association studies (Sham et al. 2002). There are at least two possible explanations for the existence of an association between an allele and a disease: 1) the associated allele itself is the genetic variant causing an increased risk for the disease; and 2) the associated allele itself is not causing the disease but rather a gene in linkage disequilibrium (LD) with it (Hodge 1994). Linkage disequilibrium exists when chromosomes with the mutant allele at the disease locus carry certain marker alleles more often than expected.

The population-based association method may produce spurious associations if the patients and controls are not accurately matched (population stratification) (Spielman

et al. 1993). Therefore, new association tests have been developed that are family-based and use an internal control group from within each family, thus avoiding the necessity to match patients and controls altogether. The most widely used family-based association test is the transmission disequilibrium test (TDT) (Spielman et al. 1993, Falk and Rubinstein 1987, Schaid and Sommer 1994). The TDT is based on comparison of parental marker alleles that are transmitted and those that are not transmitted to affected offspring. The TDT can also serve as a linkage test if there is a known LD between the tested marker and the disease.

2.6 Candidate genes for inverted teat defect

2.6.1 Transforming growth factor beta 1 gene (TGFB1)

Transforming growth factor beta belongs to a large family of at least 40 related polypeptides. There are three TGFB isoforms in mammals – TGFB1, TGFB2, and TGF B3. The three isoforms are encoding separate genes that share a common gene structure and encode highly homologous polypeptides, in which an amino-terminal signal sequence and prodomain precede the carboxyl-terminal active domain (Fitzpatrick and Bielefeldt-Ohmann 1999).

2.6.1.1 Gene structure

TGFB1 is a homodimic protein of M_r 25000 synthesized by most eukaryotic cells. Each chain of TGFB1 is first synthesized as a 390 amino acid precursor protein and later cleaved to generate the C-terminal mature protein of 112 amino acids. The porcine TGFB1 nucleotide sequence has been reported (Derynck and Rhee 1987, Kondaiah et al. 1988). The TGFB1 gene consists of 7 exons. Exon 1 exhibits the translation start and encodes the 5´-untranslated region and a part of the amino propeptide. Exons 2, 3, 4 and 5´-region of exon 5 code for the propeptide domain of the TGFB1 molecule and the 3´-end of exon 5 along with exon 6 and the 5´-region of exon 7 code for amino acids of the mature peptide. The remaining 3´-region of exon 7 encodes the 3´untranslated sequence.

Two alternate polyadenylation signals and alternative splicing of the porcine TGFB1 have been observed in the pig (Kondaiah et al. 1988).

2.6.1.2 Polymorphism in TGFB1 gene

In porcine, no polymorphism was observed for TGFB1 with endonucleases AluI, BamHI, BglII, DraI, EcoRI, HaeIII, HindIII, MspI, PstI, PvuII, RsaI, and TaqI (Hu et al. 1997). The human TGFB1 gene consists of seven exons encompassing 23.5 kb on chromosome 19q13.1. Three SNPs in the promoter region, one insertion/deletion in the 5' untranslated region, two SNPs in the signal peptide sequence, one SNP in intron4 and one SNP in exon 5 have been identified previously (Cambien et al. 1996). Polymorphism at position +869 T/C and +915 G/C in the signal protein sequence of the human TGFB1 gene change codon 10 (Leu-Pro) and codon 25 (Arg-Pro) respectively, and polymorphism in exon 5 at position+1632 C/T changes codon 263 (Thr-Ile) This study suggests that the polymorphisms within the TGFB1 gene play at most a small role in ankylosing spondylitis is involved in susceptibility to the disease (Jaakkola et al. 2004).

2.6.1.3 Mapping

The TGFB1 has been localized by *in situ* hybridisation in the q2.2----q2.4 and cen----q2.1 regions of pig chromosome 6 and the chromosomal locations of their human and murine homologues are on chromosome 19q13.1 and 7 respectively. The porcine TGFB1 has been allocated to Sscr6q11-q21 (Yerle et al. 1990a, b).

2.6.1.4 Association

Several polymorphisms in the human TGFB 1 have been identified (Cambien et al. 1996) and TGFB 1 is also a candidate gene for Alzheimer's disease which is located on chromosome 19q13.1-3 (Fujii et al. 1986). Luedecking et al. (2000) reported that the polymorphism in TGFB1 may be modestly associated with the risk of AD.

From the study of Pulleyn et al. (2001), they suggest that one or more polymorphism in the TGF beta1 gene acting to increase the level or the function of the protein may be associated with asthma severity. These results suggest that the allele is associated with the severity rather than the initiation of the asthma or atrophy, in contrast to studies where polymorphism have been compared between asthmatics and controls such as in IL-3. Zhou et al. revealed no evidence that genetic polymorphisms of the TGFB1 of the PDGF families are associated with systemic sclerosis in the Native American population (Zhou et al. 2000). The study show that certain common polymorphism influence blood levels of TGFB1. As it likely that TGFB1 has a role in a number of common important disease, predisposition to these conditions may be associated with alleles at the TGFB1 locus (Grainger et al. 1999).

Several host genetic association studies with polymorphism of the TGFB1 gene have been performed for elucidating the function of polymorphism of the TGFB1 gene. Regarding osteoporosis, the concentration of TGFB1 in human bone tissue was positively related to bone turnover and a large part of the variability of TGFB1 in bone tissue is thought to be due to differences in bone resorption (Pfeilschifter et al. 1998). The substitution proline to leucine at codon 10 in signal sequence of the TGFB1 gene was significantly associated with higher bone mineral density at the lumbar spine, lower spinal bone loss, and a lower susceptibility to vertebral fractures (Yamada et al. 1998).

2.6.2 Relaxin gene (RLN)

The peptide hormone relaxin was discovered in 1926. Hisaw was first to show that relaxin causes relaxation and softening of the pubic ligaments of the guinea pig. Relaxin is a 6000-d polypeptide, structurally related to insulin and the insulin-like growth factors. Unlike insulin, the structure of which is remarkably well conserved among the vertebrates, relaxin sequences can vary by more than 50% between different species. Despite these large sequence variations, relaxins (with few exceptions) have very similar biologic activities in animal test systems. Circulating relaxin is secreted by the corpus luteum. The placenta, decidua, or both also produce relaxin, which does not enter the circulation but may act in an autocrine or paracrine fashion. hCG is a stimulus to luteal relaxin secretion (Goldsmith et al. 1995).

The source of RLN that is secreted into the periperal blood varies among species. For example, the source of relaxin is the corpara lutea in rats (Fields 1984, Golos et al. 1984) and pig (Anderson et al. 1973, Fields and Fields 1985) the placenta in rabbits (Eldridge and Fields 1985), and the uterus in guinea pig (Pardo and Larkin 1982).

2.6.2.1 Gene structure

The porcine relaxin gene contains two exons, separated by a large intron of 5.5 kb. The preprorelaxin coding region, are comprising a signal peptide of 24 amino acids, a B chain of 32 amino acids, a large C peptide of 104 amino acids, and an A chain of 22 amino acids (Haley et al. 1987). The relaxin of all species is containing two chains, A and B covalently linked by two disulfide bonds (the C peptide is trimmed off in the processing of prorelaxin to produce relaxin (John et al. 1981).

Human, porcine and rat preprorelaxins contain a single peptide followed by a B chain 19-35 amino acids, a connecting ‘C’ peptide of approximately 105 amino acids and an A chain of 22 or 24 amino acids. Processing of preprorelaxin to the active form involves removal of the signal and C peptides, and formation of one intra and two interchain disulphide bonds. The positions of these bonds are highly conserved amongst mammalian species (Kemp and Niall 1984), but only 11 amino acids from mature relaxin are conserved in all species studied. Among species, the sequence homology of relaxin is remarkably low, with differences of 30-60%, but the localization of the disulfide bonds and the cysteines is very similar, thus suggesting similar tertiary of the different forms of relaxin (Bryant-Greenwood and Schwabe 1994).

2.6.2.2 Polymorphism

Within the coding sequence, a single base change of (C <-> T) relative to the cDNA sequence (Haley et al. 1982) is observed at position 7041, resulting in a Ser <->Leu change in the carboxyl-terminal peptide at residue 116. There are no known mutations at loci on either mouse chromosome 19 or human chromosome 9 which affect aspects of fertility, pregnancy or parturition (Fowler et al. 1991).

2.6.2.3 Mapping

By study of mouse-human cell hybrids, Crawford et al. (1984) found that both relaxin genes are on 9p (9pter-9q12). Only a single relaxin gene is found in the pig, rat and mouse. In the case of the growth hormone genes, 'extra' genes not predicted by known gene products are found in man only. Structural similarities of relaxin to insulin exist at the level of both the gene and the product. Naggert and Mu (1994) stated that the relaxin gene maps to mouse chromosome 19 near D19Mit23. The human relaxin gene encoding H1 and H2 are localized in close proximity on chromosome 9 at 9p24 whereas the H3 gene is on chromosome 19 at 19p13.3.

2.6.3 Parathyroid hormone like hormone gene (PTHLH)

A PTHLH has been purified (Burtis et al. 1987, Moseley et al. 1987, Strewler et al. 1987) and its cDNA cloned (Suva et al. 1987, Mangin et al. 1988b, Thiede et al. 1988) from malignant tumors associated with the syndrome of HHM. The PTHLH and PTH genes appear to be related on the basis of an ancient duplication event, following which they have clearly evolved separately.

2.6.3.1 Gene structure

The human PTHLH gene structure has been identified in several investigations. Initial studies of the human PTHLH gene structure reported it to be a complex transcriptional unit spanning more than 12 kilobases (kb) of genomic DNA and containing six exons. Exon 4, 5 and 6 are alternatively spliced to generate three classes of PTHLH mRNAs with distinct 3'ends, each encoding unique C-terminal PTHLH sequences (Mangin et al. 1988a). Yasuda et al. (1989) isolated a single-copy PTHLH gene from a human placental genomic library. They found that the gene spans 13 kb and contains 7 exons. The organization was closely similar to that of the parathyroid (PTH) gene, suggesting a common evolutionary origin. Exon 1 and exon 2 encode two alternative 5' untranslated regions each driven by different promoters. Exon 3 encodes a leader sequence and the majority of the pro sequence, and exon 4 encodes the remainder pro sequence and 139 amino acids representing the majority or all of the mature peptide. These two exons 6

and 7 each encode potential alternative carboxyl – terminal extensions to the peptide (of 34 and 2 amino acids respectively) as well as alternative 3' untranslated regions. Consequently, as a result of alternative splicing, mRNAs may result which encode at least 2 different 5' untranslated regions, 3 different 3' untranslated regions and 3 isoforms of the mature peptide of 139, 141, and 173 amino acids (Goltzman et al. 1992).

Mangin et al. (1990b) reported that human PTHLH gene contain 8 exons and spans more than 15 kb of genomic DNA with apparent promoter elements lying immediately up-stream of exon 1A and 2. The TATA box is present approximately 25 bp upstream of exon 1A. Various consensus sequences for transcription factors, such as AP-1, AP-2 and SP1 can be localized. All exon-intron junctions have the consensus GT-AG sequence, including the splice junction at the end of exon IV which contains a GT beginning in the Arg codon for amino acid 139 of the mature peptide.

Suva et al. (1989) investigated the alternate cDNA species isolated were representative of functional BEN cell mRNAs. Thus, BEN cells produce four major PTHLH mRNA species which are alternately spliced at both the 5' and 3' ends. Clearly, the two separate PTHLH promoters produce multiple mRNA species.

Mouse exon 1 and exon 2 correspond to 5' exon 1C and 2 of human gene and are separated by a 46 bp putative promoter region. Exon 3 encodes prepro PTHLH sequence. These organization features are similar to those of the human gene, except that the 5' flanking region of the mouse gene appears to be less complex. The organization of the remaining portions of the mouse gene was found to be simpler than that of the human gene (Mangin et al. 1990a). The bovine has also a genomic organization similar to the gene of the rat and mouse (Wojcik et al. 1998).

2.6.3.2 Polymorphism

Pausova et al. (1993) have characterized a VNTR polymorphism in the hPTHLH gene that is located in an intron 100-bp downstream of exon VI that encodes a 3' untranslated region. By PCR analysis eight different alleles were identified in a group of 112 unrelated individuals. All eight alleles were sequenced and the repeat unit was identified as the general sequence [G(TA)nC]N, where n = 4 to 11 and N = 3 to 17. This

polymorphic sequence-tagged site will be useful for mapping chromosome 12p and will aid in testing for linkage of genetic diseases to the hPTHLH gene.

Human PTHLH alternative 3'mRNA splicing results in transcripts, which encode three PTHLH isoforms and have been identified in amnion (Curtis et al. 1998). Alternative 3' splicing to exons VII, VIII and IX produces transcripts encoding three different isoforms of the protein, PTHLH 1-139, 1-173 and 1-141 respectively (Mangin et al. 1988b). As compared to the complex hybridization pattern observed on Northern analysis of human RNAs (Ikeda et al. 1988a, b, Mangin et al. 1988b, 1989), RNAs prepared from rodent tumors and tissues appear to contain a single broad hybridization band (Ikeda et al. 1988a, b, 1989, Thiede and Rodan 1988, Drucker et al. 1989, Yasuda et al. 1989), suggesting that the mouse and rat PTHLH genes might have a simpler organization than the human gene.

2.6.3.3 Association

The PTHLH gene is a candidate gene for a skin carcinogenesis susceptibility locus mapping to distal chromosome 6 (Manenti et al. 2000b). PTHLH shows a Thr166Pro amino acid polymorphism in inbred mouse strains. The $PTHLH^{Pro}$ and $PTHLH^{Thr}$ alleles are linked with high and low genetic susceptibility to two-stage skin carcinogenesis of outbred Car-S (susceptible) and Car-R (resistant) mice, respectively (Manenti et al. 2000b, Dragani 2003). Allele 2 at a VNTR polymorphism in the PTHLH gene showed borderline statistically significant associations with lung cancer risk. Furthermore, the same alleles were significantly associated with tumour prognosis. Studies on association were then performed in the Japanese and in European populations (Manenti et al. 2000a).

2.6.3.4 Mapping

Mangin et al. (1988a) assigned the human PTHLH gene to 12p12.1-p11.2 by a combination of Southern analysis of somatic cell hybrid DNA and in situ hybridization. Hendy et al. (1989) and Hendy GN and Goltzman D (1990) assigned the corresponding

gene in the mouse to chromosome 6 by means of Southern blot analysis of DNAs isolated from a panel of mouse/chinese hamster cell hybrids.

3 Material and Methods

3.1 Material

3.1.1 Experimental animals

3.1.1.1 Unrelated animals

For polymorphism screening in three candidate genes, one individual from each of pig breeds namely Duroc, Hampshire, German landrace, Pietrain and Miniature pig, was used in this study.

3.1.1.2 Bonn-Berlin DUMI resource population

The resource population was developed from a reciprocal cross between Duroc and Berlin-miniature pig (Hardge et al. 1999) as diagrammed in figure 2. The Berlin miniature pig is a cross of Vietnamese Pot Belly, Saddle Back and German Landrace pigs. Five sows of Berlin miniature pigs were crossed with a Duroc while four Duroc sows were crossed with a Berlin miniature boar to produce parental generation animals (F1). Forty seven F1 animals generated the F2 animals (n=902) of Berlin-Bonn-DUMI resource population. About half of the F2-population (n=485) were reared at the research station of the Humboldt University, Berlin (F2-Berlin-DUMI). F2 animals (F2=417), that are reared and performance tested at the research farm of Frankenfrost, Institute of Animal Science, University of Bonn, were generated from 11 sows and 3 boars of the F1 generation and were used to investigate in this study.

Animals of this population have been inspected for traits of mammary gland phenotype including the scoring of the appearance of inverted teats. Within this resource population the disorder occurs with a frequency of 42%. That provides excellent conditions for detecting of the responsible genetic factors.

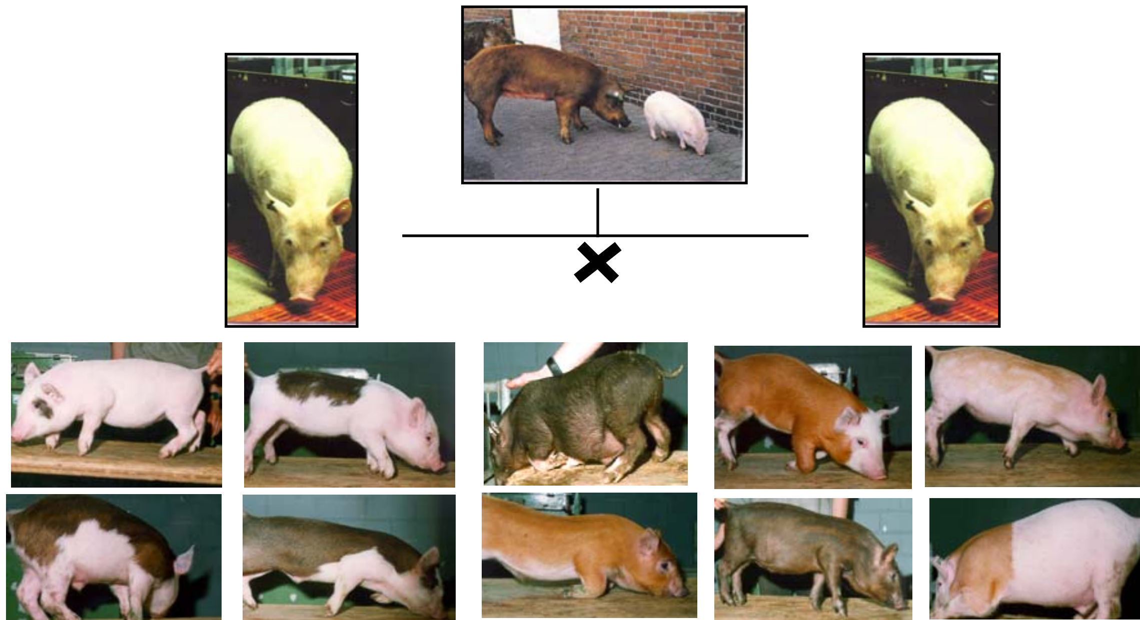

Figure 2: Bonn- Berlin DUMI resource population

3.1.1.3 Commercial pigs

Tissue samples of commercial pig breeds including the German Landrace (n=30), Large White (n=30) and Pietrain (n=30) were obtained from the Landesverband Rheinischer Schweinezüchter, Bonn Germany. The Genomic DNA was isolated and used for determining the polymorphism in this study.

