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# Candidate Gene Analysis for Loci Affecting Sperm Quality and Fertility of Boar

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**Candidate gene analysis for loci affecting sperm quality and fertility of boar**

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Dedicated to my family

# Kandidatengenanalyse für Loci, die die Spermaqualität und Eberfruchtbarkeit beeinflussen

Fruchtbarkeit ist eines der wichtigsten ökonomischen Merkmale in der Schweine-produktion.

Die Einführung der künstlichen Besamung (KB) in der Schweineproduktion erlaubt eine verbesserte Selektion der Eber für Produktionsmerkmale, aber gleichzeitig betont sie die Bedeutung der individuellen Reproduktionsleistung der Eber.

Diese Studie zielt darauf ab, den Effekt von direkten funktionellen Kandidatengenen auf Spermakonzentration (SCON), Spermabeweglichkeit (MOT), Samenvolumen je Ejakulat (VOL), Plasmatröpfchenrate (PDR), abnormale Spermienrate (ASR) und den Fruchtbarkeitsmerkmalen Non-Return-Rate (NRR) und Anzahl lebend geborener Ferkel (NBA) aufzuklären. Insgesamt wurden für die Untersuchung 18 Kandidatengenorte, *GnRHR*, *FSHB*, *LHB*, *RLN*, *PRL*, *PRLR*, *AR*, *FST*, *INHA*, *INHBA*, *INHBB*, *RBP4*, *ACR*, *OPNin6*, *OPNpro*, *ACTG2*, *ACTN1* und *ACTN4*, basierend auf deren biologischer Korrelationen und/oder physiologischer Funktionen in der männlichen Reproduktion, ausgewählt.

Mit Hilfe der vergleichende Sequenzierung von Tieren der Rassen Pietrain und Hampshire wurden Polymorphismen im Intron 18 (G>A) des porcinen *ACTN1* (AY837722) und in 3´-UTR (A>C) des porcinen *ACTN4* (AY837723) gefunden.

Eber der Rassen Pietrain (n=244) und Pietrain × Hampshire (n=112) aus einer KB Eberstation wurden für diese SNPs am Locus *ACTN1* und *ACTN4*, sowie an bereits beschriebenen Polymorphismen an anderen Genorte genotypisiert.

Varianzanalysen ergaben signifikante Assoziationen zwischen folgenden Kandidaten-genen und Merkmalen: *ACTN1* ($P < 0.05$) und *ACR* ($P < 0.05$) mit der NRR; *ACTN1* ($P < 0.01$) und *OPNin6* ($P < 0.05$) mit der NBA; *PRL* ($P < 0.01$), *INHBB* ($P < 0.05$), *ACR* ($P < 0.05$) und *FSHB* ($P < 0.01$) mit der SCON; *ACTG2* ($P < 0.01$), *RLN* ($P \leq 0.01$) und *FST* ($P < 0.01$) mit dem VOL. *ACTG2* ($P < 0.05$), *RBP4* ($P < 0.05$), *OPNin6* ($P \leq 0.05$), *ACR* ($P < 0.01$) und *GnRHR* ($P < 0.05$) mit der MOT; *GnRHR* ($P < 0.01$) und *INHBA* ($P < 0.05$) mit der PDR; *GnRHR* ($P < 0.01$), *INHA* ($P < 0.05$), *INHBA* ($P < 0.05$), *OPNpro* ($P < 0.05$) und *AR* ($P < 0.05$) mit der ASR. Es wurden keine signifikanten Effekte von *ACTN4*, *LHB* und *PRLR* auf eines der untersuchten Merkmale gefunden.

Candidate gene analysis for loci affecting sperm quality and fertility of boar

Fertility is one of the most important economical traits in swine production. Implementation of artificial insemination (AI) in swine production allowed improving selection on the boars for production traits, but at the same time, it stresses the meaning of the individual boar reproductive performance. This study aims to elucidate the effect of direct functional candidate gene loci as markers on sperm quality traits of concentration (SCON), motility (MOT), semen volume per ejaculate (VOL), plasma droplets rate (PDR), abnormal sperm rate (ASR). In addition these gene loci were also tested for association with the fertility traits, i.e. non return rate (NRR) and number of piglets born alive (NBA). In total, eighteen candidate gene loci *GnRHR, FSHB, LHB, RLN, PRL, PRLR, AR, FST, INHA, INHBA, INHBB, RBP4, ACR, OPNin6, OPNpro, ACTG2, ACTN1*, and *ACTN4* were chosen for investigation based on their biological correlations and/or physiological functions in male reproduction. Comparative sequencing of animals of the breeds Pietrain and Hampshire revealed polymorphisms in intron 18 (G>A) of porcine *ACTN1* (AY837722) and in 3′-UTR (A>C) of porcine *ACTN4* (AY837723). The pure breed of Pietrain (n=244) and the crossbred of Pietrain × Hampshire (n=112) boars of an AI boar station, born between 1990 and 1999 and used in commercial pig herds mainly of North-Western Germany, were genotyped for the SNPs of *ACTN1* and *ACTN4* and previously in the literature described polymorphisms within the other loci. Analyses of variance revealed significant association of the following candidate genes with traits: *ACTN1* ($P < 0.05$) and *ACR* locus ($P < 0.05$) with NRR; *ACTN1* ($P < 0.01$) and *OPNin6* locus ($P < 0.05$) with NBA; *PRL* ($P < 0.01$), *INHBB* ($P < 0.05$), *ACR* ($P < 0.05$) and *FSHB* locus ($P < 0.01$) with SCON; *ACTG2* ($P < 0.01$), *RLN* ($P ≤ 0.01$) and *FST* ($P < 0.01$) with VOL; *ACTG2* ($P < 0.05$), *RBP4* ($P < 0.05$), *OPNin6* ($P ≤ 0.05$), *ACR* ($P < 0.01$) and *GnRHR* ($P < 0.05$) with MOT; *GnRHR* ($P < 0.01$) and *INHBA* ($P < 0.05$) with PDR; *GnRHR* ($P < 0.01$), *INHA* ($P < 0.05$), *INHBA* ($P < 0.05$), *OPNpro* ($P < 0.05$) and *AR* ($P < 0.05$) with ASR. No significant effects of *ACTN4, LHB* and *PRLR* locus on any trait were observed.

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List of abbreviations

| | |
|---|---|
| A | : Adenosine |
| ABP | : Androgen binding protein |
| *ACR* | : Acrosin |
| *ACTB* | : Actin beta |
| *ACTG2* | : Actin gamma |
| *ACTN1* | : Actinin alpha 1 |
| *ACTN4* | : Actinin alpha 4 |
| AI | : Artificial insemination |
| *AR* | : Androgen receptor |
| ASR | : Abnormal sperm rate |
| bp | : Base pair |
| C | : Cytisine |
| °C | : Degree Celsius |
| cDNA | : Complementary deoxy ribonucleic acid |
| *CYP2* | : Steroid 21-hydroxylase |
| DMSO | : Dimethyl sulfoxide |
| DNA | : Deoxy ribonucleic acid |
| dNTP | : Deoxy nucleotide triphosphate |
| DTT | : 1, 4, Dithio theritol |
| DUMI | : Duroc-Berlin miniature pig resource population |
| EDTA | : Ethylenediaminetetraacetic acid |
| EST | : Expressed sequenced tag |
| °F | : Degree Fahrenheit |
| *FSHB* | : Follicular stimulating hormone beta |
| *FST* | : Follistatin gene |

G          : Guanine

*GnRHR*     : Gonadotropin releasing hormone receptor

*GPX-5*     : Glutathione peroxidase 5

HCl         : Hydrochloric acid

h           : Hour

*HSP 70.2*  : Heat shock protein 70.2 gene

*INHA*      : Inhibin alpha

*INHBA*     : Inhibin beta A

*INHBB*     : Inhibin beta B

*LHB*       : Luteinizing hormone beta

mg          : Milligram

min         : Minute

ml          : Milliliter

MOT         : Sperm motility

mRNA        : Messenger ribonucleic acid

Mw          : Molecular weight

NaOH        : Sodium hydroxide

NBA         : Number of piglets born alive

NRR         : Non return rate

ns          : Not significant difference

OD          : Optical density

*OPN*       : Osteopontin

*OPNin6*    : Marker within intron 6 of osteopontin gene

*OPNpro*    : Marker within the promoter region of osteopontin gene

PCR         : Polymerase chain reaction

PDR         : Plasma droplets rate

PI          : Pietrain breed

PI×HA     : Pietrain Hampshire crossbred

*PRL*     : Prolactin gene

*PRLR*     : Prolactin receptor gene

QTL     : Quantitative trait loci

RACE     : Rapid amplification of cDNA ends

RBP     : Retinol-binding protein

*RBP4*     : Retinol-binding protein 4

RFLP     : Restriction fragment length polymorphism

*RLN*     : Relaxin gene

RNA     : Ribonucleic acid

rpm     : Rotations per minute

*RYR1*     : Ryanodine receptor 1 gene

SAS     : Statistical Analysis System software

SCON     : Sperm concentration

SD     : Standard deviation of mean

SDS     : Sodium dodecyl sulfate

SM     : Sodium chloride - magnesium sulphate buffer

SNPs     : Single nucleotide polymorphisms

SSCP     : Single strand conformation polymorphism

SSLP     : Simple sequence length polymorphism

T     : Thymidine

TBE     : Tris-Boric acid-EDTA buffer

TE     : Tris-EDTA buffer

TEMED     : N, N, N´, N´-Tetramethylendiamine

*TGF*     : Transforming growth factor

TNF-$\alpha$     : Tumor necrosis factor –alpha

UTR     : Untranslated region

VIM : International vocabulary of basic and general terms in metrology

VOL : Semen volume per ejaculate

W : Watt

w/v : Weight by volume

X-gal : 5-Bromo 4-chloro-3-indolyl-β-D-galactoside

μg : Microgram

μl : Microliter

μM : micomolar

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1 Introduction

Fertility is one of the most important economical traits in swine production. Reproductive performance is controlled by the genetic make-up of the dam, boar and offspring, but is in general largely affected by environment. The reproductive efficiency of the breeding herd depends on the fertility of the boar. Boar fertility is also essential since boar's DNA is the primary mechanism through which genetic improvements can efficiently be accomplished. Implementation of artificial insemination (AI) in swine production allowed improving selection on the boars for production traits. At the same time it stresses the meaning of the individual boar reproductive performance and requires consequent evaluation of fertilization potential of a semen sample for AI in boar stations. Malmgren and Larsson (1984) proposed that semen evaluation could be used as an indicator of fertility in boars. Sperm concentration, motility and normal sperm rate have usually been used as criteria for semen quality evaluation (Colenbrander et al. 1993). However, a number of laboratory assays that examine cellular attributes of sperm are still unable to predict the fertility of a semen sample consistently (Braundmeier and Miller 2001).