3.1.2 Chemicals and equipments

3.1.2.1 Chemicals

Biozym Diagnotik (Hessisch-Oldendorf, Germany)

- Sequagel Complete Buffer Reagent
- Sequagel XR (6%) (SQG-XR-842)

Carl Roth GmbH (Karlsruhe, Germany)

- Agar-Agar

- Acetic acid
- Ampicillin sodium salt
- Chloroform
- Glacial Acetic Acid
- Nitric acid
- Phenol/Chloroform (50:50)
- Silver Nitrate
- Sodium carbonate
- TEMED ($C_6H_{16}N_2$)
- Tris (hydroxymethyl)-aminomethane
- X-Gal**:** $C_{14}H_{15}BrClNO_6$;
- IPTG dioxanfrei (Isopropyl β–D –Thiogalactosid)
- N, N'-Dimethylformamide

Gibco BRL®, Life Technologies (Karlsruhe, Germany)

- TRIzol Reagent

Sigma-Aldrich GmbH (Taufkirchen, Germany)

- Agarose
- Isopropanol

3.1.2.2 Reagents

40% acrylamide/bis-acrylamide (49:1) :

Acrylamide	39.2 g
N, N-methylene-bisacrylamide	0.8 g
Distilled water to 250 ml	

Agarose gel loading buffer :

0.25% Bromophenol blue

0.25% Xylene cyanol

120 mM EDTA

Ampicillin (10 mg/ml) :

Ampicillin powder	2 g
Sterile, distilled water,	40 ml
Filtrate with 0.45 µl filter	

Blue dextran loading buffer :

Blue dextran	50 mg
EDTA (0.5M)	50 µl
Water	950 µl
Formamide	5 ml

DEPC-treated water :

DEPC	1 ml/l water

Incubation at 37 ^{o}C and heat inactivated by autoclaving (121 ^{o}C for 30 min)

1.2% FA gel :

Agarose	1.2 g
10x FA buffer	10 ml
37% formaldehyde	1.8 ml
Ethedium bromide	1 µl

Fill up to 90 ml with DEPC-treated water and boil

Proteinase K :	10 mg/ml
SDS :	1% (w/v)

SSCP loading buffer (per 50 ml) :

Formamide	47.5 ml
Sodium hydroxide	200 mg
Bromophenol blue	125.0 mg
Xylenecyanol	125.0 mg

X-Gal (50 mg/ml) :

X-Gal	50 mg
N, N-dimethylformamide.	1 ml

Stored at -20 ^{o}C

3.1.2.3 Buffers

10x FA buffer:

MOPS	200 mM
Sodium acetate	50 mM

EDTA	10 mM

50x TAE (per litre)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100 ml

10x TBE (per litre)

Tris borate, pH 8.0	90 mM
EDTA, pH 8.0	20 mM

10x TE (per 500 ml)

1 M Tris pH 8.0	50 ml
0.5M EDTA pH 8	0.5 ml
ddH_2O	445 ml

Autoclave to sterilize (121 ^oC for 30 min)

3.1.2.4 Competent cells

E.coli strain DH5α (Stratagene, Amsterdam), JM109 (Promega, Mannheim)

3.1.2.5 Medium

LB-agar plate

Sodium chloride	8.0 g
Peptone	8.0 g
Yeast extract	4.0 g
Agar-Agar	12.0 g

Sodium hydroxide (40.0 mg/ml)	480.0 µl
Water added to	800.0 ml
Autoclave (121 °C for 30 min)	

LB-broth

Sodium chloride	8.0 g
Peptone	8.0 g
Yeast extract	4.0 g
Sodium hydroxide (40.0 mg/ml)	480.0 µl
Water added to	800.0 ml
Autoclave (121 °C for 30 min)	

3.1.2.6 Commercial kits

SequiTherm EXCEL™II (Biozym Diagnostic)

PGEM®-T and PGEM®-T Easy Vector Systems (Promega)

Gen Elute™ Plasmid Miniprep Kit (Sigma)

Recombinant RNasin®Ribonuclease Inhibitor (Promega)

RNeasy Mini kit (Qiagen GmbH)

RQ1 RNase-Free DNAse (Promega)

SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech USA)

3.1.2.7 Enzymes

SuperScript™ II Rnase H⁻Reverse Transcriptase (Gibco BRL®, Karlsruhe), (Invitrogen life technologies, Karlsruhe)

Restriction enzyme: CfoI (Promega)

Pfu DNA polymerase (Promega)

Taq DNA polymerase (Sigma)

PLATINUM® Taq DNA polymerase (GibcoBRL®)

AccuTaq DNA polymerase (Sigma)

3.1.2.8 Equipments

- Centrifugation

  HERMLE Z 323K, Wehingen

- Electrophoresis/DNA Analyzer: Automated sequencer

  LI-COR, DNA Analyzer, GENE READER 4200 (MWG-BIOTECH)

- Electrophoresis system

  For agarose gel: BioRad, Göttingen

  Vertical apparatus: Consort, Turnhout

  Power supply: PAC 3000 BioRad

- Gel Dry (BioRad, München)

- Spectrophotometer

  PM 2K (ZEISS, Germany)

  Beckman, Unterschleissheim-Lohhof

-Thermocycler:

PTC-100 Programmable Thermal Controller (MJ Research, Inc. USA)

-UV Transilluminator (Uvi-tec)

Uni Equip, Martinsried

3.1.2.9 Used software

BLAST program

http://www.ncbi.nlm.nih.gov/BLAST/

DNA alignment

http://searchlauncher.bcm.tmc.edu/

FBAT statistic analysis

http://www.biostat.harvard.edu/~fbat/fbat.htm

Image analysis program (Version 4)

Li-COR Biotechnology, USA

Multiple Sequence Alignment

http://pbil.ibcp.fr/htm/index.php

Restriction enzyme

http://rebase.neb.com/rebase/rebase.html

Tm determination

http://alces.med.umn.edu/rawtm.html

3.2 Methods

3.2.1 RNA isolation and cDNA synthesis

3.2.1.1 RNA isolation

Liver tissue samples of five pig breeds (Hampshire, Duroc, German Landrace, Pietrain and Miniature pigs) were used for RNA isolation. Freezed tissue samples (1 to 10 mg) were powdered by mortar and pestle, 1 ml of TRIZOL ® reagent was added and homogenised by using syringe. The homogenised samples were incubated for 5 min at room temperature. Then 0.2 ml of chloroform was added to homogenised samples, mixed thoroughly by shaking and incubated for 15 min at 15 – 30 °C. Samples were centrifuged at 12,000 g for 15 min at 2 to 8 °C. The RNA remains exclusively in the upper aqueous phase which was transferred to a fresh tube. The RNA in the aqueous phase was precipitated by adding 0.5 ml of isopropyl alcohol. The samples were incubated at room at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 2 to 8 °C. The supernatant was removed and the RNA pellet was washed once with 1 ml of 75 % ethanol. After centrifugation and removal of supernatant the RNA pellet was air dried and dissolved in 50 µl RNase-free water and stored at –80 °C for further use. The RNA was treated with DNase to remove residual DNA. The DNA digestion was performed by mixing following components in a 0.5 ml RNase free tube: 25 µl RNA, 5 µl 10X buffer, 2 µl RQ1 RNase-Free DNase, 1 µl RNase inhibitor (40 U/µl), and 17 µl RNase-free water and incubated at 37 °C for 1 hour. After incubation, the RNA was purified by RNeasy Mini Kit (Qiagen). To assess the quality of RNA, 2 µl RNA sample was electrophoresed in duplicate on 0.66 M formaldehyde/1.2 % agarose gel. RNA was checked for any DNA contamination by performing PCR with Beta-actin primer (beta actin Fw -GAG AAG CTCT GCT ACG TCG C, beta actin Rw-CCA GAC AGC ACC GTG TTG GC). The purified RNA was used to synthesize cDNA.

3.2.1.2 cDNA synthesis

The total RNA was used to synthesize first strand cDNA. The reaction was performed in a nuclease-free microcentrifuge tube, 1 µl oligo dT (11)N (500 µg/ml), 1 ng to 5 ng total RNA, 1 µl 10 mM dNTP mix and 12 µl sterile water was added in 0.2 ml PCR tube. The mixture was heated at 70 °C for 10 min and immediately chilled on ice. The contents of the tube were collected in bottom by brief centrifugation and 4 µl 5 X first-strand Buffer (Gibco BRL, Karlsruhe Germany), 2 µl 0.1 M DTT, and 1 µl RNase OUT Recominant Ribonuclease Inhibitor (40 U/µl) was added. The reaction was gently mixed and incubated at 40 °C for 2 min. One microlitre (200 units) of Superscript^{TM} II RNase H reverse transcriptase was added and further incubated at 42 °C for 90 min. The reaction was inactivated by heating at 70 °C for 15 min. The cDNA was diluted 1:4 in RNase free water and was used as a template for subsequent PCR amplifications. The cDNA samples were stored at -20 ^{o}C for further use.

3.2.2 DNA isolation from agarose gel

The band of interest was excised with a sterile razor blade, placed in a microcentrifuge tube, frozen at –20 ^{o}C for at least 1 hour. Chopped by blue pipet tip and 500 µl TE buffer was added. The gel was homogenized by repeated pipeting using syringe attached with a needle. Five hundred microlitres of phenol-chloroform (1:1) was added to the DNA solution and vigorously vortexed for a few seconds to form an emulsion. After centrifugation at 12,000 g for 15 min the upper aqueous layer was carefully removed to a new tube. Equal volume of chloroform was added to the aqueous phase, vortexed and centrifuged for 15 min at 12,000 g to allow phase separation. The aqueous layer was transferred to a new tube. One-tenth volume of 3M sodium acetate (pH 5) and two volumes of cold 100 % ethanol were added to the DNA sample in a microcentrifuge tube and mixed by inversion. This precipitation was performed by incubation at –20 ^{o}C overnight or at –80 ^{o}C for at least 30 min. To recover the precipitated DNA, the mix was centrifuged at 12,000 g in a microcentrifuge for 20 min at 4 ^{o}C. After the supernatant was discarded, the DNA pellet was washed with a cold 75 % ethanol (corresponding to about two volume of the original sample) being first incubated at room temperature for 5-10 min and centrifuged again for 5 min. The supernatant is

drained being inverted on a paper towel until DNA pellet is air dried. Finally, DNA pellet is dissolved with 5 µl of distilled H_2O.

3.2.3 Ligation

The PCR fragment was ligated with pGEM-T vector (Promega, Mannheim Germany). The reaction was performed in 5 µl total volume, which contained 2.5 µl 2 X Ligation buffer, 0.5 µl pGEM® – T Vector, 1.5 µl PCR products and 0.5 µl T 4 DNA Ligase. The reaction was gently mixed and incubated for 1 hour at room temperature or at 4 °C overnight.

3.2.4 Transformation

Competent *E.coli* DH5α was aliquoted to chilled polypropylene tubes and 3 µl of pGEM ligation reaction mix was added per 60 µl cells. The mixture was incubated on ice for 30 min. The cells were then heat shocked at 42 ^oC for 90 sec, incubated on ice for a further 2 min, and 700 µl LB medium was added at room temperature. The culture was incubated at 37 ^oC in shaking incubator for 90 min. For each ligation reaction, two LB plates supplemented with ampicillin (100 µg/µl) were prepared by adding 0.10M IPTG and 20 µl of X-Gal and spreaded with glass pipett being allowed to absorb for 20 min prior to use. The transformed bacterial culture was plated to the prepared plates in duplicate and incubated at 37 ^oC overnight. Successful cloning of the insert will produce white colonies as it interrupts the coding of ß-galactosidase as against the blue colonies which does not have any insert. The presence of DNA insert in these colonies was confirmed by PCR of the supernatant following lysis (cell suspension was boiled at 95 ^oC for 15 min). Each of the 20 µl PCR reaction contained 10 µl lysated supernatant as template DNA, 0.2 µM of M13 primer (Fw: TTGTAAAACGACGGCCAGT, Rw: CAGGAAAC AGCTATGACC), 50 µM of each dNTP and 0.5 U of Taq polymerase (Sigma) in 1 X PCR reaction buffer with 1.5 mM $MgCl_2$ (Sigma). The PCR was performed at 94 ^oC for 5 min, followed by 35 cycles of 94 ^oC for 30 sec, 65 ^oC for 30 sec and 70 ^oC for 30 sec following by final extension at 70 ^oC for 5 min. The extension time may be longer than 30 sec for a longer fragment.

3.2.5 Sequencing

Each positive clone was sequenced by chain-termination procedures using SequiTherm Excel II DNA sequencing Kit (Epicentre Technologies, Biozym). The 4 termination mixtures were prepared by dispensing 1 µl of the ddGTP, ddATP, ddTTP, ddCTP mix into four correspondingly marked tubes. The 2 µl reaction of premix (3.6 µl of 3.5X Sequencing Buffer, 0.25 µl 10 µM SP6 primer-800 IRD-labeled (AATTAACCTCACTAA AGGG), 0.25 µl 10 µM T7 primer-700 IRD-labeled (TAATACGACTCACTATAGGG), 0.5 µl Taq DNA polymerase, 3 µl template DNA, 0.9 µl deionized water) was added into the different termination mix. PCR was performed for 29 cycles at 94 ° C for 30 sec, 59 - 60 °C for 30 sec, 70 °C for 1 min and 1.5 µl of the stop solution was added to all samples, after the thermal cycling is completed. If the samples cannot be analysed immediately, the samples were stored at -20 °C. About 1.5 µl of heat denatured samples were loaded on a standard 6 % polyacrylamide sequencing gel (30 ml SequaGel® XR, 7.5 ml SequaGel Complete Buffer, 400 ml DMSO, 300 µl 10 % APS). Electrophoresis was performed on LI-COR model 4200 automated DNA sequencer in 1 X TBE buffer at a power of 1500 V, 50 mA, 50 W and 50 ^{o}C temperature. Sequence data were analyzed by Image Analysis program, version 4.10 (Li-COR Biotechnology).

3.3 Screening for polymorphism in genes

3.3.1. Transforming growth factor beta 1 gene (TGFB1) screening

The porcine TGFB1 gene has been published by Kondaiah et al. in 1988. Specific primers were designed based on the published TGFB1 sequence (Accession number AF461808) that reveal overlapping fragments of about 400 bp in size covering the cDNA sequence from exon 1 to the 3´-UTR. Primer sequences are shown in table 3 below. The cDNA fragments obtained from liver of five pig breeds namely: Hampshire, Duroc, Pietrain, German Landrace and Berlin-Miniature pig –were comparatively sequenced which were described in section 3.2.1-3.2.5. The sequences were aligned by using BLAST or Multiple alignment programs to find the polymorphism.

3.3.1.1 PCR condition

Each of the 25 µl of PCR reaction contained 50 ng genomic DNA, 0.2 µM of each primer, 50 µM of each dNTP, 0.5 U of Taq polymerase (Pfu) and 1.5 mM $MgCl_2$ in 1 X PCR buffer. PCR was performed in the thermal cycler PTC 100 (MJ Research USA) at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 62 °C for 30 sec, 72 °C for 30 sec and final extension at 72 °C for 5 min.

Table 3: Primer sequence for SNP identification in TGFB1 gene

Primer	Sequence	Annaling Temperature (°C)	Product size (bp)
Tgf1Fw	TGCTGTGGCTGCTAGTGCTG	62	501
Tgf1Rw	CAATGATTCCTGGCGCTACC		
Tgf2Fw	AAGTGGAGCAGCACGTGGAG	62	469
Tgf2Rw;	GAACCCAAGGGCTACCATGC		
Tgf3 Fw	ACTTCCGGAAGGACCTGG	60	260
Tgf2066Rw	GCAGGAACGCACGATCATGT		
Tgf2047 Fw	ACATGATCGTGCGTTCCTGC	60	186
Tgf32346Rw	GTTCACCAGGAGTACCTGCT		
Tgf4Fw	GTCTTCCTTCGGACGTTACC	62	480
Tgf4Rw	CCACTATGGGCTTCCTTTTC		
Tgf5Fw	TTCCATATGTCTCAGGTGCG	62	389
Tgf5Rw	ATTTACAGAAACAGGCAGCG		

3.3.1.2 PCR condition and single strand conformation polymorphism (SSCP) analysis

Primers covering the polymorphism site (or SNP) were designed to amplify a fragment length of 200 bp to perform SSCP. Each 10 µl of PCR reaction contained 25 ng genomic DNA, 0.2 µM of each primer (Fw: CTACTCATCCATCTGAGTG, Rw: GAAGCAGT AGTTGGTATCCA), 50 µM of each dNTP, 0.5 U of Taq polymerase (Sigma) in 1 X PCR reaction buffer with 1.5 mM $MgCl_2$ (Sigma). PCRs were performed at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 65 °C for 30 sec, 72 °C for 30 sec and final

extension at 72 ^{o}C for 5 min. The PCR products were diluted 1:1 with loading buffer (95 % formamide, 10 mM NaOH, 0.25 % bromophenol blue, and 0.25 % xylene cyanol), denatured at 95 ^{o}C for 5 min, chilled on ice and loaded on 12 % polyacryamide gel (acrylamide:bisacrylamide = 49:1) containing 5 % glycerol/formamide (3:1). Gels were run at 15 W for 3 hours at room temperature in 0.5 X TBE. The DNA was visualized with silver staining technique.

3.3.1.3 Silver staining procedure

Electrophoresis was performed till bromophenol blue runs to the end of gel. The gel was fixed for 20 min in 10 % glacial acetic acid. Then moved to 0.2 % nitric acid for 15 min and rinsed 2 times with deionized water. The gel was stained for 20 min with 0.2 % silver nitrate (w/v in H_2O), rinsed in deionized water and developed in 3% sodium carbonate containing 8 ml/l of 37 % formaldehyde and 520 µl/l of 0.1 N sodium thiosulfate which were cooled down to 4°C before use. The development times were between 3-5 min. After visualization of the bands, the reaction was stopped by immersion of the gel in 10 % glacial acetic acid for 3 min. Then the gel was washed with distilled water 3 times and dried at 80 ^{o}C for 2 hours on Gel-Blotting paper (Roth, Karlsruhe Germany) for analysis and documentation.

3.3.2 Relaxin gene screening

The Relaxin gene has been published by Haley et al. in 1987. Three pairs of primers were designed based on the published relaxin sequence (Accession number J02792) (Table 4) that reveal overlapping fragments of about 300-500 bp in size covering the DNA sequence from part of 5´-UTR to the 3´-UTR. Genomic DNA from 5 breeds (Hampshire, Duroc, Pietrain, German Landrace and Berlin-Miniature pig) were used for screening a polymorphism of this gene. All DNA fragment were cloned and comparatively sequenced as described in section 3.2.1-3.2.5.

Table 4: Primer sequence for SNP identification in RLN gene

primer	sequence	annealing temperature (°C)	Product size (bp)
RLN6880Fw	CACAGGAGCTGAAGGCAACA	59.4	216
RLN7076Rw	CGCTCAGTGTCATACGGAAC		
RLN1070Fw	TGAAACGCCTGGAGCAGAAG	59.4	331
RLN1381Rw	CAGCCAATTAGGTCTCGAGC		
RLN7070Fw	GAGACTGTTCCGTATGACAC	57.3	541
RLN7561Rw	GAAGACTTTGGGCATCAGGT		
RLN1070Fw	TGAAACGCCTGGAGCAGAAG	59.4	228
RLN1278Rw	GTTTCCAGCTGAGGCTCTTC		

3.3.2.1 PCR condition

PCR amplification was performed in 20 µl reaction volume using 25 ng of genomic DNA, 0.2 µM of each primer (Table 4), 50 µM of each dNTP, 0.5 U of Taq polymerase (Sigma) in 1 X PCR reaction buffer with 1.5 mM $MgCl_2$ (Sigma). Thermalcyling was performed using the following touchdown program; initial denaturation for 5 min at 95 °C, 10 cycles each 30 sec at 95 °C, 30 sec at 65-60 °C (-0.5 per cycle), and 30 sec at 72 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C and 5 min final extension at 72 °C.

3.3.2.2 Genotyping the porcine RLN gene polymorphism

Single strand conformation polymorphism (SSCP)

The SSCP analysis was performed to detect polymorphism in PCR product. The PCR was performed by using Exn22 primer (Table 5) and a standard PCR condition. The products were diluted 1:1 with loading buffer (95 % formamide, 10 mM NaOH, 0.25 % bromophenol blue and 0.25 % xylene cyanol), denatured at 95 °C for 5 min, chilled on ice and loaded on 12 % polyacryamide gel (acrylamide:bisacrylamide = 49:1). Gels

were run at 12 W for 2.5 hours at room temperature in 0.5 X TBE buffers (4 ^oC). The DNA was visualized by silver staining technique.

Table 5: The primer and method of genotyping for RLN gene

Primer		sequences	Method
Int9	Fw:	TGAAACGCCTGGAGCAGAAG	RFLP
	Rw:	CAGCCAATTAGGTCTCGAGC	
Exn 22	Fw:	TGAAACGCCTGGAGCAGAAG	SSCP
	Rw:	GTTTCCAGCTGAGGCTCTTC	

Restriction fragment length polymorphism (RFLP)

The fragment covering the polymorphic site was amplified by using specific primer (Table 5) and standard PCR condition. Restriction digestion of the product was carried out in 10 µl reaction volume containing 1 U of CfoI enzyme per µg of starting DNA. 1 µl of 10 X restriction buffer and incubated at 37 ^oC overnight to ensure complete digestion. The digestions were assayed by 2 % agarose gel electrophoresis versus non-digested DNA and marker.

3.3.3 Identification of parathyroid hormone like hormone gene (PTHLP)

3.3.3.1 Isolation of porcine PTHLP gene by RACE-PCR

Reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of 5'- and 3'-cDNA ends (5'- and 3'- RACE)-PCR were performed on mRNA of porcine ovary and muscle tissue. The first-strand cDNA synthesis was performed by SMART^{TM} RACE cDNA Amplification Kit (BD Biosciences Clontech, USA) as the manufacture's protocol. The preparation of 5'/3'RACE cDNAs synthesis, the reactions contained with 1-3 µl RNA ovary sample (1 µg/µl) then, following to add 1 µl of 5'-CDS primer and 1 µl SMART II A oligo in 5'-RACE cDNA reaction. For the 3'-RACE cDNA synthesis, the reaction was used 1 µl of 3'-CDS primer A. The sequences of primers were shown

in table 6. Final reaction volume of each reaction was made up to 5 µl with sterile H_2O and both reactions were incubated at 70 °C for 2 min and placed on ice for 2 min. Then 2 µl 5X First-Strand Buffer, 1 µl DTT (20 mM), 1 µl dNTP Mix (10 mM), and 1 µl PowerScript Reverse Transcriptase were added to each tube. The contents were gently mixed, and spined briefly to collect the contents at bottom. The reactions were incubated at 42 °C for 1.5 hr in an air incubator. The first-strand reaction product was diluted with Tricine-EDTA buffer, and heated at 72 °C for 7 min. The cDNA was stored at –20 °C for further use.