Boar reproductive performance made limited progress through traditional selection and crossbreed system due to low heritability of fertility traits (Rothschild et al. 1997, Urban and Kuciel 2001) and expressing late in life to serve as useful criteria of selection (Hugo 1998) such as sperm quality traits. With the advancement in molecular techniques it is possible to increase rate of response to selection. It has been proposed that candidate gene analysis can be used to identify individual genes responsible for traits of economic importance (Linville et al. 2001).

For selecting fertility traits one can take the genotype into consideration besides the phenotype, which are the externally visible and measurable traits. The phenotype expression of fertility depends on the underlying genotype that is modulated by environmental deviations by the means of phenotypic traits leads inevitable to inaccuracies as the genotypic effects can only be distinguished from the environmental deviations by the means of simplified statistical models. The accuracy and intensity of selection can be improved by including the genotypic information. Also the selection decision can take place early in life of boar due to the time to phenotypic expression of

fertility and sperm quality traits has not to be bided for. The possibility of realizing selection criteria on a molecular genetic level shortens the generation interval. Particularly for fertility traits, this is of greatest interest. Genotyping can be performed by using blood or tissue samples of new-born piglets; it is not necessary to wait for the first parities of a sow or the results obtained by the offspring of a boar.

Spermatogenesis is a complex process that involves stem-cell renewal, genome reorganization, genome repackaging, and that culminates in the production of motile gametes (Cooke and Saunders 2002). The process of spermatogenesis is regulated by reproductive hormones in gonadotropin axis and controlled by large number of genes. Therefore, hormone and their receptors are presumed to be good candidate genes for reproductive traits (Vincent et al. 1998a).

For instance, in mouse the deletion in *GnRH* gene is responsible for the phenotype of hypogonadism with an autosomal recessive inheritance (Mason et al. 1986). Male homozygous FSHB knockout mice have normal levels of serum testosterone, small testes, and oligospermia but are still fertile (Kumar et al. 1997). Synergistic effects of androgen and prolactin stimulate growth and secretory activity of accessory sexual glands (Rothschild et al. 1997). More than 300 different mutations (commonly missense mutations) in the *AR* gene have been reported to cause androgen insensitivity (Layman 2002). Relaxin levels in seminal plasma have been shown to be significantly correlated with sperm motility traits (Sasaki et al. 2001). An association was found between inhibin and sperm concentration, suggesting that alpha-inhibin in the human seminal fluid could be a marker for some aspects of spermatogenesis (Matorras et al. 1998). A significantly positive correlation is also observed between testis volume and inhibin B level, as well as between sperm count and inhibin B level. The inhibin B is a direct product of the seminiferous tubules, reflecting the total testicular tissue (Hu and Huang 2002). Booth (1970, 1974) reported the occurrence of vitamin A in testicular tissue of the boar. Retinoic acid (vitamin A) is derived from endogenous retinol, which is delivered to target cells by RBP. Wemheuer et al. (1996) showed supplementing of diet of boar with vitamin A can influence the ejaculation parameters. *ACTN1*, *ACTN4* and *ACTG2* genes are expressed in sperm and potentially involved in membrane changes during acromome reaction with important implication on sperm function (Waters et al. 1985, Yagi and Paranko 1992 and 1995, Casale et al. 1988).

In this study, the candidate genes gonadotropin-releasing hormone receptor (*GnRHR*)*,* follicle-stimulating hormone beta (*FSHB*), luteinizing hormone beta (*LHB*)*,* follistatin (*FST*)*,* inhibin alpha (*INHA*), inhibin beta A (*INHBA*) and inhibin beta B (*INHBB*)*,* relaxin (*RLN*), prolactin (*PRL*) and prolactin receptor (*PRLR*)*,* androgen receptor (*AR*), retinol binding protein 4 (*RBP4*), acrosin (*ACR)*, actin gamma (*ACTG2*)*,* actinin alpha 1 (*ACTN1*) and actinin alpha 4 (*ACTN4*) were as direct functional candidate genes investigated for association with sperm quality traits and fertility traits of boars.

The objective of this study is to elucidate effects of these direct functional candidate genes on sperm concentration (SCON), motility (MOT), semen volume per ejaculate (VOL), plasma droplets rate (PDR), abnormal sperm rate (ASR). In addition, these genes were also tested for association with boar fertility traits, i.e. non return rate (NRR) and number of piglets born alive (NBA), that reflect the success rate of artificial inseminations.

2 Literature review

2.1 Boar reproductive biology

2.1.1 Boar reproductive tract anatomy

The boar reproductive tract consists of testes, epididymis, spermatic cord, accessory gland and penis that is supported by the pelvis, and is housed internally in the abdomen and outside the abdominal cavity in the region of the groin (Hafez and Hafez 2000). The testes of the boar are paired and produce sperm and hormones. Sperm cells are produced only within the seminiferous tubules of the testes. The epididymus is highly coiled and when laid out end to end is about 54 meters in length (Hafez and Hafez 2000). The testicles are contained within the scrotum that serves the important functions of protection and temperature regulation. Spermatic cord serves as a passageway for blood vessels and nerves, which allows entry and exit for necessary substances to sustain cell life and supply hormones for reproductive regulation. The cord also contains blood, nervous and muscular systems involved in temperature regulation and movement of sperm out of the epididymis. Accessory glands consist of prostate, vesicular and the bulbourethra gland. The boar penis is composed of tough fibroelastic tissue supplied with blood and nerves, which is extended through an opening called the prepuce located on the abdomen.

2.1.2 Boar reproductive physiology

Boar reproductive physiology includes sexual development, spermatogenesis, sperm maturation and storage, accessory glad secretion, which are all regulated by hormone.

2.1.2.1 Hormone control

The reproductive process in boar is initiated and regulated by hormones at the level of the brain (Figure 2.1). As both a neural and endocrine organ, the hypothalamus is

located near the base of the brain. In response to many different stimuli, the hypothalamus releases the hormone GnRH. This hormone is important and responsible for inducing the release of FSH and LH from the pituitary gland, which is located just below the hypothalamus (Hafez and Hafez 2000, Kraus et al. 2001).
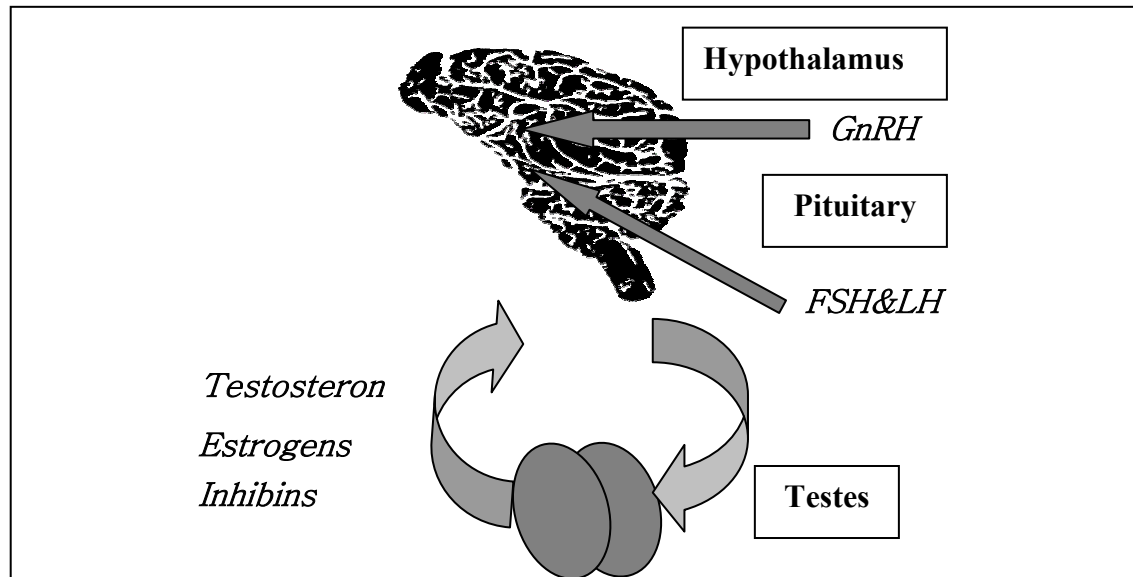


Figure 2.1: Hypothalamus, pituitary and testes axis adapted from the figure described by Knox (2003)

The release of GnRH is regulated by neural and hormonal inputs. These inputs indicate physiological age, weight, nutritional level, season of the year, environmental temperature and reproductive status of the testes (Clark and Althouse 2002). All of these factors can collectively or individually influence the release of GnRH and increase or reduce the amount of FSH and LH releasing into the bloodstream to alter the activity of the testicles. FSH stimulation of the testes starts the process of spermatogenesis by initiating sperm cell division and development. The FSH molecule actually binds to Sertoli cells, which serve as nurse cells for the forming sperm cells (Figure 2.2). The Sertoli cells line the seminiferous tubules that produce sperm. Sperm cells are actually nursed inside these cells. These nurse cells also produce proteins that function to accumulate testosterone, which happens to be produced outside of the tubules. Testosterone is needed in high levels inside the tubule to allow sperm cell production. The other gonadotropin, LH, is responsible for initiating testosterone

production by the Leydig cells. These cells are located just outside of the seminiferous tubules. Testes stimulation with FSH and LH regulates hormone production. These hormones act locally and also enter the blood stream where they act on other tissues and organs in order to allow normal reproductive function. The hormones produced by the testes, include androgens, estrogen and inhibin. These hormones are important for regulating the release of GnRH, FSH and LH at the level of the brain. This feedback regulation for hormone release between the testes and the brain, allows for positive and negative control of spermatogenesis and reproductive activity.
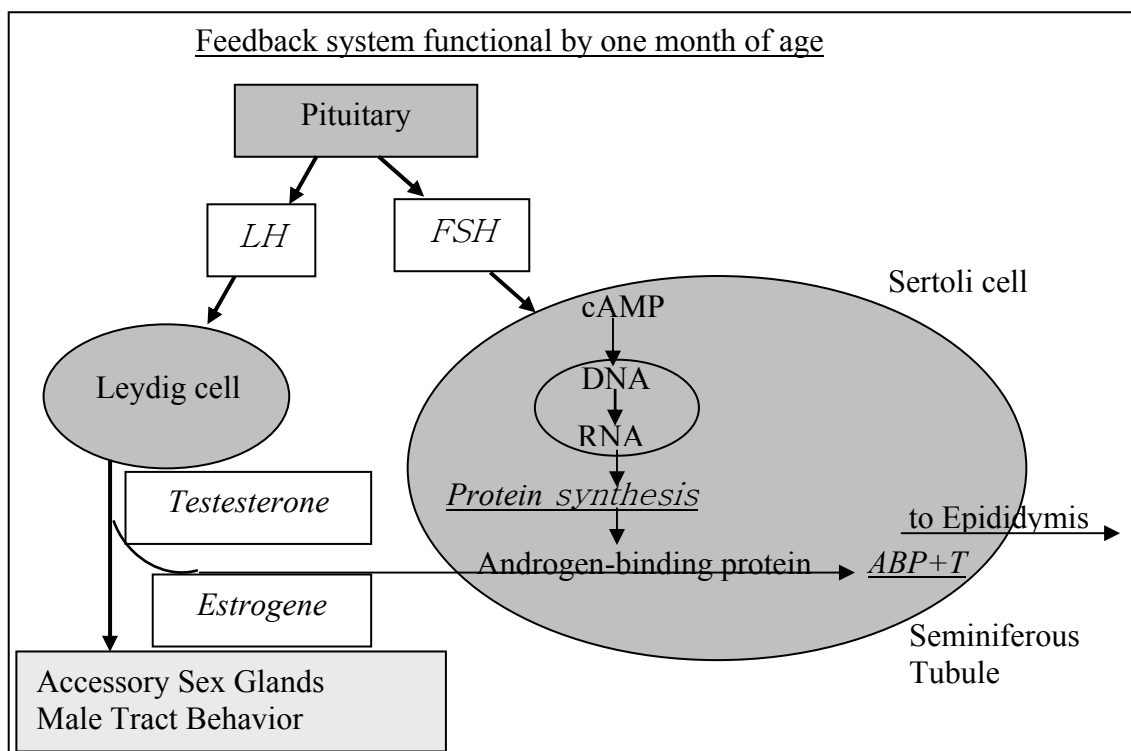