The RACE-PCR reactions were performed in 25 µl volume, 10X Buffer (minus Mg), 10mM dNTP, 50 mM $MgCl_2$, 10 X universal primers A Mix (UPM) (Table 6), 0.2 µM primer (GSP) (Table 6), and 0.5 U PLATINUM® Taq DNA polymerase (GibcoBRL®, Life Technologies). The 5'-RACE PCR amplification PCR were followed by the thermalcycling at 95 °C 3 min, 7 cycles of 94 °C 20 sec, 71 °C 20 sec, 72 °C 2min, and 38 cycles of 94 °C 20 sec, 68 °C 20 sec, 72 °C 2 min, and final extension by 72 °C for 5 min. The 3'-RACE PCR amplification were performed by the initiation 95 °C 5 min, 38 cycles of 94 °C for 30 sec, 68 °C for 30 sec, 72 °C for 2 min and final extension at 72 °C for 5 min.

Table 6: List of primers employed for RT-PCR and 5'-and 3'-RACE-PCR

Primer	sequence
SMART II A oligo	AAGCAGTGGTATCAACGCAGAGAGTACGCGGG
5'-CDS primer	5'-$(T)_{25}N_{-1}N$-3' (N=A, C, G, or T; N_{-1}=A, G, or C)
3'-CDS primer A	AAGCAGTGGTATCAACGCAGAGAGTAC$(T)30N_{-1}N$ (N=A, C, G, or T; N_{-1}=A, G, or C)
UPM Long	TACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGAGT
Short	ATACGACTCACTATAGGGC
GSP : PTHLH-RACE-3'	TGTCTGAACACCAGCTCCTCCATGACAAG
: PTHLH-RACE-5'	GGTTTCCTGAGTCAGGTATCTGCCCTC

* GSP (Gene specific primer) primers were designed based on EST Accession number BI181671 and BI181482

Table 7: PTHLH primers for intron isolation

primer	sequence	Annealing temperature (^{o}C)
PTHLP5-73FW	TGCCCTGCTCCCTGAAC	58.6
PTHLP5-393RW	GAGCTCCTCCACCGAGC	59.3

3.3.3.2 Linkage mapping analysis by radiation hybrid panel

The sequence from the 3′UTR of the gene was used for primer design. Primer pair was first optimized on pig and hamster DNA for determining the annealing temperature that reproducibly amplified pig DNA without an interfering product from the hamster. PCR was performed on the 118 radiation hybrid cell lines from the RHDF-5000.2 panel. PCR reactions were performed in 15 µl reaction volume using 25 ng DNA from each clone in a final volume of 15 µL containing 10 pmol of each primer (Pthrp_map3'Rw GATTCATTAGAATCAACC, Pthrp_map3'Fw CAGAGACCTTCAGAGACGT), 50 µM dNTPs, 1.5 mM $MgCl_2$, and 0.5 U Taq DNA Polymerase (Promega). PCR cycles were performed using the following touchdown program; initial denaturation for 5 min at 95 ^{o}C, 10 cycles each 30 sec at 95 ^{o}C, 30 sec at 60-55 ^{o}C (-0.5 per cycle), and 30 sec at 72 ^{o}C, followed by 35 cycles of 30 sec at 95 ^{o}C, 30 sec at 55 ^{o}C and 30 sec at 72 ^{o}C and 5 min final extension at 72 ^{o}C. PCR products were electrophoresed on 2 % agarose gels stained with ethidium bromide and visualized under UV light.

The statistical analysis for placement of marker was done with the whole RH data set by using the MultiMap software (http://imprh.toulouse.inra.fr/). Assignment of new marker to chromosomes was done by two-point linkage analysis in a two-step process.

Table 8: Primer and probe sequences used for SNP genotyping of PTHLH gene

primer/probe	sequence	label
PTHLP-FW	GAGCGTCGCGGTGTTC	-
PTHLP-RW	AGCGCCCGCAGGAG	-
PTHLP-V2	CTGAGCTATTCGGTGCC	VIC/NFQ
PTHLP-M2	CTGAGCTATTTGGTGCC	FAM/NFQ

3.3.3.3 Genotyping method for PTHLH gene

TaqMan allelic discrimination using real-time PCR

Primers and probe for genotyping polymorphism in the exon2 of PTHLH gene were synthesised by 'Assay by design service of SNP genotyping of Applied Biosystems.
Two alleles were discriminated by two differentially labeled hydrolysis probes:
Probe #1 – VIC-labeled probe specific for C allele at position 550 in PTHLP
Probe #2 – FAM-labeled probe specific for T allele at position 550 in PTHLP

For SNP Genotyping, an allelic discrimination assay was designed to genotype this polymorphism using primer PTHLH-FW and RW, primer extension with PTHLH (V2&M2) probe (Table 8) (TaqMan® MGB probes, FAM™ and VIC® dye-labeled) by Assay-by-Design℠ service (ABI PRISM, Applied Biosystems). Polymerase chain reactions were performed on an ABI PRISM 7000 Sequence Detection System in 25 µl volume containing 12.5 µl TaqMan Universal PCR Master Mix (2X), 0.625 µl 40X Assay Mix, and 25 ng Genomic DNA. Amplification was performed using a thermal cycling program of AmpErase UNG activation 2 min 50 °C, AmpliTaq Gold Enzyme activation to 10 min 95 °C and 40 cycles of 15 sec at 92 °C and 1 min at 60 °C. The reaction mixture was prepared and added to 96 well plate having 0.5 µl genomic DNA of 2 positive control for each of the allele, samples to be genotyped and 2 no template control. Plates were 'preread' in ABI prism 7000 instrument and PCR was performed (initial denaturation of 95 °C for 10 minutes and 40 cycles of 92 °C for 15 sec and 60 °C for 1 min). After PCR amplification endpoint plate read was performed. The SDS software calculates the fluorescence measurements made during the plate read and plots

Rn values based on the signals from each well. Using the software the SNPs were determined for each sample

The process for analyzing data for SNP assays involves the following procedures:

1. Creating and setting up an allelic discrimination plate read document
2. Performing an allelic discrimination plate read on an SDS instrument
3. Analyzing the plate read document
4. Making manual allele calls
5. Confirming allele types

3.4 Linkage mapping

Two-point and multiple procedures of the CRI-MAP package version 2.4 were used for linkage mapping (Green et al. 1990).

3.5 Association analysis between the markers and mammary gland trait

3.5.1 Family Based Association Tests (FBAT)

The Family-Based Association Test (FBAT) is recently developed software (http://www.biostat.harvard.edu/~fbat/fbat.htm) that can analyse for association pedigrees with heterogeneous family structure. Furthermore, FBAT allows the analysis of the phenotype as a qualitative trait. The FBAT statistic is based on a linear combination of offspring genotypes and traits; it is calculated under the null hypothesis of no association, conditioning on traits and on parental genotypes. In the present study, the FBAT program (version 1.4) was used to perform the qualitative and quantitative family-based analysis for the Bonn-Berlin DUMI resource population. The quantitative analyses were done under the condition of an additive model (Horvarth et al. 2001). Significant results were determined by p – value less than or equal to 0.05 for the disease allele.

4. Results

4.1 Transforming growth factor beta 1 gene (TGFB1)

4.1.1 cDNA sequence of porcine TGFB1 and screening SNP in TGFB1 gene

The porcine TGFB1 sequence was obtained from liver mRNA on primer derived from information of Gen Bank (Accession number M23703). The fragments reveal overlapping about 400 bp in size covering the cDNA sequence from exon 1 to the 3'-UTR (Accession number AF461801) (Figure 4). The cDNA fragment was obtained from five pig breeds - Hampshire, Duroc, Pietrain, German Landrace and Berlin-miniature pig – which were comparatively sequenced.

4.1.2 Polymorphism in the porcine TGFB1

Comparative sequencing of TGFB1 cDNA fragment obtained from individual of five breeds (Duroc, Hampshire, German landrace, Pietrain and Miniature pig) revealed 'A/G' transition in exon 5 at position 797 of the coding sequence (Figure 3).

Figure 3: Sequence analysis of porcine TGFB1 gene revealed single nucleotide polymorphism in exon 5 at position 797 of the coding sequence

4.1.3 Development of methods for genotyping the allelic polymorphism

For genotyping a single strand conformation (SSCP) protocol was established being diagnostic for the polymorphism detected within exon 5 at position 797 of the coding sequence. A forward primer located in intron 4 (Accession number AF461809) (Figure 4) and a reverse primer positioned in exon 5 were derived to produce a 218 bp PCR product from porcine genomic DNA.

```
  1  TGAGGCCCGC CTCCCTGGCC CAGCCCTGTG CCCAGCAGTG ACTCTGTGCG
 51  TGTGTGTGCA CACGGCGTGC GCACGTGCGC GCGGTGGGCG GGTTCTTCCC
101  CTGCCCACCC CCTACTCATC CATCTGAGTG TGTGTGTGTA TGTCTCCCCC
151  AACCCTATCC GCTCCCTGAC TCGTAAACCA AAGCAG
```

Figure 4: Sequence of intron 4 of TGFB1 gene (Accession number AF461809)

4.1.3.1 Genotyping method by using SSCP analysis

The PCR fragments of 218 bp were amplified with primer (Table 3) that covers the polymorphic sites at position 797 in the exon 5 of the porcine TGFB1. The allelic variation was detected by SSCP analysis. Figure 6 shows the SSCP banding pattern of this polymorphic sites which are in agreement with the results obtained by DNA sequencing analysis.

4.1.4 Genetic variation of the porcine TGFB1 in the DUMI resource population

The SNP was genotyped in 400 animals of the F2-DUMI resource population including the animals of the grandparental generation (Duroc and Berlin miniature pig) and the F1 parental generations. Allele frequencies obtained are shown in table 2. Within the grandparental generation of the Berlin-Bonn-DUMI resource population all Berlin Miniature pigs (four sows, one boar) were found to be homozygous ‘GG’. The 3 animals of Duroc grandparents (five sows, one boar) were be homozygous ‘AA’ and the others 3 animals grandparents were be heterozygous.

```
   1  ATGCCGCCTT CGGGGCTGCG GCTCTTGCCG CTGCTGCTGC CGCTGCTGTG
  51  GCTGCTAGTG CTGACGCCTG GCCGGCCGGC CGCCGGACTG TCCACCTGCA
 101  AGACCATCGA CATGGAGCTG GTGAAGCGGA AGCGCATCGA GGCCATTCGC
 151  GGCCAGATTC TGTCCAAGCT TCGGCTCGCC AGCCCCCCGA GCCAGGGGGA
 201  CGTGCCGCCC GGCCCGCTGC CTGAGGCCGT ACTGGCTCTT TACAACAGTA
 251  CCCGCGACCG GGTAGCCGGG GAAAGTGTCG AACCGGAGCC CGAGCCAGAG
 301  GCGGACTACT ACGCCAAGGA GGTCACCCGC GTGCTAATGG TGGAAAGCGG
 351  CAACCAAATC TATGATAAAT TCAAGGGCAC CCCCCACAGC TTATATATGC
 401  TGTTCAACAC GTCGGAGCTC CGGGAAGCGG TGCCGGAACC TGTATTGCTC
 451  TCTCGGGCAG AGCTGCGCCT GCTGAGGCTC AAGTTAAAAG TGGAGCAGCA
 501  CGTGGAGCTA TACCAGAAAT ACAGCAATGA TTCCTGGCGC TACCTCAGCA
 551  ACCGGCTGCT GGCCCCCAGT GACTCACCGG AGTGGCTGTC CTTTGATGTC
 601  ACCGGAGTTG TGCGGCAGTG GCTGACCCGC AGAGAGGCTA TAGAGGGTTT
 651  TCGCCTCAGT GCCCACTGTT CCTGTGACAG CAAAGATAAC ACACTCCACG
 701  TGGAAATTAA CGGGTTCAAT TCTGGCCGCC GGGGTGACCT GGCCACCATT
 751  CACGGCATGA ACCGGCCCTT CCTGCTCCTC ATGGCCACCC CGCTGGAGAG
 801  GGCCCAGCAC CTGCACAGCT CCCGGCACCG CCGAGCCCTG GATACCAACT
 851  ACTGCTTCAG CTCCACGGAG AAGAACTGCT GCGTGCGGCA GCTCTACATT
 901  GACTTCCGGA AGGACCTGGG CTGGAAGTGG ATTCATGAAC CCAAGGGCTA
 951  CCATGCCAAT TTCTGCCTGG GGCCCTGTCC CTACATCTGG AGCCTAGACA
1001  CTCAGTACAG CAAGGTCCTG GCTCTGTACA ACCAGCACAA CCCGGGCGCG
1051  TCGGCGGCGC CGTGCTGCGT GCCGCAGGCG CTGGAGCCAC TGCCCATCGT
1101  GTACTACGTG GGCCGCAAGC CCAAGGTGGA GCAGCTGTCC AACATGATCG
1151  TGCGTTCCTG CAAGTGCAGC TGAGGCCCCA AGCCCACTTG GGATCGATTA
1201  AAGGTGGAGA GAGGACTGGG TCTCCGTGTG TTGGGCACCT GACTGGGGTC
1251  TTCTTCGGAC GTTACCGGAC CCCCACTCCC AGCCTCCGCC TGCCTCCGCC
1301  TGTGTCTGTC CACCATTCAT TTGTTCCTCC TCCTCATGCA AACGCGTCCT
1351  GAGCAGGTAC TCCTGGTGAA CTCTACTTAG ATTTACTTAC TGAGCATCTT
1401  GGACCTTATC CTGAATGCCT TATATTAATT AACTCATTTA ACCACCATAA
1451  CAAAGCTAAA AGGGACTCTG ATAACACCCA CTTTAAAAAG GAAACGGAAG
1501  CTGGAGTTTC CATTGTGGCT CAGTGGTAAC CTACCCGACT GGTATCCTTG
1551  AAGACACAGG TTCAATCCCT GGCCCTGTTC TGTAAGTTAA AGGTCCGGCT
1601  GTGGCGGCTG TGGTATAGGC CGGCAGCTGC AGCTCCGATT TAACCCCTAG
1651  CCTGGGAACT TCCATATGTC TCAGGTGCGG CCCTAAAAAG ACAAAAAGAA
1701  AGGAAAAGGA AGCCCATAGT GGTTAAGGGA ATAATTCCTG CCCACCAAGA
1751  ACCTGCTTTC GGCTTTCTGG TGGGGAGACA GACATAGCAA AGTTGTGTGA
1801  AAACAGGAAG GCAGTGTGGG TCAGAGAGGG CTTTGGGAGG TGGGAGGGCT
1851  TCTTGGAGGA GGTGGCACCT GGGCCTTGAA GGAAGCCAAG AAAGCAGCCT
1901  AGGGGAGCAT GGGGGAGGGT GTTCATGGTA GGAGGACAAA AGCAAAGTCC
1951  TGGAAGTGAA GATGAATTTG GGGTGAGCTA CACCGGCGGG AAAGAGGCCA
2001  GTGCGGTTGG AAGGGAGGGG CAAGGGGAAA AGTGTTGGGA TCTGAGTCAG
2051  AAAGTAACAC TCACAGGCCA GAGAGTAATA ACAGTTCTCC AGGCTAGGTA
2101  TGGAGCTACT AGCTCAAGGC ATTCTTCCCA CAGCCCAGCA GAGCAGAGGT
2151  TGTTAAACTA TTGCCTGCAG GCACATTCTG ACCCGCTGCC TGTTTCTGTA
2201  AATAAAGTTT TATTGGAGAA C
```

Figure 5: Nucleotide sequence of TGFB1 gene (Accession number AF461808)

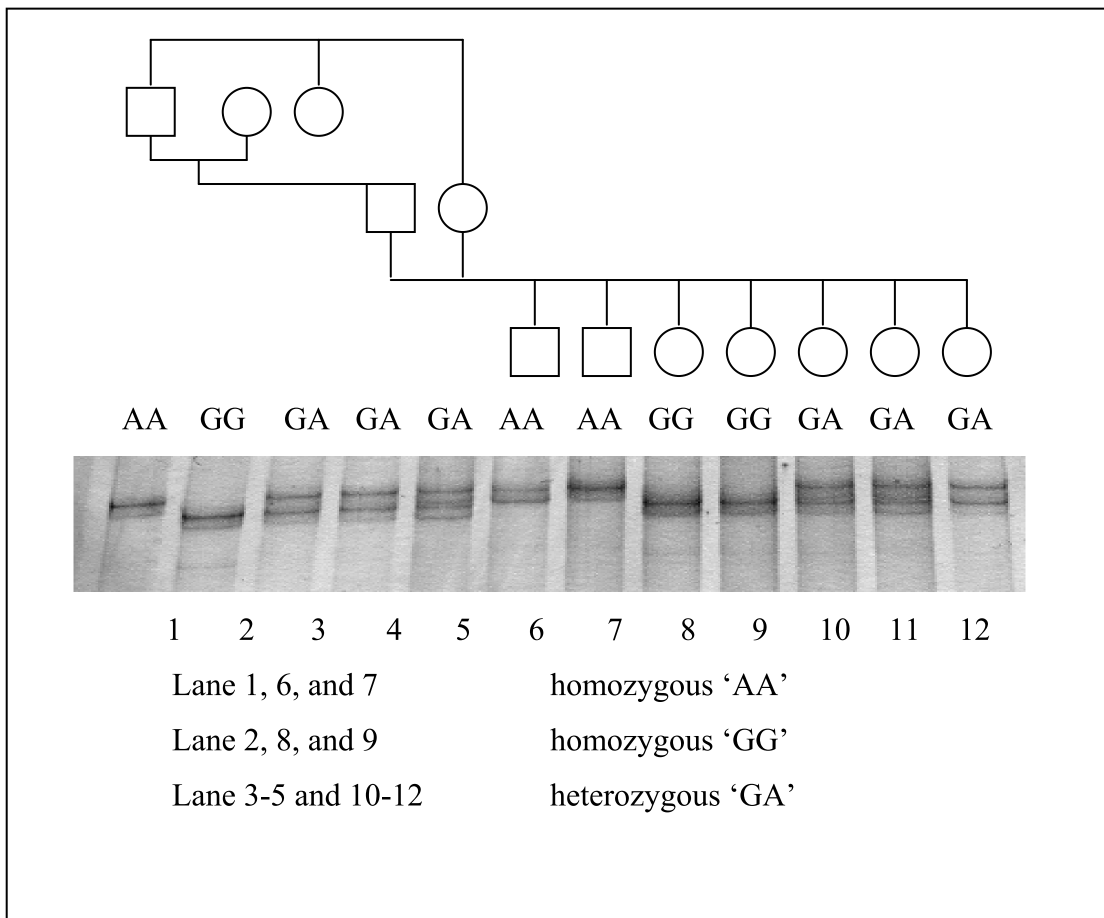

Figure 6: Mendelian inheritance of porcine TGFB1 at exon 5 on position 797 using SSCP analysis

Twelve out of the 14 animals of the F1 generation were found to be heterozygous animals and only 2 animals were homozygous ‘GG’. Among the 400 F2 animals of the DUMI families that were genotyped, 173 (43.25%) animals were found to be homozygous ‘GG’, 16 (4%) were homozygous ‘AA’ and 211 (52.75%) were heterozygous. Frequencies of the allele ‘G’ and ‘A’ were 0.7 and 0.3 respectively.

4.1.5 Genetic variation of the porcine TGFB1 in the commercial breeds.

No polymorphism was found among commercial breeds German Landrace (n=39) and Large White (n=44) for this SNP in TGFB1 gene. Within the breed Pietrain segregation of the TGFB1 was found in 67 % of the animals being homozygous GG, and 33% being heterozygous.

Table 9: Frequencies of alleles of the TGB1 gene

Breeds	No. of animals	Allele Frequency	
		Allele 'G'	Allele 'A'
German Landrace	39	1.00	0.00
Large white	44	1.00	0.00
Pietrain	30	0.82	0.18
DUMI Resource population	400	0.70	0.30

4.1.6 Linkage mapping

Linkage mapping analysis was performed using CRI-MAP package (version 2.4). Multipoint linkage map was established using the BUILD and FLIPS options. Genotyping of 380 F2-individuals from 21 families of the Berlin-Bonn-DUMI resource population and subsequent two-point and multipoint procedures of the CRI-MAP revealed linkage to loci SO300, SW193, and SW1067 (proximal) and SO220 (distal) with distances of 7.3, 13.8, 24.5 and 31.5 cM, respectively, on the sex average map (Table 10). SW1067 has been assigned to Sscr6q11-q22 (Yerle et al. 1990a, b). TGFB1 gene position assigned is in agreement with the published genetic and physical map.