Figure 2.2:    Male hormone pathways adapted from the figure described by Knox (2003)

2.1.2.2 Sexual development

Sexual development in the boar is determined by the presence of the Y chromosome and androgens (Hafez and Hafez 2000). At approximately 20 to 40 days of fetal age, genes are expressed on the Y chromosome that allows germ cells and the male

reproductive tract cells to begin to grow and differentiate. Androgens and forms of testosterone are responsible for male associated characteristics such as libido, aggressiveness and muscling. It is also essential for the growth and development of the reproductive tissues such as the penis, testes and accessory sex glands. The testosterone production is also essential for sperm production process in the testes and maturation in the epididymis. Physiological maturation in boars after birth is an ongoing process. At about four months of age, sperm first appear in the seminiferous tubules and erection can be accomplished in the immature boar. At 5.5 months of age, puberty begins and sperm now appear in the ejaculation. Over the next 6-18 months, the testes increase in size and both concentration and ejaculation volumes continue increasing. By 18 months of age no appreciable improvements in fertility are observed and the boar is considered fully mature (Leman and Rodeffer 1976).

## 2.1.2.3 Spermatogenesis

Spermatogenesis is a continuous process. Inside the testicle, spermatogonia begin to mature near the outside wall of the seminiferous tubes. As the cell matures, it moves closer to the centre of the tube where it will be released into the sperm passageway (lumen). During maturation inside the Sertoli cell, the sperm cell changes its shape from nearly a round shape to the elongated sperm head with a tail. Within any Sertoli cell, there are many sperm cells in different stages of development. The time period required to change from a round cell on the outer wall of the tube to its release into the seminiferous tubule as a sperm with a tail takes approximately 34-36 days (Franca and Cardoso 1998). Sperm cells production in the testes is not synchronized within the tubules so that sperm cells can be produced for ejaculation on a daily basis. Therefore, along the entire length of the seminiferous tubules there are segments that contain different stages of sperm cell development (Franca and Cardoso 1998).

## 2.1.2.4 Sperm maturation and storage

Sperm enters to the head of the epididymis, these cells are very concentrated and in this

form, they are not motile. In this portion of the epididymis, the head of the sperm incorporates proteins into the membrane, which are thought to be essential to fertilize the egg. As they are moving down the length of the epididymis by fluid and smooth muscle contractions, they enter into the midpiece where they now acquire the capability for tail movement. However, although they are capable of movement, they are still too concentrated to permit motion. As the sperm moves into the tail of the epididymis, additional proteins are added which are important for sperm fertility. Sperm is stored immotile in the tail region in concentrated form. The sperm will acquire full motility and fertilizing capability when diluted with seminal plasma in the ejaculate (Hafez and Hafez 2000).

## 2.1.2.5 Accessory glands

The accessory glands are important for adding substances to the ejaculate that serve to increase volume, protect sperm cells and provide nutrients needed for ensuring sperm fertility. Recently much attention has been directed toward the importance and function of these fluids (Manaskova et al. 2002). The prostate gland is the first gland to meet the pelvic urethra and responsible for flushing out urine and any bacteria in the tract before entry of sperm into the urethra. The vesicular gland produces most of the volume, energy sources, buffers and ions. The bulbourethral glands produce the gelatine plug. In general, the accessory glands are responsible for providing volume to dilute and stimulate motility, for providing a fluid medium for ejaculation, for energy in the form of citric acid and fructose, and pH buffering (Hafez and Hafez 2000).

2.2 Semen and sperm quality

2.2.1 Semen characteristics

Semen is the liquid cellular suspension containing spermatozoa, the male gametes and secretion from the accessory organs of the boar reproductive tract. The fluid portion of this suspension, which is formed in the ejaculation, is known as seminal plasma. The seminal characteristics of boars are listed in Table 2.1.