Table 10: Two-point link for TGFB1

	Sex Averaged	
	Recombination fraction	LOD scored
SO300	0.08	44.8
SW193	0.07	19.2
SW1067	0.17	21.4
SO220	0.29	12.9

4.2 Relaxin gene (RLN gene)

Primers were designed from the published RLN gene sequence (Accession number J02792) and used to amplify overlapping fragments of about 300-500 bp in size covering part of 5'- untranslated region (UTR), exon 1, part of intron 1, exon 2 and part of 3'-UTR.

4.2.1 Polymorphism in the porcine RLN gene

Using PCR with primers encompassing the exon 1 to exon 2, DNA fragment of RLN gene were amplified from genomic DNA of five pig breeds (Duroc, Hampshire, Pietrain, German Landrace and Berlin-Miniature pig). Amplified fragments were analysed for polymorphism using the denaturing gel electrophoresis.

Sequence comparison of the exon1-intron1 of the RLN gene revealed two polymorphisms (Figure 7). First, a transversion (C > A) at position 1 of codon 8 (nt 22) in the first exon leading to a amino acid exchange was detected. Another transversion (T>G) was found at position 9 of the intron 1.

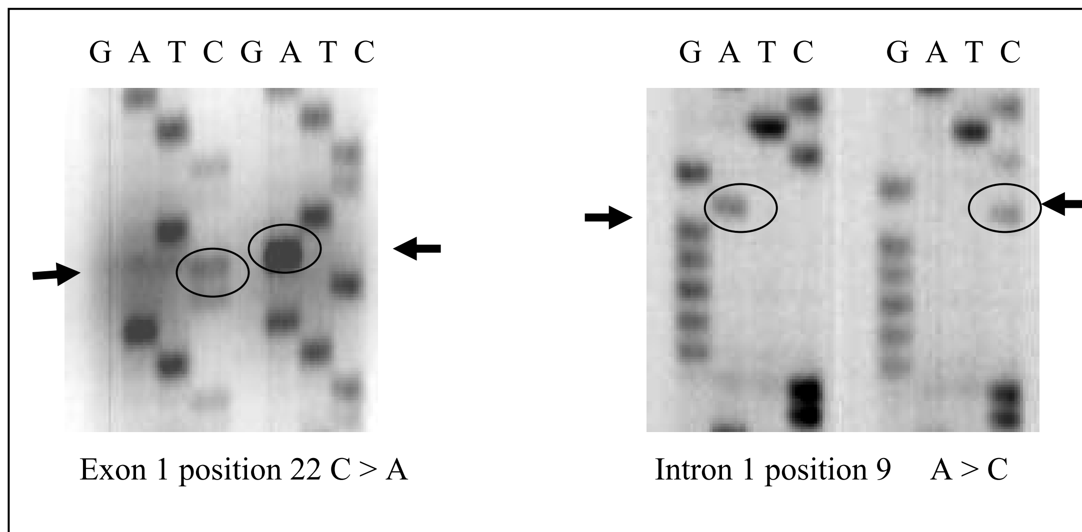

Figure 7: Sequence analysis of porcine RLN gene revealed single nucleotide polymorphism

4.2.2 Development of methods for genotyping the allelic polymorphism

4.2.2.1 Genotyping by using SSCP analysis

The SSCP analysis was established for exon1 (C/A) SNP genotyping. Banding patterns were detected by silver staining (Figure 9).

4.2.2.2 Genotyping by using RFLP analysis

Another transversion (T>G) was found at position 9 of the intron 1 altering the recognition site of CfoI. For genotyping of this SNP, PCR product of 331 bp was incubated with CfoI at 37 ^{o}C overnight. The polymorphism were detected in agarose gels with 2 alleles, with major band at 298 and 42 bp in length for allele ‘T’ or 209, 80 and 42 for allele ‘G’.

4.2.3 Genetic variation of the porcine RLN gene in the DUMI resource population

A Mendelian inheritance of both polymorphic sites were observed in full-sib families (21 offspring) of Bonn Berlin-DUMI resource population (Figure 8). The SNP in exon 1 position 22 was genotyped within the grandparental generation of the Berlin-Bonn-DUMI resource population. All Berlin Miniature pig grandparents (four sows, one boar) were homozygous 'CC'. In 4 animals of Duroc grandparents (five sows, one boar) were found homozygous 'AA' and one animal was found heterozygous. In parental generation (F1), 2 boars were homozygous 'CC' and 11 sows were heterozygous. Among the 384 in F2 animals of the DUMI families that were genotyped 12 animals were found to be homozygous 'AA', 175 were homozygous 'CC' and 197 were heterozygous. Frequency of the A and C alleles at position exon 22 was 0.29 and 0.71 respectively, in the F2 generation of Bonn-Berlin-DUMI resource population. Genotyped frequencies were as follows: 45.57 % CC, 51.30 % CA and 3.13 % AA.

Another SNP in intron 1 at position 9 was genotyped within the grandparental generation of the Bonn-Berlin-DUMI resource population (Figure 9). All Duroc grandparents (five sows, one boar) were homozygous (TT), Berlin-Miniature pigs (Four sows, one boar) were found to be 4 homozygous (GG) and 1 heterozygous in sow. In all parental generation (F1), 14 animals were heterozygous. Among 388 F2 animals of the DUMI families that were genotyped, 100 (25.77%) animals found to be homozygous 'T', 87 (22.42%) were homozygous 'G' and 201 (51.8%) were heterozygous. Frequency of the allele 'G' and 'T' were 0.48 and 0.52 respectively.

Table 11: Frequencies of alleles of the RLN gene

Breeds	No. of animals	Allele Frequency	
		Allele 'A'	Allele 'C'
German Landrace	31	0.06	0.94
Large white	27	0.02	0.98
Pietrain	35	0.11	0.89
DUMI Resource population	384	0.29	0.71

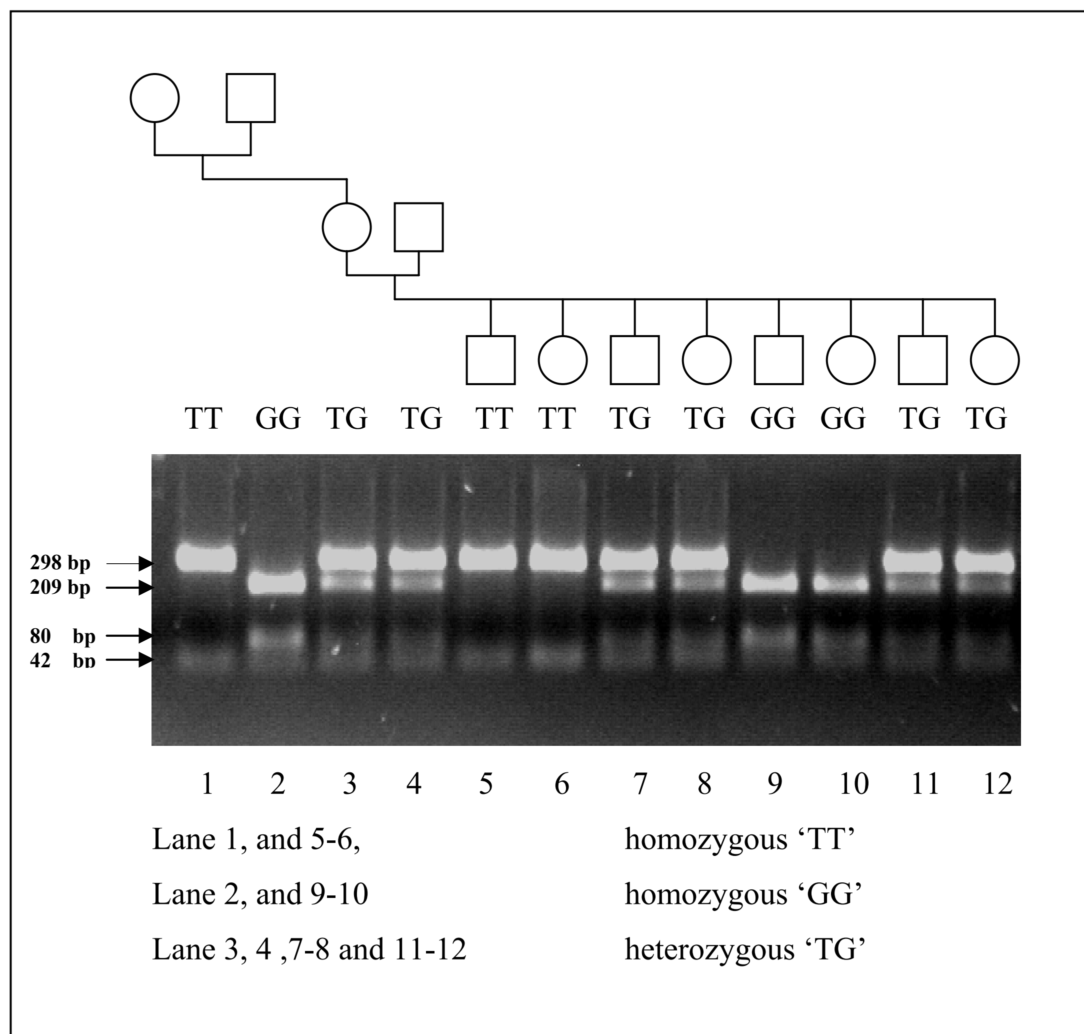

Figure 8: Mendelian inheritance of RLN gene in intron1 position 9 using RFLP analysis

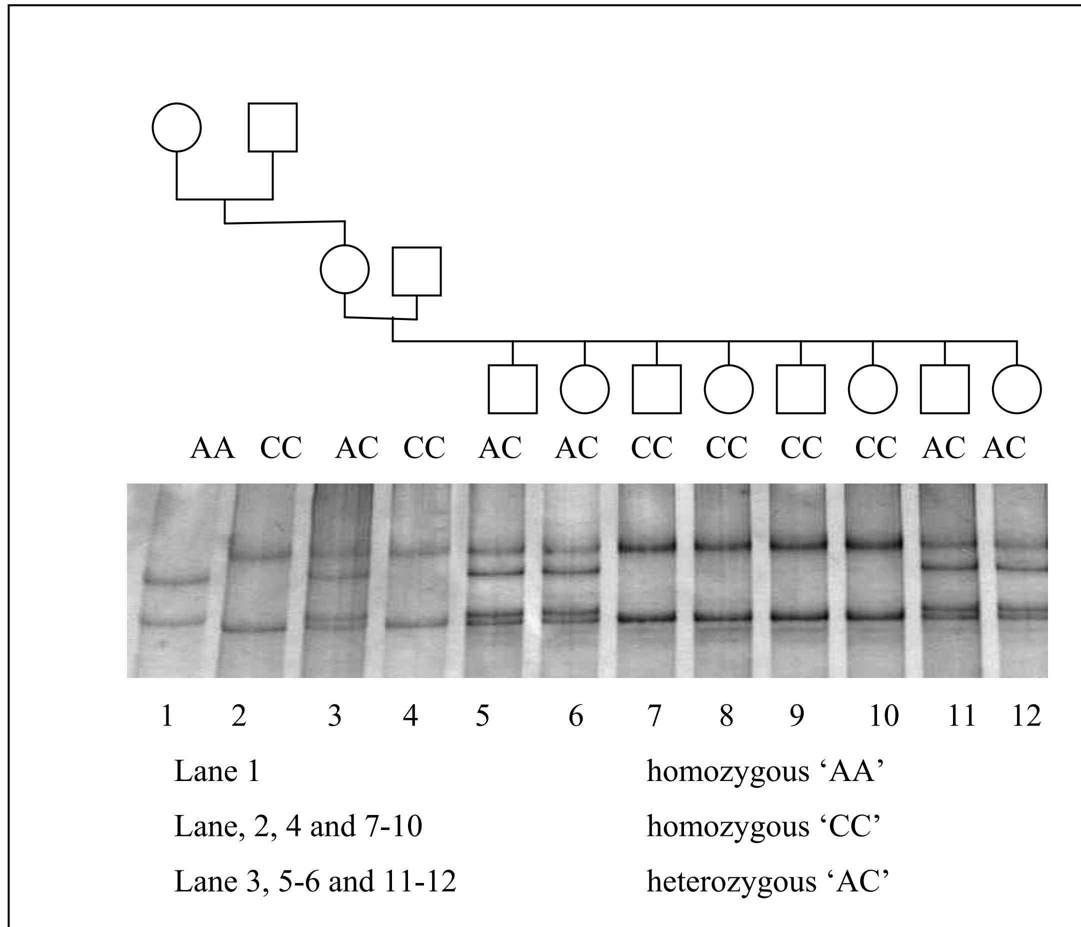

Figure 9: Mendelian inheritance of relaxin gene SNP in exon 1 position 22 using SSCP analysis

4.2.4 Genetic variation of the RLN gene in the commercial breeds

The SNP in exon 1 position 22, polymorphism in commercial breed was found among 93 commercial pigs of the breeds German Landrace (n=31) and Large White (n=27), Pietrain (n=35). Allele frequencies are shown in table 12.

At position 9 on the intron 1, no polymorphism was found among 93 commercial of breeds German Landrace (n=31) and Large White (n=27), Pietrain (n= 35) (allele 'T' =1.00, allele 'G' = 0.00) (Table 12).

Table 12: Allele frequencies of the RLN gene in the commercial breeds

		Allele Frequency			
		SNP22 Exon1		SNP9 Intron1	
breeds	number of animals	allele 'A'	allele 'C'	allele 'G'	allele 'T'
German Landrace	31	0.06	0.94	0.00	1.00
Large White	27	0.02	0.98	0.00	1.00
Pietrain	35	0.11	0.89	0.00	1.00
Resource population	384	0.29	0.71	0.48	0.52

4.2.5 Linkage mapping

Linkage mapping analysis was using CRI-MAP package (version 2.4). Multipoint linkage map was established using the BUILD and FLIPS options (SW1515-33.0 cM - SW1815-31.4 cM SO155-7.6 cM-RLN 56.5cM-SW1301; sex averaged distances are given in Kosambi centimorgan). Two-point linkage analysis revealed a recombination fraction of 0.08 between RLN and SO155 (LOD=18.3). The position of RLN gene is in good agreement with the published physical assignment to Sscr1q28-29.

4.3 Parathyroid hormone like hormone gene (PTHLH)

4.3.1 cDNA sequence of porcine PTHLH

The complete cDNA sequence of the porcine PTHLH gene was obtained starting with heterologous primers designed from conserved regions of the human PTHLH (accession number NM_002820) and porcine EST (Accession numbers BI181671 and BI181482), and subsequent 5' and 3'-RACE using homologous primers (SMART™ RACE cDNA Amplification Kit, CLONTECH). The cDNA consisted of a 1,336 bp coding region and a 455 bp 3' UTR (Figure 11). In addition 5'UTR was sequenced in 319 bp lengths and separated by intron. This intron sequence is 175 bp in length (Figure12) and showed 83% homology with human PTHLH intron 2. The C' terminal portion of PTHLH is

94% homologous to exon 6 of human PTHLH (Yasuda et al. 1989) and 94% homologous to canine C'terminal (Rosol et al. 1995).

A comparison with PTHLH sequences from other species indicated a high level of sequence conservation. Comparison of the translated sequence of PTHLH with other published sequences showed the 91, 87, 92, 91, 92 and 89% similarity to human, mouse, bovine, rabbit, canine and equine, respectively (Figure 13).

4.3.2 SNP screening in PTHLH gene

Primers were designed from the sequence flanking every 400-500 bp over the entire gene sequence to screen for polymorphism. The PCR fragments amplified from liver cDNA of five pig breeds-Duroc, Hampshire, German Landrace, Pietrain and Berlin-Miniature pig were sequenced. The single nucleotide polymorphism was identified within coding region.

4.3.3 Polymorphism in the porcine PTHLH gene

A T/C (S19L) non-synonymous SNP was detected at nucleotide position 375 of the porcine PTHLH cDNA (Figure 10).

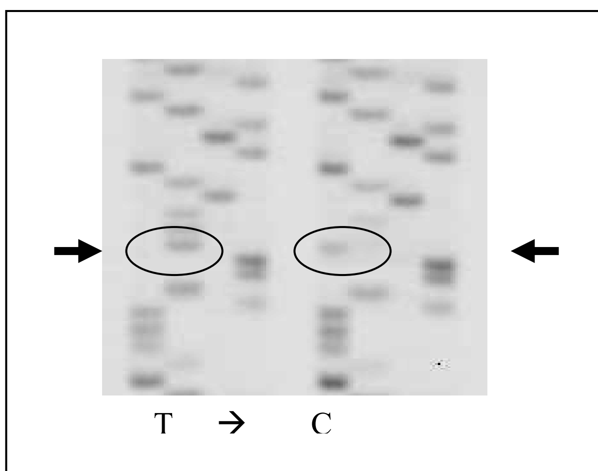

Figure 10: Sequence analysis of porcine PTHLH gene revealed single nucleotide polymorphism at position 375 of coding sequence

```
DNA: GGGAGCAAGACGGCCTAGTTTGCAAAGAAGCTGACTTCGGAGGGGAAAACT
   :
DNA: TTCTTTTTTTCAGGAGGGGTTTAGCCCTGCTCCCTGAACCCAGGATAACTG
   :
DNA: CCGGCCAGATTAATTAGACATTGCTATGGGAGACGTGTAAACACGTTGCTC
   :
DNA: ATCATTGATGCCTATATAAAACCATTTCATTTGGGTGATTATTTCGGAGGA
   :
DNA: AGCGCCTCTGATTATTTTTTCTTCTCCTTTTTGGTCCCTTTTTGCCTGTGC
   :
DNA: GGTTTGGAAAAAGCACAGTTGGAGTAACTGGTTCCTAAATAAGTCCCCAGA
   :
DNA: GCCAGAGGATACGATGCTGTGGAGGCTGGTTCAGCAGTGGAGCGTCGCGGT
 AA:              M  L  W  R  L  V  Q  Q  W  S  V  A  V
DNA: GTTCCTGCTGAGCTATTCGGTGCCCTCCTGCGGGCGCTCGGTGGAGGAGCT
 AA:  F  L  L  S  Y  S  V  P  S  C  G  R  S  V  E  E  L
DNA: CGGCCGCCGACTCAAAAGAGCCGTGTCTGAACACCAGCTCCTCCATGACAA
 AA:  G  R  R  L  K  R  A  V  S  E  H  Q  L  L  H  D  K
DNA: GGGGAAGTCCATCCAAGATTTACGGCGACGATTCTTCCTTCACCACCTGAT
 AA:  G  K  S  I  Q  D  L  R  R  R  F  F  L  H  H  L  I
DNA: CGCAGAAATCCACACAGCTGAAATCAGAGCTACCTCGGAGGTTTCCCCCAA
 AA:  A  E  I  H  T  A  E  I  R  A  T  S  E  V  S  P  N
DNA: CTCCAAGCCTGCTCCCAACACCAAGAACCACCCTGTCCGATTTGGGTCTGA
 AA:  S  K  P  A  P  N  T  K  N  H  P  V  R  F  G  S  D
DNA: CGATGAGGGCAGATACCTGACTCAGGAAACCAACAAGGTGGAGACGTACAA
 AA:  D  E  G  R  Y  L  T  Q  E  T  N  K  V  E  T  Y  K
DNA: AGAGCAGCCACTGAAGACACCGGGCAAGAAAAAGAAAGGCAAACCTGGGAA
 AA:  E  Q  P  L  K  T  P  G  K  K  K  K  G  K  P  G  K
DNA: ACGCAAGGAGCAGGAAAAGAAGAAACGGCGAACTCGATCGGCCTGGCTGAA
 AA:  R  K  E  Q  E  K  K  K  R  R  T  R  S  A  W  L  N
DNA: CTGCAGCATGGTCGGGAGTGGGCTGGAAGTGGACCACGTGTCTGATGACTC
 AA:  C  S  M  V  G  S  G  L  E  V  D  H  V  S  D  D  S
DNA: GGAGACCTCACTGGAGCTCAATTCAAGGAGACATTGAAATTTTCAGCAGAG
 AA:  E  T  S  L  E  L  N  S  R  R  H  *
DNA: ACCTTCAGAGGACGTATTGCAGAATTCTGTAATAGTGAAAAGTATTAGAAA
   :
DNA: TATTTATTGTCTGTAAATACTGTAAATGCATTGGAATAAAACTGTCTCCCC
   :
DNA: ATTGCTCTATGAAACTGCACATTGGTCATTGTGAATATATATTTTTTTGGC
   :
DNA: CCAGGCTAATCCAATTATTATTATCACATTTACCATAATTTATTTTGTCAA
   :
DNA: CTGATGTATTTATTTTGTAAATGTATCTTGGTGCTGCTGAATTTCTATATT
   :
DNA: TTTTGTAACATAATGCACTTTAGATATACATATCAAGTATGTTGATAAATG
   :
DNA: ACACAATAAAGTGTCTCTATTTTGTGGTTGATTCTAATGAATGCCTAAATA
   :
DNA: TAATTATCCAAACTGATTTTCCTCTGTGCATGTAAAAATAGCAGTATTTTA

DNA: AATTTGTAAAGAATGTCTAATAAAATATAATCCAAAAAAAAAAAAAAAAAA

DNA: AAAAAAAAAA
```

Figure 11: Nucleotide and amino acid (AA) sequences of porcine PTHLH gene

```
  1  AAGTGCCGAG AGGCTCCAGA GAAAGTTTTC TTCTTCTTCT TTGCAACTTT
 51  GTAGATGCCC TTGAAGTTGA AGAGGCTATT TGAGAGCAAG TGAAGGGGTG
101  GAGGGGAGCG GGGTAATGAG GGGAAGTGGA GATGCTGGAT TGACCTTTAG
151  GGCCCCTTTC CTTTCCGTCG CAGGT
```

Figure 12: Sequence of intron 2A of the PTHLH gene

```
                   10        20        30        40        50        60
                    |         |         |         |         |         |
Porcine   MLWRLVQQWSVAVFLLSYSVPSCGRSVEELGRRLKRAVSEHQLLHDKGKSIQDLRRRFFL
Bovine    MLWRLVQQWSVAVFLLSYSVPSCGRLVEELGRRLKRAVSEHQLLHDKGKSIQDLRRRFFL
Canine    MLRRLVQQWGVAVFLLSYSVPSCGRSVEELGRRLKRAVSEHQLLHDKGKSIQDLRRRFFL
Humanx1   MQRRLVQQWSVAVFLLSYAVPSCGRSVEGLSRRLKRAVSEHQLLHDKGKSIQDLRRRFFL
Rabbit    MQRRLVQQWSVAVFLLSYAVPSCGRSVEGLSRRLKRAVSEHQLLHDKGKSIQDLRRRFFL
Mousex2   MLRRLVQQWSVLVFLLSYSVPSRGRSVEGLGRRLKRAVSEHQLLHDKGKSIQDLRRRFFL

                   70        80        90       100       110       120
                    |         |         |         |         |         |
Porcine   HHLIAEIHTAEIRATSEVSPNSKPAPNTKNHPVRFGSDDEGRYLTQETNKVETYKEQPLK
Bovine    HHLIAEIHTAEIRATSEVSPNSKPAPNTKNHPVRFGSDDEGKYLTQETNKVETYKEQPLK
Canine    HHLIAEIHTAEIRATSEVSPNSKPAPNTKNHPVRFGSDDEGRYLTQETNKVETYKEQPLK
Humanx1   HHLIAEIHTAEIRATSEVSPNSKPSPNTKNHPVRFGSDDEGRYLTQETNKVETYKEQPLK
Rabbit    HHLIAEIHTAEIRATSEVSPNSKPSPNTKNHPVRFGSDDEGRYLTQETNKVETYKEQPLK
Mousex2   HHLIAEIHTAEIRATSEVSPNSKPAPNTKNHPVRFGSDDEGRYLTQETNKVETYKEQPLK

                  130       140       150       160       170       180
                    |         |         |         |         |         |
Porcine   TPGKKKKGKPGKRKEQEKKKRRTRSAWLNCSMVGSGLEVDHVSDDSETSLELNSRRH---
Bovine    TPGKKKKSKPGKRKEQEKKKRRTRSAWLTSYVAGTGLEEDYLSDISATSLELNSRRH---
Canine    TPGKKKKGKPGKRKEQEKKKRRTRSAWLNSGVAESGLEGDHPYDISATSLELNLRRH---
Humanx1   TPGKKKKGKPGKRKEQEKKKRRTRSAWLDSGVTGSGLEGDHLSDTSTTSLELDSR-----
Rabbit    TPGKKKKGKPGKRKEQEKKKRRTRSAWLDSGVTGSGLEGDHLSDTFTTSLGARFTYSTSV
Mousex2   TPGKKKKGKPGKRREQEKKKRRTRSAW--PSTAASGLLEDPLPHTSRTSLEPSLRTH---

                      190       200
                        |         |
Porcine     ----------------------
Bovine      ----------------------
Canine      ----------------------
Humanx1     ----------------------
Rabbit      GFEKKKGKQQKNTSYATNDLII
Mousex2     ----------------------
```

Figure 13: Comparative amino acid sequence of different species

4.3.4 Development of method for genotyping the allelic polymorphism

4.3.4.1 Genotyping by using Taqman assay

The genotyping method for the PTHLH gene is an allelic discrimination reporter assay developed on the ABI PRISM 7000 (Applied Biosystems, USA). The polymorphism of interest in the PTHLH gene involves a single nucleotide change at the 550 position. This method involves two reporter oligonucleotide probes (TaqMan® MGB probes, labelled with FAM™ or VIC® dyes) that are specific to the polymorphic site. Each probe is bound with a unique fluorescent molecule. If an individual is homozygous, only one of the two unique reporter molecules will be detected. If an individual is heterozygous, both of the unique reporter molecules will be detected. The PCR fragment of 50 bp was amplified that cover the polymorphic site (Figure 14).

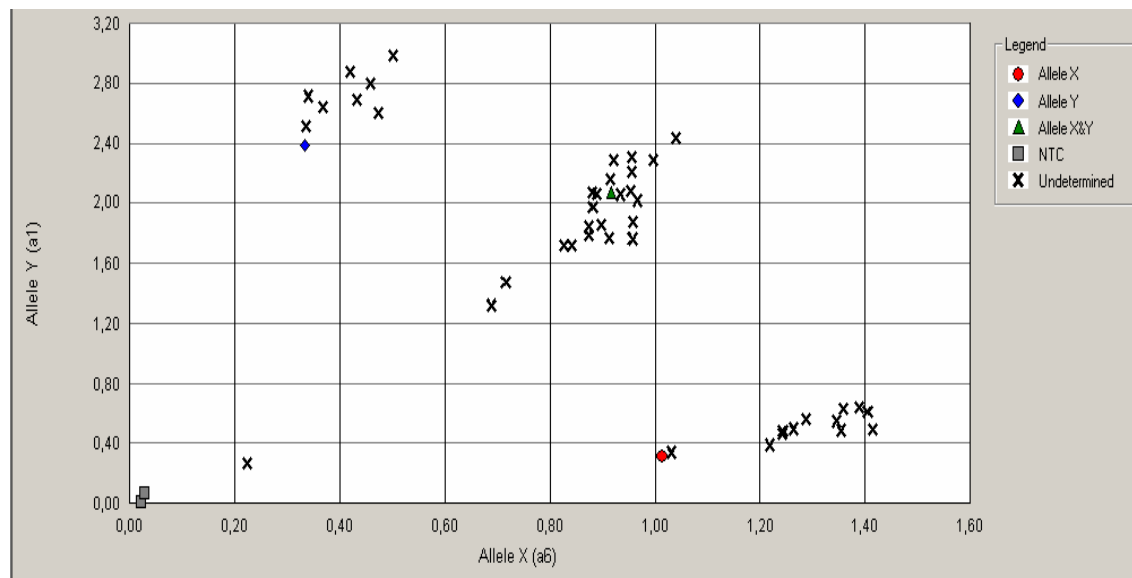

Figure 14: Scheme presentation using Taqman assay genotypes of porcine PTHLH gene in DUMI resource population

4.3.5 Genetic variation of the porcine PTHLH gene in the DUMI resource population

Mendelian inheritance of this polymorphic site was monitored in 347 individuals of 21 families of the three generation F2-DUMI resource population. Within the grandparents of the DUMI resource population, all Berlin-Miniature pig grandparents (n=4) were homozygous for allele ‘TT’, four Duroc grandparents were homozygous for allele ‘CC’. This polymorphism was heterozygous in all of 15 F1 generations. Among 347 F2 animals were genotyped 77 (22.19%) animals found to be homozygous ‘CC’, 76 (21.9%) were homozygous ‘TT’, and 194 (55.91%) animals were heterozygous. Frequency of the ‘C’ and ‘T’ alleles were 0.5 (Table 15).

4.3.6 Genetic variation of the porcine PTHLH gene in the commercial breeds

The SNP was found to segregate among pigs of three commercial breeds (German Landrace, Large white and Pietrain) with allele ‘C’ being the prominent one (Table 13).

Table 13: Frequencies of alleles of the PTHLH gene

Breeds	Number of animals	Allele Frequency	
		Allele ‘C’	Allele ‘T’
German Landrace	29	0.93	0.07
Large White	30	0.95	0.05
Pietrain	31	0.98	0.02
DUMI resource population	347	0.5	0.5

4.3.7 Linkage mapping

Radiation hybrid mapping was performed using INRA-Minnesota 7000 rads radiation hybrid panel (IMpRH), consisting of 118 hamster-porcine hybrid cell lines. PCR products were separated on 2 % agarose gel and visualized by ethidium bromide staining (Figure15). The primers designed on the porcine 3'UTR PTHLH sequence enabled the amplification of a 358-bp product from porcine genomic DNA and no amplification with mouse or hamster DNA. Amplification results were submitted to the IMpRH database (http://imprh.toulouse.inra.fr). Two-point linkage analysis identified the most significant linkage of PTHLH with SW1134 and SW2033 (LOD=2.74 and 1.94). The most significantly linked marker (2pt analysis) is SW1319 on chromosome 5 (73 cR; LOD 4.85).

For genetic mapping, two-point and multiplepoint procedures of the CRI-MAP package version 2.4 revealed linkage to loci SW1134 (proximal) and IGF1 (distal) with distance of 24.4 cM (recombination fraction = .23, lods = 17.41), and 48.2 cM (recombination fraction = .37, lods = 3.43), respectively, on the sex averaged map. IGF1 has been assigned to Sscr5q23. Microsatellites SW1319 and SW1134 mapped in close proximity at 49.3 cM on the second release genome-wide linkage map developed by the USDA Meat Animal Research Centre (Rohrer et al. 1994, 1996, Alexander et al. 1996). The results of RH and genetic mapping are in agreement and correspond with published genetic and physical map.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 H P

18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 H P

35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 H P

52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 P H

69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 P H

86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 P H

103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 P H

Figure 15: Physical mapping of PTHLH gene using radiation hybrid panel (IMpRH)

4.4 Family based association tests (FBAT)

The Family-Based Association Test (FBAT) is recently developed software that can analyse association pedigrees with heterogeneous family structure. Furthermore, FBAT allows the analysis of the phenotype as a qualitative trait. In the present study, we made use of the FBAT program (Version 1.4) to perform the qualitative and quantitative family-based analysed of the Bonn-Berlin DUMI resource population. For all the quantitative analysis were done under the condition of an additive model. The *p*-values from FBAT for multiple allelic tests are reported in the table 14, 15.

No significant association was observed between TGF1 and affection of inverted teats (p = 0.521) (Table 14). Significant association was found at *p* value of 0.014, 0.012, and 0.013 between TGFB1 and the number of teats in the left hand side, number of teats in the right hand side and total number of teats, respectively. In addition, the association analysis between TGFB1 and number of inverted teats in the left hand side, number of inverted teats in the right hand side and total number of inverted teats showed significant in *p* value of 0.004, 0.003, and 0.036, respectively (Table 15).

The association between PTHLH and affection was highly significant with *p* value of 0.000099 (Table 14). Furthermore, the significant association was found at *p* value of 0.037, 0.036, and 0.037 between PTHLH and the number of teats in the left hand side, number of teats in the right hand side and total number of teats, respectively. In addition the significant association was shown between PTHLH and number of inverted teats in the left hand side, number of inverted teats in the right hand side and total number of inverted teats with a *p* value of 0.003, 0.0008, and 0.007, respectively (Table 15).

No significant association was shown between RLN1 and inverted teats affecting trait (p = 0.673) (Table 14). The significant association was found at *p* value of 0.021, 0.020, and 0.020 between RLN2 and the number of teats in the left hand side, number of teats in the right hand side and total number of teats, respectively. Moreover, the association between RLN1 and number of inverted teats in the left hand side, number of inverted teats in the right hand side and total number of inverted teats were significant at *p* value of 0.002 (Table 15).

The association analysis between RLN2 and affection in inverted teats defect showed significant in *p* value of 0.006 (Table 14). Furthermore, the significant association was found at *p* value of 0.011 between RLN1 and the number of teats in the left hand side, number of teats in the right hand side and total number of teats, respectively. In addition the association analysis between RLN2 and number of inverted teats in the left hand side, number of inverted teats in the right hand side and total number of inverted teats showed significant in *p* value of 0.005, 0.006, and 0.006, respectively (Table 15).

Table 14: Result of the FBAT done considering of inverted teat as the affection trait

Marker	Allele	Freq	S	E(S)	Z score	*p* value	
TGFB1	G	0.70	-0.582	1.062	-0.641	0.521	NS
(A797G)	A	0.30	2.706	1.062	0.641	0.521	
RLN1	C	0.71	-0.658	0.356	-0.422	0.673	NS
(A22C)	A	0.29	1.370	0.356	0.422	0.673	
RLN2	T	0.52	26.00	61.979	-2.762	0.0057	**
(T9intG)	G	0.48	116.00	80.021	2.762	0.0057	**
PTHLH	C	0.50	-8.673	8.360	-3.893	0.000099	**
(C375T)	T	0.50	34.820	17.787	3.893	0.000099	**

NS = not significant ($p > 0.05$), ** $p < 0.01$

Table 15: Association of each marker in DUMI resource population by FBAT analysis

Characteristic	TL	ITL	TR	ITR	TT	TIT	Affection
Marker	Z (*p*)	Z (*p*)	Z (*p*)	Z (*p*)	Z (*p*)	Z (*p*)	χ^2(df=2) (*p*)
TGFB (A797G)	12.448 (0.014*)	2.873 (0.004**)	2.487 (0.012*)	2.931 (0.003**)	2.469 (0.013*)	2.908 (0.0036**)	NS
RLN1 (A22C)	2.309 (0.021*)	3.128 (0.002**)	2.330 (0.020*)	3.103 (0.002**)	2.320 (0.020*)	3.120 (0.002**)	NS
RLN2 (T9intG)	2.554 (0.011*)	2.765 (0.005**)	2.528 (0.011*)	2.744 (0.006**)	2.541 (0.011*)	2.758 (0.006**)	7.630 (0.006**)
PTHLH (C375T)	2.081 (0.037*)	2.711 (0.006**)	2.095 (0.036*)	2.635 (0.008**)	2.088 (0.037*)	2.680 (0.007**)	8.1 (0.004**)

TL : left teat number
TR : right teat number
TT : total teat number
ITL : left inverted teat number
ITR : right inverted teat number
TIT : total inverted teat number
NS : non significant.

NS = not significant (p >0.05), *p < 0.05, ** p < 0.01

5. Discussion

5.1 Motives to address the inverted teat defect

The regulation of mammary growth involves complex interactions of many hormones and growth factors, of which some are growth–stimulating and others growth-inhibitory. In particular the influence of the sex hormones and the metabolic hormones on the development of the mammary gland have been investigated in different animal species. It can be assumed that the emergence of teat anomaly is to due to a disregulation of the hormonal control. Hence the genes responsible for these hormones (proteohormones), hormone receptors and growth factors can act as candidate genes for inherited disorders of the mammary gland.

Inverted teats are of the great concern in modern pig industry. Teat defects, which reduce mostly the number of functional teats, belong to the hereditary defects with a substantial influence on the operating economic result in the pig breeding. They lead to the reduction of the raising performance (Wiesner and Willer 1978, Matzke 1980) and restriction of the selection intensity with other productivity characteristics (Mayer 1994). Inverted teat is a condition characterised by failure of teats to protrude from the udder surface. The teat canal is held inward, forming a small crater so that normal milk flow is prevented. This abnormality has a genetic background, but the number of pairs of genes involved is unknown. Caution must be exercised in evaluating underlines since nipples surrounded by a ring of loose skin are not inverted if the nipple tip is present but are due to injuries. Teats located near the sheath of boars are often falsely classified as inverted. Because of the uncertainty of the genetics of this trait, culling of parents and littermates is not recommended. But individuals with a high proportion of inverted nipples should not be placed in the breeding herd.

The most frequent and most important teat defect represents the inverted teat (Blendl et al. 1980). In different investigations in German pig populations affection frequency between 7.6 and 20 % were found (Niggemayer 1993, Brevern et al. 1994, Mayer and Pirchner 1995). In the DUMI Bonn-Berlin resource population, proportion of mammary

gland abnormalities was 53.6 %, the proportion of inverted teats was 42.2 % and of extra teats was 17.9 %.

The hereditary of the teat anomaly is not clearly enlightened and is controversially discussed in the literature. The heritability values for the number of inverted teat lies in the middle range between 0.2 and 0.5 (Hittel 1984, Mayer 1994, Brevern et al. 1994). In comparison to other hereditary caused deformations, inverted teat cannot be determined *in praxi* with same security (Steffens 1993). Therefore the identification of the genetic causes of this anomaly an interesting perspective for selection measures for the improvement of the quality of teats.

5.2 Strategies to identify genes controlling economically important traits

The identification of genes controlling economically important traits provides the basis for new progress in genetic improvement of livestock species, complementing traditional methods based only on measured performance. The identification of these genes, whether they are major genes or quantitative trait loci (QTL), directly affecting variability in traits to be improved, is an important objective to be pursued.

Two approaches have been pursued to identify genetic markers for inverted teat traits. First, genome scans employing anonymous DNA markers have been used to identify quantitative trait loci (QTL) influencing this trait. However, a genome scan will fail to detect trait loci with smaller effects if they do not reach the stringent significance thresholds that must be applied when doing number of tests in a full genome scan (Andersson 2001). Second, physiological candidate gene approaches have utilized polymorphisms within or close to genes that are known to play a role during mammary gland development in tests for associations with this trait. This candidate gene approach might fail to identify a major trait locus simply because of the gap on the present knowledge about gene function (Andersson 2001). Hence, in this study, the strategy combines these two approaches, identifying QTL through genome scans using interval mapping and testing genes identified as candidates on both positional and physiological grounds.

Another approach to detect candidate genes is based on the analyses of differences of the expression profile in particular subsets of cells and/or individuals with certain phenotypes. These genes are functional candidate genes because of their temporo-spatial distribution of expression or their expression in certain phenotypes. The functional candidate approach benefits from the fact, which it only deals with cDNA, devoid of intronic and intergenic sequences, which represent only a few percent of the total genome (about 3% in mammals). Differential expression screening approaches are therefore more closely associated to gene function. It is foreseen to also apply this functional genomics approach in order to elucidate the aetiology of inverted teats.

5.3 Gene mapping approach

For gene mapping there are currently two main approaches that have been used in this study. One is to use a radiation hybrid panel (IMpRH). The general principle of radiation hybrid mapping is to test for the co-retention of genome markers in very small chromosome fragments, obtained by X-ray irradiation of donor cells of the species of interest and rescued by fusion to recipient cells of another species. IMpRH are an attractive tool for mapping arbitrary sequences (such as ESTs) without the need for polymorphism and high resolution, and have been important in making a very gene-rich human map (Deloukas et al. 1998). This study used the IMpRH panel (7000 rds) developed by Yerle et al. (1998), including 118 hybrid clones. The results of radiation hybrid PCR products were easily analyzed with the IMpRH mapping tool developed by Milan et al. (2000). One limitation of this approach is that RH maps tend to have more uncertainty in the order of closely spaced markers than genetic maps, but an important advantage is that mapped sequences need not be polymorphic.

The second approach for mapping gene is called ‘linkage mapping’. The result would be developed and mapped markers and test if a marker that is polymorphic "co-segregates" with the SNP in the gene. Genetic linkage maps have become powerful research tools in many organisms (Dietrich et al. 1996, Dib et al. 1997). The development of genetic maps in livestock species has allowed the detection of genomic regions contributing to the genetic variation of quantitative traits, such as growth, body

composition, meat quality, or reproduction (Bidanel et al. 2001, Malek et al. 2001a, b). A complete linkage map is necessary in order to efficiently carry out molecular-based analyses such as marker-based selection (Cho et al. 1994), quantitative trait locus (QTL) analysis (Lander and Botstein 1989) and loss of heterozygosity (LOH) studies in tumorigenesis (Dietrich et al. 1994) and for comprehensive investigations of genome evolution between lineages (Morizot et al. 1977, Morizot 1983, Lyons et al. 1997). In order to link our results of QTL studies and candidate gene analyses it is necessary to link genetic map which is mainly based on microsatellites.