Table 2.1:      Characteristics and chemical components of boar semen[‡]

| Component | Mean | Component | Mean |
|---|---|---|---|
| Ejaculate volume (ml) | 150-500 | Citric acid* | 173 |
| Sperm concentration (million/ml) | 200-300 | Inositol* | 380-630 |
| Sperm/ejaculate (billion) | 30-60 | Sorbitol* | 6-18 |
| Motile sperm (%) | 50-80 | Ergothioneine* | 17 |
| Morphologically normal sperm (%) | 70-90 | Sodium* | 587 |
| Protein (g/100 ml) | 3.7 | Potassium* | 197 |
| pH | 7.3-7.8 | Calcium* | 6 |
| Fructose* | 9 | Magnesium* | 5-14 |
| Glyceryl phosphoryl choline* | 110-240 | Chloride* | 260-430 |

[‡]Adapted from Hafez and Hafez (2000): "Reproduction in Farm Animals"

*The unit of chemical components: mg/100 ml

2.2.2 Factors affecting semen and sperm quality

Factors that influence semen and sperm quality include genetic factors (like breed) and environmental factors (social, light, season, temperature, etc).

2.2.2.1 Genetic factors

There are reports of different breeds which have different ejaculate volumes,

concentration, motility and even percent of live/dead cells between European breeds of swine (Duroc, Yorkshire, and Hampshire), but the results appear inconclusive (Ciereszko et al, 2000). The size of the testes is moderately heritable (Rothschild and Ruvinsky 1998) and has been related to early puberty in both male and female siblings and offspring (Rathje et al. 1995). The weight of the testes at puberty and in the adult boar is related to sperm output and even testosterone levels (Huang and Johnson 1996). Sperm production of boar correlates positively with testicular size (Fent et al. 1983, Young et al. 1986, Lubritz et al. 1991, Rathje et al. 1995, Huang and Johnson 1996). Knox (2003) reported that boars less than 9 months of age have both lower ejaculate volumes and concentrations compared to boars of 18 months of age or older. Many boars are only in stud for a period of 2-4 years before culling. Daily sperm production over a seven-month period of time fluctuated between 12 and 14.5 billion cells produced per day (Akingbemi and Makinde 1995).

## 2.2.2.2 Environmental factors

Effects of season and social environment on sperm quality of boars were reported (Trudeau and Sanford 1986, Knox 2003). Libido and ejaculate volumes are lower in boars raised in physical isolation from other males or females (Trudeau and Sanford 1986). Semen quality appears not to be greatly influenced by the effects of supplemental light during short days. However, increasing or decreasing light length in the opposite season can advance the age of puberty (Trudeau and Sanford 1986). In the domestic boar, sperm production seems to be highest between September and February compared to March through August. Low temperatures have not been found to be a problem for boar fertility. Temperatures above 95 °F for 3 days have increased abnormalities in ejaculates beginning 2-6 weeks after the stress. Heat stressed boars also have higher levels of basic proteins in their ejaculates and these proteins are associated with reducing sperm life of extended semen at cool storage temperatures (Knox 2003).

## 2.2.3 Semen evaluation

Malmgren and Larsson (1984) proposed that semen evaluation could be used as an indicator of fertility in boars. Sperm concentration, motility and normal sperm rate have usually been used as criteria for semen quality evaluation (Colenbrander et al. 1993). The only measure of semen viability is to observe the fertility of inseminated females. However, a number of semen characteristics can be used to evaluate the general quality of the sample and to estimate the extent to which it may be extended. The evaluation of the ejaculate is an important part of examination a male. Simple and important conclusions can be obtained from the results.

### 2.2.3.1 Appearance

A properly collected semen sample should be near the characteristic volume. The colour should be opaque in appearance, indicating a high sperm concentration and nothing indicative of contamination. Semen with a crude appearance, containing chunks of material, like the gelatinous fraction of boar's semen, should not be used.

### 2.2.3.2 Sperm concentration

Sperm concentration in semen is estimated using a calibrated electronic cell counting equipment, spectrophotometer or haemocytometer.

Accurate determination of concentration, semen volume, and percentage of living cells is essential for estimating the maximum dilution of sperm which can be prepared for artificial insemination and how many females can be inseminated. Sperm concentration of AI must be $2 \times 10^9$ or greater per insemination (Xu et al. 1998).

### 2.2.3.3 Sperm motility

Sperm motility is one of the most widely used tests for semen quality. Motility is essential for fertility. However, motility, while it is an essential feature of healthy

spermatozoa, is not necessary indicative of fertilizing capacity (Xu et al. 1998, Vyt et al. 2004).

Sperm motility is estimated visually using light microscopy. In measuring this, two aspects are usually considered, the percentage of motile sperm and the motility quality of individual spermatozoa by subjective judgement. Other ways of assessing motility include computer assisted image analysis and the swim-up technique.

### 2.2.3.4 Sperm morphology

Stained slides are used to examine the morphology of sperm and the ratio of living to dead cells, using the eosin-negrosin stain. Abnormalities of the head, midpiece, and tail may be due to defective mechanisms in the reproductive tract of the male or to changes caused during collection and processing. These are usually not associated with lower fertility rates until the proportion of abnormal sperms exceeds about 20%. Morphological evaluation is done by light microscopy.

### 2.2.3.5 Acrosome integrity

Acrosome integrity is regarded as an important semen quality parameter (Zou and Yang 2000). The acrosome is an essential part of the sperm cell and it contains enzymes which play important roles in the penetration of the zona pellucida and therefore fertilization. Acrosome damage removes the chance to fertilise an egg.