5.4 Single nucleotide polymorphism (SNP) and genotyping method

Regardless of the study design used, single nucleotide polymorphisms (SNPs) may provide an important alternative to conventional markers, for genetic mapping studies of complex traits. SNPs are sites in the genome that have nucleotide differences. These polymorphism are highly abundant, occurring approximately ~ 1/1,000 bp (Wang et al. 1998). Methods for the genotyping of SNPs are more easily automated and potentially less expensive per marker than are conventional methods such as microsatellite markers (Nickerson et al. 1990, Pease et al. 1994). Given the large number of markers and individuals that must be genotyped for studies of complex traits, SNPs could substantially reduce the cost of a genetic mapping study. For these reason, SNPs may become a key component in the studies of complex traits. Several studies have evaluated SNP characteristics that are important for both association mapping studies, including the allele frequencies and the LD between markers.

The technological and economic goal is accurate, easy, cheap and fast large-scale SNP genotyping. Several methods are currently being developed, and it is unclear which one(s) will turn out to be the best. The commonly used detection methods such as sequencing of an amplified PCR product (e.g. by radiolabelled - or fluorescence-based polymerase chain reaction), single-strand conformation polymorphism (PCR - SSCP) or restriction fragment length polymorphism (RFLP) are time consuming and therefore not suitable for the routine screening of large numbers. PCR - RFLP can be genotyping

procedure that is easy to set up in any molecular laboratory. The RLN (T9intG) loci was performed this method. The recognition site of CfoI restriction enzyme is GCG▼C.

Single strand conformation polymorphism (SSCP) is based on the specificity of folding conformation of single stranded DNA when placed in non - denaturing condition. The TGFB1 loci and RLN (A22C) loci were genotyped by this method, because there were non recognition sites of any restriction enzymes.

In this study a polymorphism in the PTHLH gene was found in coding sequence (TTG/TCG: Leu/Ser). A TaqMan assay was used to perform genotyping. It can be used for rapid, precise and unambiguous detection and analysis of biallelic SNPs. The determination of an optimal condition for the analysis was facile, and typing results were judged to be accurate since they were consistent with results obtained by sequencing method. Here, we report the use of the Taqman probe in the genetic analysis of the PTHLH gene polymorphism to test for association with inverted teat. This protocol enabled the fluorescence genotyping of 96 samples in 2 hours without need for electrophoresis.

The SNP assay contains two primers for amplifying the sequence of interest and two TaqMan® MGB probes for distinguishing between two alleles. Each TaqMan MGB probe contains a reporter dye at the 5′ end of each probe. VIC ™ dye is linked to the 5′ end of the Allele ‘C’ probe, 6-FAM ™ dye is linked to the 5′ end of the Allele ‘T’ probe and a minor groove binder (MGB) in both the probe. This modification increases the melting temperature (Tm) without increasing probe length (Afonina et al. 1997, Kutyavin et al. 1997), which allows the design of shorter probes. This results in greater differences in Tm values between matched and mismatched probes, which produce more accurate allelic discrimination.

During PCR, each TaqMan ® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by energy transfer (Lakowicz, 1983). AmpliTaq Gold® DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the

reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Thus, the fluorescence signal generated by PCR amplification indicates which alleles are present in the sample. Mismatches between a probe and target reduce the efficiency of probe hybridization. Furthermore, AmpliTaq Gold DNA polymerase is more likely to displace the mismatched probe rather than cleave it to release reporter dye. The detail of SNP assay is given below in figure 16.

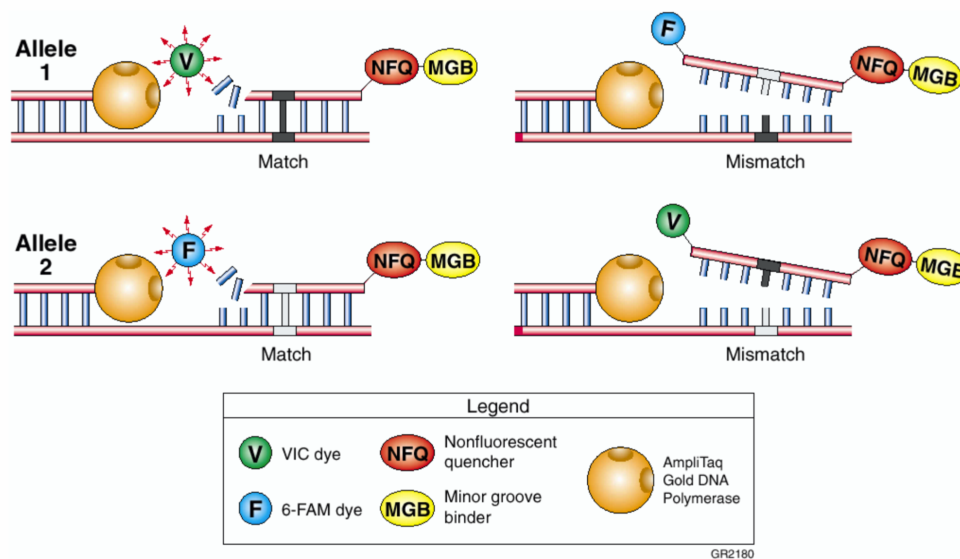

Figure 16: Overview of SNP assay using TaqMan probe

5.5 Quantitative trait loci for inverted teat defect (and number of teats)

QTL identification is based on animals from `DUMI´ three generation F_2 resource population found by reciprocal crossing of Berlin Miniature pig and Duroc at the Institute of Animal Science of the Humboldt University of Berlin (Hardge et al. 1999). Animals of the DUMI resource population were kept and performance tested at the research farm Frankenforst (Institute of Animal Breeding Science, University of Bonn). With close to 1000 F_2-animals of this experimental population a genetic map has been established covering 72 microsatellite markers and some type I marker loci. The

evidence of linkage of markers with the appearance of inverted teats was reported in a whole genome scan experiments (Ün 2002, Oltmanns 2003).

The non-parametric analysis of the data of the genome scan within the DUMI population identified six QTL regions with significant NPL values (> 4.0) (Table 16). The highest linkage was found with marker S0220 on chromosome 6 (NPL score 8.8). TGFB1 and RLN are located on chromosome 6q11-q22 and 1q28-29, respectively. Therefore, the pervious study proposed TGFB1 gene and RLN gene as candidate genes for inverted teat based on the QTL analysis and gene function (Ün 2002). Strong evidence of linkage to chromosome 6 was also identified in a linkage analysis for the inverted teat defect in commercial breeds (DL, DL*DE). The highest NPL score on chromosome 6 was 6.8. On chromosome 2 a NPL score of 2.4 was found when analysing affected sibpairs of the crossbreed DL*DE. The results are summarized in table 17 (Wimmers, unpublished data).

The teat number is an important trait with regard to the mothering ability of sows, the pig industry has traditionally applied selection pressure to teat number (Pumfrey et al. 1980). In particular, teat number plays an important role when the number is less than the litter size. Nevertheless, information about the inheritance of teat number is limited in comparison to other reproductive traits of pigs. Number of teats has not been investigated in most previous porcine reproductive QTL studies (Rathje et al. 1997, Wilkie et al. 1999, Rohrer et al. 1999). However, QTL affecting number of teats was reported on the p arm of SSC8 at the genome-wide significance level ($P<0.05$) (Cassady et al. 2001). The additional evidence for teat number QTL was found in a Meishan X Dutch cross on chromosome 10 and 12, with the beneficial alleles from the Meishan breed and a QTL on chromosome 2 with a negative effect of the Meishan allele (Hirooka et al. 2001). Rohrer (2000) found significant evidence for a QTL for teat number in a Meishan X Large White cross on porcine chromosome 10. Neither of these studies found evidence for QTL on SSC8 influencing teat number. A suggestive QTL for teat number was revealed on the short arm of SSC8. Moreover, Wada et al. (2000) reported a putative QTL affecting teat number on *Sus scrofa* chromosome 1, and 7 in a Meishan x Göttingen miniature cross.

Table 16: QTL regions for affection of inverted teat within DUMI resource population

Chromosome	1	2	3	4	5	6
No. of loci	3	6	4	7	4	11
max. NPL	4,7	7,8	4,5	5	3,0	8,8
Position cM	160	20	70	100	0	120
Microsatellite	SW1301	S2443	S0164	S0097	SW1482	S0220

Chromosome	7	8	9	10	11	12
No. of loci	8	5	5	3	4	4
max. NPL	2,3	3,9	2,8	2,2	3,0	1,5
Position cM	140	101	27	72	96	51
Microsatellite	S0115	SW61	SW911	S0070	SW703	SW874

Chromosome	13	14	15	16	17	18
No. of loci	3	5	4	3	3	3
max. NPL	2,1	4,8	2,1	4,0	1,2	3,5
Position cM	79	20	81	54	0	69
Microsatellite	SW398	S0007	SW936	S0026	SW335	SWR414

Table 17: QTL-regions for inverted teat affection within commercial breeds German Landrace (DL), Large white (DE), and F1 reciprocal cross German Landrace and Large white (DL * DE)

		Sscr1	Sscr2	Sscr6
All	max. NPL	0,4	1,8	2,6
(70 sibpairs)	Microsatellite	SW1301	S0227	S0035
DL	max. NPL	-	1,9	1,9
(45 sibpairs)	Microsatellite		S0227	S0220
DL*DE	max. NPL	1,1	2,4	6,8
(15 sibpairs)	Microsatellite	SW1515	S0227	S0035

Quantitative traits are regulated by many genes and affected by interactions among them, and thus, a candidate gene associated with a trait in one population may have a different effect, or show no effect at all, in another population due to negative effects of other genes and epistatic interactions of the candidate gene with other genes in the population (Pomp 1997). This theory is supported by many association studies, in which a polymorphism was significantly associated with performance traits in one family or breed (Casas et al. 1998, Feng et al. 1997, Knorr et al. 1997). A more complex system must be developed to model QTL effects before a genetic marker can be broadly applied to breeding schemes in different populations.

In summary, QTL for inverted teat defect trait have been found in multiple regions of the genome (Ün 2002, Oltmanns 2003). TGFB1 and RLN represent biological candidate genes for the inverted teat defect. The genes TGFB1 on chromosome 6 as well as RLN on chromosome 1 map within QTL regions identified here and thus also represent positional candidate genes. This study aims to identify polymorphisms in the candidate genes, which were found in the QTL regions. In addition, focus was on a third direct biological candidate gene, PTHLH.

In this study, detailed analyses of three candidate genes were carried out in the Bonn-Berlin DUMI resource population. The TGFB1 and RLN gene were selected as the functional positional candidate genes for molecular investigation, based on the findings of the QTL analysis and biological evidence in both human and rodent models. The TGFB1 and RLN sequence gene were available in databank. In addition, PTHLH has also been characterised in this study. The PTHLH is located on Sscr5q23. Oltmanns (2003) identified no significant QTL region on chromosome 5; maximum NPL value on chromosome 5 was 3.2. Nevertheless many studies show that the PTHLH gene is functionally related in mammary gland development, and hence PTHLH can act as a functional candidate gene. Researchers usually choose genes to be "candidate genes" or likely genes for a trait because a very similar trait was shown to be caused by that gene in another species, such as mouse or humans. It could be that a good functional candidate gene for inverted teat defect is PTHLH because that is the gene that effects the formation of mammary gland in mice (Foley et al. 2001).

5.6 Candidate gene analysis

5.6.1 Transforming growth factor beta 1 gene (TGFB1)

The TGFB1 gene contains 7 exons separated by six non-coding intron sequences ranging in size from 1.9 to 40 kb in human (Derynck et al. 1987). In this study, porcine TGFB1 intron 4 was sequenced which is 186 bp in length (Accession number AF461809). Recently, intron 6 of TGFB1 gene was sequenced (819 bp) by Kopecny et al. (AJ621785: Unpublished).

5.6.1.1 Experimental and functional evidence for an aetiological role in the inverted teat defect

The TGFB1 locus is addressed as a candidate gene because of its location near the QTL region, and also based on its role in mammary gland developmental. Developmental roles of TGFB1 are reflected in its apparent involvement in the maturation and function of the mammary gland (Robinson et al. 1991, Silberstein et al. 1992). Implants containing TGF family protein have been introduced into the mammary glands of subadult virgin mice directly in front of the mammary end buds so that the effect of the peptide on the ductal development of the gland might be studied (Silberstein and Daniel 1987, Robinson et al. 1991). The implant locally and reversibly inhibited ductal penetration of the fatty stromal tissue in juvenile mice. In contrast, introduction of similar TGFB implants into hormonally or pregnancy induced mammary glands failed to overtly affect lobuloalveolar development (Daniel et al. 1989) and Soriano et al. (1996) reported that the low concentrations of TGFB1 promote mammary gland ductal morphorgenesis. Furthermore, TGFB inhibited ß-casein expression in HC11 mammary epithelial cells and in mammary explants from mid-pregnant mice, providing evidence for a role in regulating functional differentiation and lactogenesis (Mieth et al. 1990, Robinson et al. 1993). The growth inhibitory effect of TGFB1 in mammary epithelial cells was confirmed by study in transgenic mice. The transgenic mice were generated by injecting one cell embryos with simian TGFB1 mutated to produce a constitutively active product under the control of the MMTV enhancer/promoter. Tissue-specific

expression of the transgene in mouse mammary glands resulted in marked ductal hypoplasia, suppression of ductal branching, and a concordant reduction in bromodeoxyuridine labelling in mammary epithelium. Interestingly, during pregnancy alveolar outgrowths developed from the hypoplastic ductal tree and transgenic females were able to lactate (Pierce et al. 1993).

5.6.1.2 Polymorphism

Alternative splicing of the TGFB1 transcript has been described in human and pig. Although the frequency of occurrence of the alternate splice from pig is considerably less than the dominant splice form, where the exons are retained, the precise frequency and distribution of the alternate splice pattern are not well described (Kondaiah et al. 1988). No evidence for similar splicing patterns in other animal species has been defined, nor have the biological implications of this splice variant been extensively explored in the pig. An alternative splicing pattern of the amino terminal of the TGFB RNA transcript seen in the pig could not be confirmed in the equine TGFB1 precursor, despite considerable effort to identify its presence (Nixon et al. 2000). A previous report on polymorphism of the porcine TGFB1 was based on Southern blot analysis that revealed a polymorphism after hybridisation of DraI digested genomic DNA with a human cDNA probe. Two variable fragments, 5.0 kb and 3.6 kb were detected. In six three-generation pedigrees of Meishan x Yorkshire pig (n=90) RFLP allele segregation was consistent with codominant, autosomal inheritance of the marker (Feltes et al. 1993). Consistent to the importance of this gene polymorphism study of this gene was carried out. A SNP ‘A/G’ transition in exon 5 at position 797 of the coding sequence was found. Until now, no polymorphisms causing amino-acid substitution were detected in the porcine TGFB1 gene. Furthermore Hu et al. (1997) reported that no polymorphism was observed for porcine TGF beta 1 with endonucleases AluI, BamHI, BglII, DraI, EcoRI, HaeIII, HindIII, MspI, PstI, PvuII, RsaI, and TaqI.

5.6.1.3 Candidate gene for disease in other species

Human TGFB1 plays an important role in the modulation of cellular growth and differentiation in a wide variety of cell types and in the production and degradation of the extracellular matrix. Several polymorphism of the human TGFB1 gene were identified and elucidated the predisposition to various diseases. Over eight novel polymorphisms have been identified (Cambien et al. 1996, Keen et al. 2001), and these polymorphisms of TGFB1 might be correlated with atherosclerosis, bone disease, or various forms of cancer (Grainger et al. 1999). Two studies have shown the presence of an association between polymorphisms of TGFB1 and either osteoporosis or bone turnover in the white and Japanese population (Langdahl et al. 1997, Yamada et al. 1998). Yamada and other (1998) demonstrated that a T to C transition at nucleotide 29 was associated with both BMD at the lumbar spine and the rate of bone loss in postmenopausal Japanese women. A similar association was obtained in an Italian population, although a polymorphism (718-8delC) in the gene showing the association was different (Bertoldo et al. 2000). Recently, several host genetic association studies with polymorphisms of the human TGFB1 gene have been performed for elucidating the function of polymorphism of the human TGFB1 gene. Camurati-Engelmann disease (CED), or progressive diaphyseal dysplasia, is a rare, sclerosing bone dysplasia inherited in an autosomal dominant manner. Recently, the gene causing CED has been assigned to the chromosomal region 19q13 (Ghadami et al. 2000). Because this region contains the gene encoding transforming growth factor-beta 1 (TGFB1), an important mediator of bone remodelling, they evaluated TGFB1 as a candidate gene for causing CED. A lack of growth inhibition by TGFB leads to carcinogenesis (Massague´ 1998) and excess TGFB activity may play a significant role in the pathogenesis of fibrotic disorders in the kidney (Border et al. 1992) and inflammatory disorder (Shull et al. 1992, Kulkarni et al. 1993).

5.6.1.4 Gene Mapping

For linkage mapping of TGFB1 the CRI-MAP package (version 2.4) was used. Multipoint linkage map was established using the BUILD and FLIPS options. Genotyping of 380 F2-individuals from 21 families of the Bonn-Berlin-DUMI resource population and subsequent two-point and multipoint procedures of the CRI-MAP revealed linkage to loci S0300, SW193, and SW1067 (proximal) and S0220 (distal) with distances of 7.3, 13.8, 24.5 and 31.5 cM, respectively, on the sex average map. SW1067 has been assigned to Sscr6q11-q22 (Yerle et al. 1990a). TGFB-1 gene position is in agreement with the published genetic and physical map.

The TGFB1 has also been mapped to human chromosome by somatic cell hybridization and *in situ* hybridization. TGFB1 has been assigned to Hsap 19q13.1-q13.3 in man and to chromosome 7 in the mouse (Dickinson et al. 1990, Fujii et al. 1986). Concerning the comparative mapping discussion, it is based on comparison with the human genome. This result was also consistant with human comparative map result in which TGFB1 gene was localized to chromosome 6 (Figure 17).

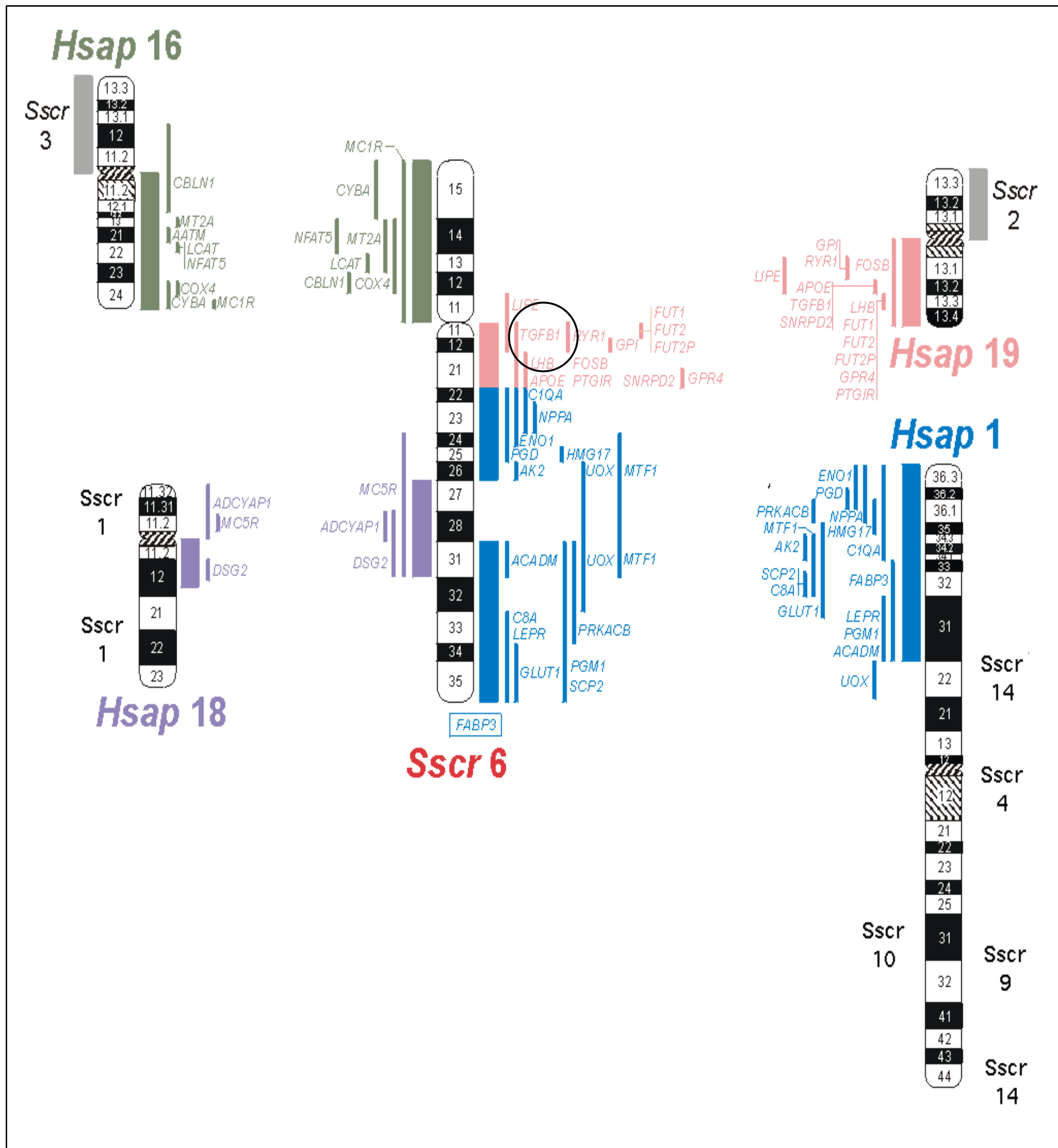

Figure 17: A comparative map of TGFB1 and the human genome

(http://www.toulouse.inra.fr/lgc/pig/compare/SSCHTML/SSC6S.HTM)

5.6.2 Relaxin gene (RLN)

In this study primers were derived based on the published relaxin sequence (Haley et al. 1987) (Accession number J02792) that reveal overlapping fragments of about 300-500 bp in size covering the DNA sequence from part of 5′-UTR to the 3′-UTR. DNA fragments obtained from five pig breeds - Hampshire, Duroc, Pietrain, German Landrace, Berlin-Miniature pig–were comparatively sequenced.

5.6.2.1 Experimental and functional evidence for an aetiological role in the inverted teat defect

Relaxin was chosen as a positional candidate gene. The mammary gland is a target organ for Relaxin. In the rodent study, it was confirmed that relaxin promotes growth and differentiation of the mammary parenchyma (epithelial and myoepithelial cells) and the mammary stroma (fibroblsts, adipocytes and collagen) (Bani et al. 1985, Bianchi et al. 1986). Relaxin has been reported to reduce milk yield and decrease mammary RNA in lactating rats. Relaxin lengthens the mammary gland ducts in rats and cause growth of ducts and alveola in synergy with other mammotropic substances and also relaxin stimulates the growth of mammary gland malignancies *in vitro* in rats (Weiss 1984). Relaxin was demonstrated to be essential for development of normal mammary nipples and nipple function in pregnant rats (Hwang et al. 1991, Kuenzi et al. 1995). Furthermore, the relaxin immunoreactivity was detected in normal as well as in neoplastic human mammary tissue (Mazoujian and Bryant-Greenwood 1990) the question arose whether relaxin influences the growth of breast neoplasm. In the knockout mice experiment, the rln -/- mothers exhibited normal nursing behaviour; however pups were not able to suckle milk from the breast because of the underdeveloped nipples (Zhao et al. 1999).

5.6.2.2 Polymorphism

The porcine genome was shown to contain only a single relaxin gene in contrast to the human, which contains two non allelic genes. The porcine relaxin gene comprises two exons separated by a 5.5-kilobase intron. Within the coding sequence, a single base change of ‘C → T’ relative to the cDNA sequence (Haley et al. 1982, 1987) is observed at position 7041, resulting in a Ser→ Leu change in the carboxyl-terminal peptide at residue 116. The amino acid sequences of pig, rat and shark relaxin have been published. Rat preprorelaxin has been shown to contain a 105 amino acid connecting peptide linking the A and B chains (Hudson et al. 1981), and similarly porcine preprorelaxin has been shown to comprise a signal peptide of 24 amino acids, a B chain of 32 amino acids, and an A chain of 22 amino acids (Haley et al. 1982). A comparison of the sheep nucleotide sequence with exon II of pig relaxin revealed homology of 72% (Roche et al. 1993). In this study, a transversion (C>A) at position 1 of codon 8 (nt 22) in the first exon leading to a amino acid exchange (Leu → Ile) was detected and another transversion (T>G) was found at position 9 of the intron 1 altering the recognition site of CfoI.

Human RLN is a peptide hormone that is encoded by two genes referred to as H1 and H2, both located into chromosome 9p24.1. D'Elia et al. (2003) searched for polymorphisms in the 5'-flanking sequence of these genes. Both genes possess a CT repeat followed by a GT repeat. CT and GT repeats of the H2 gene are longer than those of the H1 gene. Moreover, CT and GT repeats of the H2 gene, but not those of the H1 gene, show length polymorphism.

5.6.2.3 Gene Mapping

For RLN gene linkage mapping analysis was shown SW1515-33.0 cM - SW1815-31.4 cM SO155-7.6 cM-RLN 56.5cM-SW1301; sex averaged distances are given in Kosambi centrimorgan. Two point linkage analysis revealed a recombination fraction of .08 between RLN and SO155 (LOD=18.3). The position of RLN gene is in good agreement with the published physical assignment to Sscr1q28-29. By study of mouse-human cell hybrids, Crawford et al. (1984) found that both relaxin genes are on 9p

(9pter-9q12). Naggert and Mu (1994) stated that the relaxin gene maps to mouse chromosome 19 near D19Mit23. Furthermore, a comparison with the human genetics map shows that it corresponded to linkage map (Figure 18).

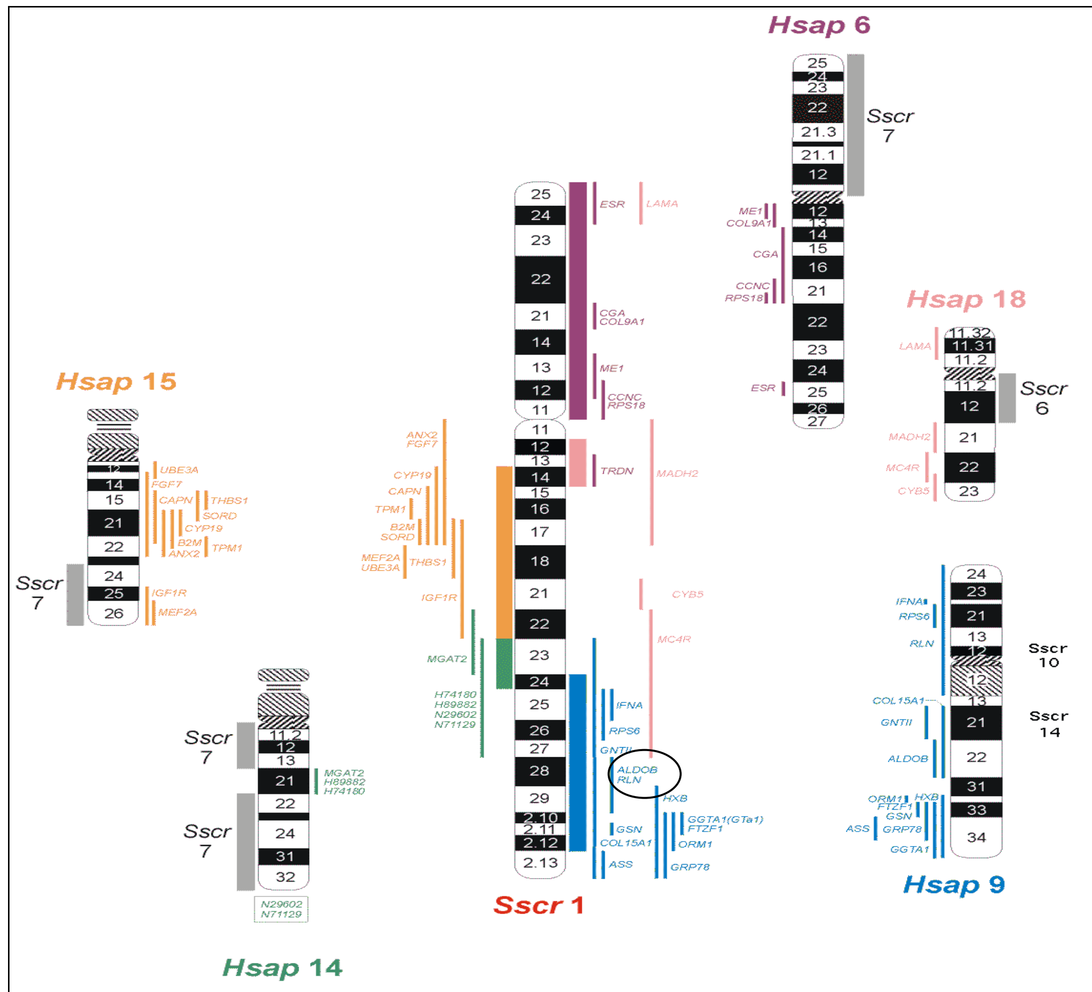

Figure 18: A comparative map of RLN and the human genome

(http://www.toulouse.inra.fr/lgc/pig/compare/SSCHTML/SSC1S.HTM)

5.6.3 Parathyroid hormone like hormone gene (PTHLH)

This study presents the first isolation of the PTHLH gene in the pig. The identification of porcine PTHLH gene was based on the human published sequence and information of ESTs of various species that matched the human PTHLH sequence. PCRs with homologoue and heterologoue primers and RACE-PCR were used to isolate gene from porcine ovary RNA and to characterise it by nucleotide sequencing. A comparison with PTHLH sequences from other species indicated a high level of sequence conservation.

5.6.3.1 Cloning and characterization of cDNA for porcine PTHLH

Suva et al. (1987) and Mangin et al. (1988a) identified a cDNA clone that encodes human PTHLH. The cDNA encodes a protein of 177 amino acids, containing a precursor sequence of 36 amino acids followed by the mature peptide of 141 amino acids. Eight of the first 13 amino acids in the mature peptide are identical to those of PTH. The sequence diverges completely after amino acid 13, and it is this subsequent region that must account for the distinctive biologic actions of the 2 peptides. An ORF of 177 aa was verified by the presence of 5' in-from stop codon (TGA) at bp 851-853. The porcine 5'-UTR (bp 1-319) is 85 % homologous to the 5'-UTR of exon 2 and 3 of human PTHLH (Yasuda et al. 1989) or exon 1b and 2 (Suva et al. 1989). The 5'UTR is homologous to exon 1A of human PTHLH homology to this region has not been identified in rat, mouse, or chicken sequence. In addition 319 bp of 5'untranslated region was sequenced which was separated by intron. This intron sequence is 175 bp in length and has 83% homology with human PTHLH intron 2. The C' terminal portion of PTHLH is 94% homologous to human PTHLH exon 6 (Yasuda et al. 1989) and 94% homologous to canine C'terminal (Rosol et al. 1995).

The porcine 3'UTR (bp 854-1308) is 94 % homologous to exon 6 of human PTHLH. The 3'UTR portion of PTHLH is highly conserved among reported species. There is 91 % and 98% homology in the coding region of porcine PTHLH to the exon 3 and 4 human PTHLH respectively. The biological function of the C-terminal PTHLH peptide is not clearly understood and may have divergent functions in different species (Mangin et al. 1989). The predicted mature peptide is 91 % homologous to human PTHLH.

Comparison of the translated sequence of PTHLH with other published sequences showed high similarity with human (91%), mouse (87%), bovine (92%), rabbit (91%), canine (92%) and equine (89%).

5.6.3.2 Experimental and functional evidence for an aetiological role in the inverted teat defect

Parathyroid hormone like hormone gene (PTHLH) was chosen as a candidate gene based on its role in physiology of reproduction. PTHLH is present in mammary epithelial, the maternal circulation during lactation and in the mothers' milk of various species (Moseley and Gillespie 1995, Philbrick et al. 1996). The proposed functional roles of PTHLH during lactation include: stimulation of mammary and neonatal cell growth and differentiation, increasing calcium transport from blood to milk, influencing mammary blood flow and myoepithelial cell tone and regulation of maternal and neonatal calcium homeostasis (Philbrick et al. 1996). Wlodek et al. (2003) indicated that milk and mammary PTHLH are regulated by different mechanisms but that they are influenced by the maternal lactation environment. Recently, overexpression of PTHLH has been shown to disrupt branching morphogenesis during mammary gland, PTHLH is produced in alveolar epithelial cells and the PTH/PTHLH receptor is expressed in adjacent stromal cells (Dunbar et al. 1998, Wojcik et al. 1999). Indeed targeted overexpression and knockout studies have revealed the critical importance of PTHLH in normal branching morphogenesis and mammary epithelial development. PTHLH mRNA and protein expression in rat mammary tissue is dependent upon the suckling induced rise in prolactin (Thiede 1989). Furthermore, Foley et al. (2001) used a combination of loss-of-function and gain-of function models and reported that PTHLH regulates a series of cell fate decision that are central to the survival and morphogenesis of the murine mammary epithelium and the formation of the nipple. PTHLH acts as an epithelial signal that induces the mesenchyme around the epithelial bud to become mammary specific. As a result, the mammary mesenchyme acts on the epithelial bud maintain the mammary identity of the epithelium and to support ductal morphogenesis. It also acts upon the epidermis around the mammary bud to suppress hair follicle formation and trigger nipple sheath formation.

5.6.3.3 Polymorphism

The human PTHLH gene comprises eight exons spanning more than 15 kb of genomic DNA. At its 5’ end, the human PTHLH gene contains two apparent promoter elements and four exons; at its 3’ end, the gene contains alternatively spliced exons which give rise to mRNAs encoding three different deduced products, each with a unique C terminus (Suva et al. 1989, Mangin et al. 1990a, b, Thiede et al. 1988, Yasuda et al. 1989). As compared to the complex hybridization pattern observed on Northern analysis of human RNAs (Mangin et al. 1989), RNAs prepared from rodent tumors and tissues appear to contain a single broad hybridizing band (Thiede and Rodan 1988, Yasuda et al. 1989) suggesting that the mouse and rat PTHLH gene might have a simpler organization that the human gene. The completed structure of porcine PTHLH is still not available.

A polymorphism of the variable number of tandem repeat (VNTR) type is located 97 bp downstream of exon VI of the parathyroid hormone-related peptide (PTHrP) gene in humans (Pausova et al. 1993). The repeat unit has the general sequence G(TA)nC, where n equals 4-11. Intra-species variability of the locus was demonstrated only in humans and gorilla. The divergence of the TA-dinucleotide repeat sequence and the variable mutation rates observed in different primate species are in contrast to the relative conservation of the flanking sequences during primate evolution. This suggests that the nature of the TA-dinucleotide repeat sequence, rather than its flanking sequences, is responsible for generating variability (Pausova et al. 1995). The mouse parathyroid hormone-like hormone $Pthlh^{Pro}$ and $Pthlh^{Thr}$ variants are linked with susceptibility and resistance to skin carcinogenesis of Car-S and Car-R mice, respectively, and with in vitro effects (Manenti et al. 2000b). The HindIII and TaqI RFLPs were performed in Human PTHLH gene (Hendy and Goltzman 1990). HindIII and TaqI identify a multiple allele polymorphism with at 35 unrelated North Americans of mixed ethnic origin. No polymorphism for EcoRI, PstI, and BclI was performed by RFLP. In this study, C/T non-synonymous SNP was detected at nucleotide 375 of the porcine PTHLH cDNA (Accession number AY193782). That only one mutation causes an amino acid change from leucine to serine and, therefore, is classified as a

conservative mutation. As no structure-function analysis is available, the possible functional effects of this mutation are unknown.

5.6.3.4 Gene mapping

In this study, two-point RH linkage analysis identified the most significant linkage of PTHLH with SW1134 and SW2033 (LOD=2.74 and 1.94). The most significantly linked marker (2pt analysis) is SW1319 on chromosome 5 (73 cR; LOD 4.85).

For PTHLH genetic mapping, twopoint and multipoint procedures of the CRI-MAP package version 2.4 revealed linkage to loci SW1134 (proximal) and IGF1 (distal) with distance of 24.4 cM (recombination fraction = .23, lods = 17.41), and 48.2 cM (recombination fraction = .37, lods = 3.43), respectively, on the sex averaged map. IGF1 has been assigned to Sscr5q23. Microsatellites SW1319 and SW1134 map in close proximity at 49.3 cM on the second release genome-wide linkage map developed by the USDA Meat Animal Research Centre. The results of RH and genetic mapping are in agreement and correspond with published genetic and physical maps.

Mangin et al. (1988a) assigned the PTHLH gene to 12p12.1-p11.2 by a combination of Southern analysis of somatic cell hybrid DNA and in situ hybridization. Hendy et al. (1989, 1990) assigned the corresponding gene in the mouse to chromosome 6 by means of Southern blot analysis of DNAs isolated from a panel of mouse/Chinese hamster cell hybrids. This assignment is in agreement with syntenic localization of PTHLH to porcine chromosome 5, mouse chromosome 6 and human chromosome 12, and the physical assignment of PTHLH to porcine chromosome 5q23. The comparative mapping between human and pigs, it was consistent with RH mapping result (Figure19).

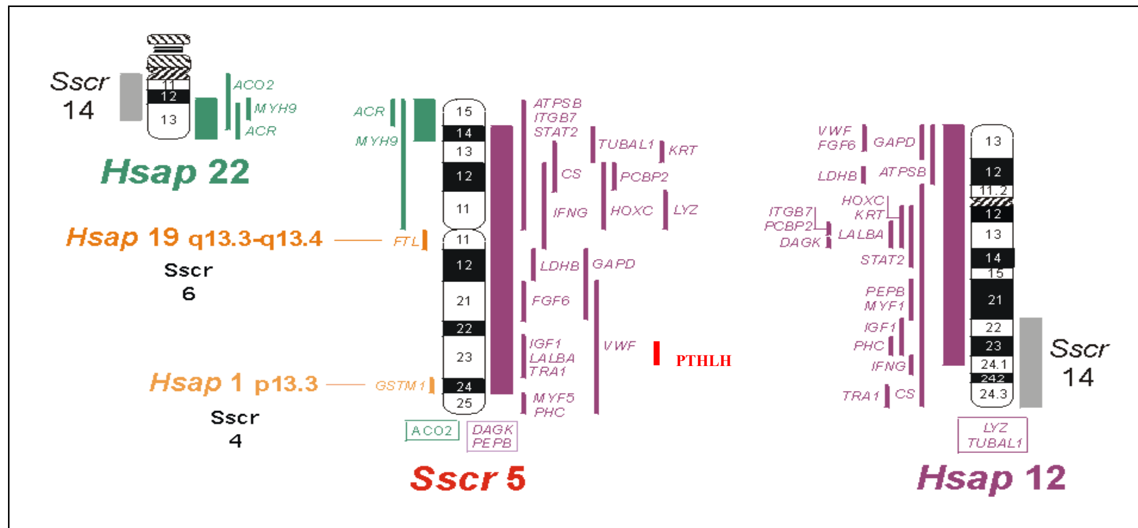

Figure 19: A comparative map of PTHLH and the human genome

(http://www.toulouse.inra.fr/lgc/pig/compare/SSCHTML/SSC5S.HTM)

5.7 Association and statistic analysis

The combinations of population genetics and molecular genetics have brought the livestock genomic field to a revolutionary time. The association between genotype and phenotype can be more completely understood. Some studies of the monogenic traits were successful in associating genotype with phenotype such as the porcine ryanodine receptor gene with porcine malignant-hyperthermia-susceptible skeletal-muscle (Fujii et al. 1991), the protein kinase, AMP-activated, gamma (3)-subunit (PRKAG3) gene with muscle glycogen content in pig (Milan et al. 2000) and the myostatin gene with excessive muscling in double-muscled cattle (Grobet et al. 1997). By contrast, complex traits have proved to be more challenging, because it is impossible to follow all genomic regions that are responsible for the complex variation of the trait without some further idea of how these regions segregate. The use of association studies to detect QTLs is an important component of most strategies for finding the genes for complex traits. Complex traits are generally multifaceted and may, in some case, be best described by one or more quantitative traits. Although linkage mapping has a long history for quantitative traits, interesting association studies with quantitative traits, especially

those studies using family-based designs, is more recent. Family-based association tests (FBATs)-in particular, those based on the transmission/disequilibrium test (TDT), popularized by Spielman and other (1993) are attractive because of their simplicity and robustness to spurious association, which can arise with population heterogeneity (Lange et al. 2002).