2.3 Important reproductive traits for boar breeding

The reproductive efficiency of natural service boars may be characterized by their age at sexual maturity, their mating ability, the conception rate, the litter size resulting from their mating and the longevity. Important component traits are libido, aggressiveness, semen and sperm quantity and quality. Traits of interest are rather similar for artificial insemination boars, but with a greater emphasis on semen and sperm quantity and quality and on the ease and frequency of semen collection (Rothschild and Ruvinsky 1998). The reproductive traits of current or potential interest for boar breeding are described in Table 2.2.

Table 2.2:      Main reproductive traits of interest for boar breeding[*]

| Reproductive function | Main components or predictive traits |
| --- | --- |
| Sexual maturity | Age at first mating or sperm collection |
|  | Testes size |
|  | Size of Cowper's gland |
| Sexual behaviour | Ability to mount |
| Gonadocyte production | Sperm quantity: |
|  | Semen volume (per ejaculate, per day) |
|  | Sperm concentration |
|  | Testes size (length, width, circumference, volume and weight) |
|  | Sperm quality: |
|  | Sperm motility |
|  | Proportion of: dead spermatozoa |
|  | abnormal spermatozoa |
| Fertility | Conception rate of mates |
| Prolificacy | Litter size of mates |
| Hormone levels | Testosterone level |

[*]Adapted from Rothschild and Ruvinsky (1998) "The Genetics of the Pig"

2.4 Molecular genetic approach to improved pig reproduction

Geneticists have made limited progress in improving reproductive traits in pigs through traditional selection and crossbreeding systems because of low heritability and also due to fact that fertility traits are expressed very late in the life to serve as useful criteria of selection (Rothschild et al. 1997). Molecular techniques allow detecting variation or polymorphisms existing among individuals in the population for specific region of the DNA. These polymorphisms can be used to build up genetic maps and to evaluate differences between markers in the expression of reproductive traits that might indicate a direct effect of the difference in terms of genetic determination on the trait. Moreover, they can prove some degree of linkage of the QTL affecting the trait and the marker (Motaldo et al. 1998). Molecular genetic approaches to improved pig fertility, not only contribute to conservation of pig specials, but also increase the efficiency of fertility selection due to the increase of the particular trait selection precision and the decrease of pig breed generations (Dekkers 1998).

2.4.1 Female reproductive traits

Candidate gene analysis for reproductive traits has shown considerable merit. Results have clearly demonstrated that estrogen receptor (*ESR*) gene is significant associated with litter size (Rothschild et al. 1996). Estimates of allelic effects vary from 1.15 pig/litter of Meishan synthetic to 0.42 pigs/litter of Large White line. These results have not been confirmed by QTL scans using divergent crosses involving Meishan and Large White pigs, perhaps due to small sample sizes or the fact that the *ESR* gene allele was not segregating in some of the populations involved in the QTL scans. The *ESR* marker was incorporated successfully into the PIC selection indices for Large White based dam lines, resulting in an increase in the rate of genetic response in its nucleus herds (Rothschild 2003). Furthermore, the increase in average litter size is observed in crossbred products derived from these lines. Other effects have been reported for retinoic acid receptor gamma (*RARG*), melatonin receptor 1A (*MTNR1A*), and follicle stimulating hormone beta (*FSHB*) genes, researching with PIC, prolactin receptor

(*PRLR*) locus is significantly associated with litter size (Vincent et al., 1998a), which was confirmed in two smaller studies. Retinol binding protein 4 (*RBP4*) was investigated using nearly 2500 litters, and shown to be associated with an increase of about 0.25 pigs per litter (Rothschild et al. 2000). Jiang et al. (2001) reported that gonadotropin releasing hormone receptor (*GnRHR*) was significantly associated with the number of corpora lutea in European Large White pig. Most candidate gene analyses have involved considerably more sows and litters than the QTL analyses and this might explain the lack of QTL scan confirmation of the regions in which there were candidate gene effects.

On the other site, results of PIC line QTL scans for pig reproductive traits are limited due to the requirements to obtain resource and time (Bidanel and Rothschild 2002). Initial scans have revealed promising results on chromosome 8. Possible QTL for uterine length and ovulation rate were reported, though in different chromosomal positions. A sizeable QTL for ovulation rate (+3.07) on chromosome 8 was reported but at a different location. However, latter work in the same laboratory did not confirm this finding. In the French QTL experiment a QTL for increased litter size of one piglet was found in the same location on chromosome 8. The large ovulation rate or litter size QTL on chromosome 8 is of interest as it is mapped to the region which is syntonic to the Booroola fecundity gene (a BMP receptor gene) in sheep. Further evidence exists for additional QTL for litter size components on chromosome 8 and other reproductive traits on chromosomes 4, 6, 7, 13, and 15 (Bidanel and Rothschild 2002, Cassady et al. 2001, Rohrer et al. 1999c, Wilkie et al. 1999, Milan et al. 1998, Henderson et al. 1998).


2.4.2 Male reproductive traits


Candidate gene approach to boar sperm quality traits are firstly reported by Hardeg et al. (1995). In recent experiments, it was observed that heat shock protein 70.2 gene (*HSP70.2)* (Huang et al. 2001), steroid 21-hydroxylase gene (*CYP2)* (Kmiec et al. 2002), glutathione peroxidase 5 gene (*GPX-5*) (Mackowski et al. 2004), ryanodine receptor 1 gene (*RYR1*) (Urban and Kuciel 2001), retinoic binding protein 4 gene, prolactin receptor (*PRLR*) gene and osteopontin gene (*OPN)* (Steinheuer et al. 2002,

2003) are significantly associated with sperm quality traits or litter size of different breed boars (Table 2.3), but the decision to include a given genetic marker to the MAS schema must be based on reliable information about the additive and dominance effect of each marker in the population examined.