This study is the first to demonstrate the association of SNPs for TGFB1, RLN and PTHLH genes with inverted teat defect in pigs. Individual quantitative phenotypes were analysed with the Family-Based Association test (FBAT). In this setting, the phenotypes are regarded as fixed variables, and tested for excess transmission of a particular allele from parents and offspring. Under the null hypothesis, Mendelian laws are used to define the distribution of transmissions. Quantitative traits were analysed in groups relating number of teat and number of inverted teat in each side using the FBAT statistic. Pfeiffer and Gail (2003) have reported that additive scores applied to the marker data can actually be more efficient than dominant scores with dominant inheritance, or recessive scored with recessive inheritance. Thus the use of additive scores, which do not require knowing which marker allele is in positive linkage disequilibrium with the putative disease allele, can be advantageous in many settings. One advantage of testing these composite null hypotheses in a family – based test is that they protect against type I error arising from population stratification. Rejecting the composite null hypothesis of no linkage and no association requires that both condition be met, as the test has no power to detect the alternative hypothesis if either condition is not met (Laird et al. 2000). When the genetic effect and environmental effects are independent, a susceptibility residual method of adjustment for environmental covariates reduces the power of the association test (Poisson et al. 2003).

Association between single SNPs and the inverted teat phenotypes were tested using the FBAT program (Horvath et al. 2001). FBAT was used to evaluate all of the markers on chromosome 1, 5 and 6 for each of the phenotypes. Multiple-allelic tests were performed using an additive genetic model to identify markers with evidence for both linkage and association with any of the phenotypes or for association in the presence of linkage. Markers significant at $P < 0.05$ in a multi-allelic test were then evaluated with a di-allelic test under an additive genetic model. The di-allelic test compares each allele

individually against all others collapsed into a single category to determine which specific allele(s) at the marker show association. This study found significantly evidence of family-based association in RLN (T9intG) loci (Z = 2.762, P = 0.0057) and inverted teat defect affection in the DUMI resource population. No significant differences were detected in the affection and TGFB1 (A797G) loci and RLN (A22C) loci. In addition parathyroid hormone like hormone gene (PTHLH) was also associated affection with PTHLH (C375T) loci (Z = 3.893, P = 0.000099). High significant association was also found between TGFB1, RLN and PTHLH loci and number of teat (P < 0.05) and number of inverted teat (P < 0.05). Thus far, no other investigations are present the candidate in gene association with the inverted teat defect in the pig.

Although the TGFB1, RLN and PTHLH genes are candidates for the inverted teat on positional and physiological arguments, the confidence intervals for the QTL identified in the previously study were large, harbouring hundreds of genes. Also a DNA test based on the causal genetic variation provides the most powerful tool for marker-assisted selection, the markers that define the litter-size and prenatal-survival QTL can be used for this purpose in the meantime.

There is no good evidence that identified variants at TGFB1, RLN and PTHLH locus are actually the causative mutations. In fact, the location of the site makes it seem unlikely that it affects protein structure or expression. Furthermore, the large differences in the effect between populations also suggest that it is not the causative mutation. The prediction for amino acid changes of interest were analysed by website http://blocks.fhcrc.org/sift/SIFT.html. SIFT is based one the premise that important amino acids will be conserved among sequences in a protein family, so changes at amino acid conserved in the family should affect protein function. SIFT uses sequence homology to predict whether an amino acid substitution in a protein will affect protein function (Ng and Henikoff 2002). In this study, the substitution of RLN gene at position 8 from L to I is predicted to affect protein function with a score of 0.00. There is low confidence in this prediction. In the same result of PTHLH prediction was analysed by SIFT. The substitution PTHLH at position 19 from S to L is not affecting protein function.

5.8 Future prospects for investigation of candidate genes for inverted teat defect in pigs

The main goal of genome research in farm animals is to map and characterize trait loci that control various phenotypic characters. In addition to the candidate genes study for inverted teat defect, the genome wide scanning should be applied to indicate the other strongly QTL regions for inverted teat which could be used for selecting more candidate genes in this DUMI resource population. Further investigation could be aimed to address this inverted teat trait by a unique combination of features, including comprehensive SNP discovery in a large number of candidate genes, testing of a large number of SNPs, use of the independent or commercial populations, and analysis of haplotypes in addition to individual SNPs where possible.

The present and future approaches to the identification of candidate and disease genes will be addressed. These include whole genome-based approaches such as integrative genomics as well as functional genomics-based approaches to analyze and model complex biological and medical processes (Hieter and Boguski 1997). The animal breeding genetics field has advanced considerably in recent years, with new information being generated that has led to improved understanding of the pathobiology underlying the complex trait defect. This has also generated interest in the study of gene-gene interaction and how linkage disequilibrium blocks and haplotypes can be used as functional units to pinpoint mutations and capture relative risk of mutated genes in complex disorders.

The term of functional genomics broadly describes a set of technologies and strategies directed to determine the function of genes and understanding how the genome works together to generate whole patterns of biological function (Hieter and Boguski 1997). The most powerful of these functional genomics approaches - expression profiling or DNA microarrays - can be used to analyze the expression of thousands of genes simultaneously. The current appreciation of the degree of variability (including single-nucleotide polymorphisms, SNPs) in the human genome is described (Taussig 2003) with emphasis on the need to prove that a particular genotype is indeed the cause of a specific phenotype; this topic has been termed 'functional genomics'. Future investigations will include functional and mutational studies of the novel transcripts

mapped or sublocalized within the critical region by this study as well as cloning efforts to isolate additional candidate genes. Such technology will be changing livestock genomics dramatically and these will be increase efficiency for animal breeding in the future.

6. Summary

The candidate gene approach has emerged as a promising method of QTL analysis with the extension of data available on the cloning and characterization of genes. Here, genes potentially involved in the biochemical pathways leading to trait expression are employed as molecular markers for QTL.

This investigation was carried out the candidate genes for inverted teat. Consequently, the transforming growth factor gene (TGFB1) and relaxin gene (RLN) were proposed to be tested for association in a positional candidate gene approach which combines linkage information for inverted teat defect and mapping information of a candidate gene. Both genes map in QTL regions for inverted teats discovered in the Bonn – Berlin DUMI resource family and are involved in proliferation and differentiation processes of the mammary gland. In addition, the parathyroid hormone like hormone gene (PTHLH) was proposed as a functional candidate gene that regulates epithelial mesenchymal interactions during the formation of mammary gland. The objective of this study are (1) to identify single nucleotide polymorphism (SNPs) in the positional candidate genes for the inverted teat defect in porcine, (2) to determine the linkage and physical location of TGFB1, RLN and PTHLH gene and (3) to evaluate the association of TGFB1, RLN and PTHLH gene on inverted teat trait.

Comparative sequencing of TGFB1 cDNA fragments obtained from individuals of five pig breeds revealed A/G transition in exon 5 at position 797 of the coding sequence. The segregation of alleles was observed in 21 families of Bonn-Berlin DUMI resource population and Mendelian inheritance of the alleles could be demonstrated.

The SNP was genotyped in 400 animals of the F2-DUMI resource population, 43.25% animals found to be homozygous (GG), 4% were homozygous (AA) and 52.75% were heterozygous (GA). Frequencies of the allele ‘g’ and ‘a’ were 0.7 and 0.3 respectively. No polymorphism was found among commercial breeds German Landrace (n=39) and Large White (n=44). Within the breed Pietrain segregation of the TGFB1 was found with allele ‘g’ being the prominent one.

TGFB1 linkage mapping, using CRI-MAP package version 2.4, revealed close linkages to loci S0300, SW193 and SW1067 (distances 7.3 cM, 13.8 cM, 24.5 cM; LOD scores 44.8, 19.2 and 21.4) with the last being assigned to Sscr6q11-q22. Our results are in agreement with published genetic and physical map.

The porcine relaxin gene, RLN, comprises two exons that are separated by a 5.5 kb intron. Primers were designed from the published RLN sequence and used to amplify overlapping fragments of about 300-500 bp in size covering part of 5´- untranslated region (UTR). Screening for polymorphism of amplicons derived from pigs of five breeds (Hampshire, Duroc, Pietrain, German Landrace, Berlin-Miniature pig) reveals two SNPs in exon1 and intron 1, respectively.

A transversion (C > A) at positon 1 of codon 8 (nt22) in the first exon leading to an amino acid exchange (L8I) was detected and was used for 'SSCP' genotyping this SNP. Among the 384 F2 animals of the DUMI families that were genotyped frequencies of the 'a' and 'c' alleles were 0.29 and 0.71 respectively. The SNP was found to segregate among pigs of three commercial breeds with allele 'c' being the prominent one.

Another transversion (T > G) was found at position 9 of the intron 1 altering the recognition site of CfoI. Frequencies of the 'g' and 't' alleles were 0.48 and 0.52 respectively, in the F2 generation of Bonn-Berlin-DUMI resource population. All 93 animals of the commercial breeds were found to be homozygous for allele 't'.

RLN gene linkage analysis was performed using CRI-MAP package (version 2.4) against four microsatellite markers of chromosome 1. The multipoint linkage map was established using the BUILD and FLIPS options (*SW1515* – 33.0 cM – *SW1851*– 31.4 cM – *S0155* – 7.6 cM – *RLN* – 56.5 cM – *SW1301*). Two point linkage analysis revealed a recombination fraction of 0.08 between RLN and SO155 (LOD=18.3). The position of RLN gene is in good agreement with the published physical assignment to Sscr1q28-29.

The complete mRNA sequence of the porcine PTHLH gene was obtained starting with heterologous primers designed from conserved regions from human PTHLH and porcine EST and subsequent 5' and 3' RACE using homologous primers. The cDNA

consisted of a 1,336 nucleotide coding region and 455 nt of 3’ UTR. In addition, 5’untranslated region was sequenced in 319 bp length. The intron sequence was found between 5’ UTR with 175 bp in length. A comparison with PTHLH sequences from other species indicated a high level of sequence conservation.

A C/T non-synonymous single nucleotide polymorphism (S19L) was detected at nucleotide position 375 of the porcine PTHLH cDNA. Mendelian inheritance of this polymorphic site was monitored in 395 F2 individuals of 21 families of the DUMI resource population. The SNP was found to segregate among pigs of three commercial breeds, German Landrace, Large White and Pietrain, with allele ‘c’ being the prominent one (allele frequency =.93, .95, .98, respectively).

Radiation hybrid mapping was performed using the INRA-Minnesota 7000 rads radiation hybrid panel (IMpRH), consisting of 118 hamster-porcine hybrid cell lines. Amplification results were submitted to the IMpRH database. The most significantly linked marker (2pt analysis) is *SW1319* on chromosome 5 (73 cR; LOD 4.85). For genetic mapping, twopoint and multipoint procedures of the CRI-MAP package version 2.4 revealed linkage to loci *SW1134* (proximal) and *IGF1* (distal) with distances of 24.4 cM (recombination fraction=.23, lods=17.41), and 48.2 cM (recombination fraction=.37, lods=3.43) respectively, on the sex averaged map. *IGF1* has been assigned to Sscr5q23. Microsatellites *SW1319* and *SW1134* map in close proximity at 49.3 cM on the second release genome-wide linkage map developed by the USDA Meat Animal Research Center. The results of RH and genetic mapping are in agreement and correspond with the published genetic and physical maps.

This study is the first to demonstrate the association of SNPs for TGFB1, RLN and PTHLH genes with inverted teat defect in pigs. Individual quantitative phenotypes were analysed with the Family-Based Association Test (FBAT). Highly significant evidence was found for association of RLN and PTHLH and the inverted teat defect affection status in the Bonn-Berlin DUMI resource population. No significant effects of TGFB1 on affection status were detected. Highly significant association was also found between TGFB1, RLN and PTHLH loci and number of teats and number of inverted teats.

7. Zusammenfassung

Der Kandidatengenansatz hat sich zu einer vielversprechenden Methode für die QTL Analyse entwickelt, die mit verfügbaren Daten der Klonierung und Charakterisierung von Genen ausgeweitet werden kann. In dieser Studie wurden Gene als molekulare Marker für die QTL Analyse verwendet, die in biochemische Abläufe eingebunden sind, welche zu der Merkmalsausprägung führen.

Diese Arbeit untersucht Kandidatengene für den Erbfehler Stülpzitze. Aufgrund der Ergebnisse der QTL Analyse wurden das Transforming Growth Faktor Gen (TGFB1) und das Relaxin Gen im positionellen Kandidatengenansatz auf Assoziation getestet, welcher die Kopplungsinformation für den Stülpzitzendefekt und die Kartierungsinformation eines Kandidatengens kombiniert. Beide Gene liegen in den QTL Regionen für die Stülpzitze, welche in der Bonn- Berlin DUMI Ressourcen Population gefunden wurden und sind eingebunden in Proliferations- und Differenzierungsprozesse der Milchdrüse. Zusätzlich wurde das Parathyroid Hormon Like Hormon Gen (PTHLH) als funktionelles Kandidatengen untersucht, welches Epithel- Mesenchym Interaktionen während der Bildung der Milchdrüse reguliert. Die Ziele dieser Untersuchung sind (1) die Identifizierung von Single Nukleotid Polymorphism (SNPs) in den positionellen Kandidatengenen für den Stülpzitzendefekt im Schwein, (2) die Bestimmung der Kopplung und der physischen Lokalisation vom TGFB1, RLN und PTHLH Gen (3) und die Untersuchung der Assoziation der Gene von TGFB1, RLN und PTHLH auf das Merkmal Stülpzitze.

Die vergleichende Sequenzierung der TGFB1 cDNA Fragmente, die aus Schweinen von fünf Rassen amplifiziert wurden, ergab eine A/G Transition in Exon 5 an der Position 797 der kodierenden Sequenz. Die Segregation der Allele wurde in 21 Familien der Bonn-Berlin DUMI Ressource Population untersucht und die Vererbung der Allele nach Mendel konnte gezeigt werden.

Bei der Genotypisierung des SNP in 400 Tieren der F2-DUMI Ressource Population waren 43,25% der Tiere homozygot (GG), 4% homozygot (AA) und 52,75% heterozygot (GA). Die Frequenzen der Allele ‘g’ und ‘a’ waren entsprechend 0,7 und

0,3. Es wurde kein Polymorphismus zwischen den kommerziellen Rassen Deutsche Landrasse (n=39) und Large White (n=44) gefunden. Innerhalb der Rasse Pietrain wurde eine Segregation von TGBF1 mit dem Allel ʻgʼ als markantes Allel festgestellt.

Die TGFB1 Kopplungskartierung mit dem CRI-MAP Paket Version 2.4 ergab eine enge Kopplung zu den Genorten S0300, SW193 und SW1067 (Distanzen 7,3 cM 13,8 cM 24,5 cM LOD Scores 44,8; 19,2; 21,4) wobei letzterer Marker auf dem Genort Sscr6q11-q22 kartiert, Unsere Ergebnisse stehen in Übereinstimmung zu den veröffentlichten genetischen und physikalischen Genkarten.

Das porcine Relaxin Gen, RLN, enthält zwei Exons welche durch ein 5,5 Kb Intron getrennt werden. Primer wurden mit Hilfe der publizierten RLN Sequenz abgeleitet und verwendet, um überlappende Fragmente der Größe 300-500 bp zu amplifizieren, welche den 5´- untranslatierten Bereich (UTR) abdecken. Die Amplikons, die man von Schweinen der fünf unterschiedlichen Rassen (Hampshire, Duroc, Pietrain, Deutsche Landrasse, Berliner Miniatur Schwein) erhielt, wurden auf Polymorphismen untersucht. Als Ergebnis konnten zwei SNPs in Exon 1 und Intron 1 gefunden werden.

Ein Basenaustausch (C > A), der zu einem Aminosäureaustausch (L8I) führt, wurde an Position 1 des Codon 8 (nt22) im ersten Exon gefunden und die ʻSSCPʼ Methode wurde zur Genotypisierung verwendet. Innerhalb der 384 genotypisierten Tieren der F2 DUMI Population waren die Frequenzen der ʻaʼ und ʻcʼ Allele 0,29 bzw. 0,71. Es wurde festgestellt, dass der SNP innerhalb der Schweine von drei kommerziellen Rassen segregiert, wobei das Allel ʻcʼ das markante Allel war.

Ein anderer Basenaustausch (T > G), welcher die Schnittstelle von CfoI ändert, wurde an Position 9 des Introns 1 gefunden. Die Frequenzen von ʻgʼ und ʻtʼ Allelen waren in der F2 Generation der Bonn-Berlin DUMI Ressource Population 0,48 und 0,52. Alle 93 Tiere der kommerziellen Rassen, welche genotypisiert wurden, waren homozygot für das Allel ʻtʼ.

Die Kopplungsanalyse des RLN Gens wurde mit dem CRI-MAP Paket (Version 2.4) in Abhängigkeit von vier Mikrosatellitenmarkern auf Chromosom 1 durchgeführt. Die Multipoint Kopplungskarte wurde mit den Optionen BUILD und FLIPS erstellt

(*SW1515* – 33,0 cM – *SW1851*– 31,4 cM – *S0155* – 7,6 cM – *RLN* – 56,5 cM – *SW1301*). Die Kopplungsanalyse zwischen RLN und S0155 ergab eine Rekombinationsrate von 0,08 (LOD = 18,3) zwischen den beiden Loci auf dem Chromosom. Die Position des RLN Gens stimmt ebenfalls mit der publizierten physischen Kartierung an Sscr1q28-29 gut überein.

Die komplette mRNA Sequenz des porcinen PTHLH Gens wurde untersucht, wobei zunächst heterologe Primer, basierend auf die konservierten Regionen des menschlichen PTHLH und des EST vom Schwein, abgeleitet wurden und anschließend eine 5´ bzw. 3´ RACE-PCR mit homologen Primern durchgeführt wurde. Die cDNA besteht aus einer 1,336 Nukleotide kodierenden Region und 455 nt der 3´ UTR. Die 5´ untranslatierte Region welche durch ein Intron abgetrennt ist, wurde mit einer Länge von 319 bp, sequenziert. Die Sequenz dieses Intron ist 175 bp lang. Der Vergleich mit den PTHLH Sequenzen anderer Spezies zeigt, dass die Sequenz stark konserviert ist.

Ein C/T nicht-synonymer Single Nukleotid Polymorphism (S19L) wurde an der Nukleotid Position 375 der porcinen PTHLH cDNA gefunden. Die mendelsche Vererbung dieser polymorphen Stelle wurde in 395 F2 Individuen von 21 Familien der DUMI Ressource Population gezeigt. Dieser SNP segregiert zwischen den Schweinen der drei kommerziellen Rassen, Deutsche Landrasse, Large White und Pietrain mit dem Allel ʻcʼ als das dominierende Allel (Allel Frequenz jeweils 0,93 0,95 0,98)

Radiation Hybrid Kartierung wurde mit dem INRA-Minnesota 7000 rads Radiation Hybrid Panel (IMpRH) durchgeführt, welcher 118 Hamster-Schwein Hybrid Zell-Linien beinhaltet. Die Ergebnisse der Amplifikation wurden der IMpRH Datenbank hinzugefügt. Der Marker SW1319 auf Chromosom 5 ist mit der höchsten Signifikanz gekoppelt (2pt Analyse; 73 cR; LOD 4,85). Für die genetische Kartierung ergaben die Twopoint und Multipoint Verfahren des CRI-MAP Paketes (Version 2,4) eine Kopplung zu den Loci SW1134 (proximal) und IGF1 (distal) mit den Abständen 24,4 cM (Rekombinationsrate = 0,23 Lods =17,41) und 48,2 cM (Rekombinationsrate = 0,37 Lods =3,43) auf der geschlechtsneutralen Karte. IGF1 wurde an Sscr5q23 kartiert. Die Mikrosatelliten SW1319 und SW1134 Kartieren dazu in einer Entfernung von 49,3 cM auf der zweiten veröffentlichten Genom-weiten KopplungsKarte, welche vom USDA

Meat Animal Research Center entwickelt wurde. Die Ergebnisse von RH und genetischer Kartierung stehen in Übereinstimmung und korrespondierend mit den publizierten genetischen und physischen Karten.

Diese Untersuchung ist die erste, die eine Assoziation von TGFB1, RLN und PTHLH Gen mit dem Stülpzitzendefekt beim Schwein zeigen soll. Individuelle quantitative Phänotypen wurden mit dem Familien-basierten Assoziationstest (FBAT) analysiert. Eine hoch signifikante Assoziation von RLN und PTHLH und dem Defektstatus der Zitze in der Bonn-Berlin Ressource Population konnte bewiesen werden. Es wurden keine signifikanten Effekte von TGFB1 auf den Erkrankungsstatus gefunden. Eine hoch signifikante Assoziation wurde ebenfalls zwischen den Genorten TGFB1, RLN und PTHLH und der Anzahl Zitzen und der Anzahl defekter Zitzen gefunden.

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