On the other hand, the only QTL reported for male sexual development were obtained from genital tract measurements after either castration or slaughter in Meishan × Large White crosses (Bidanel et al. 2001, Rohrer et al. 2001) and Meishan × Duroc (Sato et al. 2003). These significant associations were detected on 7 different chromosomes (Table 2.4). The most significant results were obtained on chromosome X near the androgen receptor gene. The QTL detected had positive Meishan alleles and explained, respectively, 20 and 22% of the variance of testes and seminal vesicle weights. Three other QTL located on chromosomes 1 and 4 affected the weight of seminal vesicles. Each QTL explained 4-5% of trait variance. The effects of Meishan alleles were positive on chromosome 3, negative on chromosome 4 and family-dependent on chromosome 1. Similarly, three QTL located on chromosomes 7, 10, and 13 explained 3-7% of the phenotypic variance of testes weight, with positive effects of Meishan alleles on chromosome 13, but negative effects in the 2 other cases. Significant dominance effects were only evidence on chromosome 13.

Table 2.3:     Survey of candidate genes associated with boar reproductive traits

| Author | Boar population | | Locus | Significant effect on sperm quality traits or on boar fertility traits[2] |
| | Breed[1] | n | | |
| --- | --- | --- | --- | --- |
| Huang et al. 2001 | Du LR Y | 55 | *HSP70.2* | MOT ($P < 0.05$); VOL ($P < 0.05$) TSN ($P < 0.05$) |
| Huang et al. 2002 | Du | 37 | *EGF* | VOL ($P < 0.01$); NSR ($P < 0.01$) PPD ($P < 0.01$) |
| | | | *PTGS2* | VOL ($P < 0.01$); NSR ($P < 0.01$) PPD ($P < 0.05$); SCON ($P < 0.05$) |
| | | | *PRLR* | VOL ($P < 0.01$); MOT ($P < 0.05$) NSR ($P < 0.01$); PPD ($P < 0.01$) |
| Kmiec et al. 2002 | Mix[3] | 88 | *CYP2* | NSR ($P < 0.01$), NLS ($P < 0.01$) NSID ($P < 0.01$) |
| Mackowski et al. 2004 | Mix[3] | 140 | *GPX-5* | SCON ($P < 0.05$) |
| Urban and Kuciel 2001 | LW LR | 59 | *RYR1* | VOL ($P < 0.05$), MOT ($P < 0.01$) ASR ($P < 0.01$), NID ($P < 0.01$) |
| Steinheuer et al. 2002 | DLR | [4]ng | *OPN* | NBA (allele *148*): -1.496±0.489 ($P < 0.01$) |
| | | | *PRLR* | NBA: a=-0.289±0.529 ($P > 0.05$) d=-1.399±0.672 ($P < 0.05$) |
| Steinheuer et al. 2003 | DLR | 51 | *RBP4* | NBA: a=-0.472±0.112 ($P < 0.001$) d=0.577±0.141 ($P < 0.001$) |

[1]LW: Large White, Du: Duroc, Y: Yokshire, LR: Landrace, DLR: Deutsche Landrace. [2]VOL: Semen volume (ml), SCON: Sperm concentration (mln/ml), NSR: Normozoosperms (%), NLS: Number of live sperms in ejaculate (bln), NSID: Number of sperms in insemination doses (bln), NID: Number of insemination doses, MOT: Sperm motility (%), PPD: percentage of sperm with proximal plasma droplets, TSN: Total sperm number per ejaculate, NBA: Number of piglets born alive. [3]Polish Landrace, Polish Large White, Pietrain, Duroc × Pietrain, Hampshire × Pietrain, Duroc × Hampshire, Polish synthetic line 990, PIC (Pig Improvement Company). [4]Not given.

Table 2.4:    Survey of significant and intimate QTL for boar reproductive traits identified in crossed divergent pig breeds

| Author | Population[1] BC F$_3$ / F$_2$ | SSC[2] | Position | Genome wide significance | Traits[3] | Variant |
|---|---|---|---|---|---|---|
| Bidanel et al. 2001 | M×LW | 1 | - | <0.05 | SVWT | 4-5% |
| Rohrer et al. 2001 | M×WC | 3 | 49 | $4.8×10^{-2}$ | FSH | - |
| Bidanel et al. 2001 | M×LW | 3 | 39 | <0.001 | SVWT | 4-5% |
| Sato et al. 2003 | M×Du | 3 | 33 | <0.001 | TEWT | - |
| Bidanel et al. 2001 | M×LW | 4 | 75 | <0.05 | SVWT | 4-5% |
| Bidanel et al. 2001 | M×LW | 7 | 86 | <0.05 | LBUG | - |
| Bidanel et al. 2001 | M×LW | 7 | 66 | <0.05 | TEWT | 3-7% |
| Bidanel et al. 2001 | M×LW | 7 | 143 | <0.05 | EPWT | - |
| Rohrer et al. 2001 | M×WC | 8 | 16 | $2.9×10^{-1}$ | FSH | - |
| Bidanel et al. 2001 | M×LW | 10 | 140 | <0.05 | TEWT | 3-7% |
| Bidanel et al. 2001 | M×LW | 10 | 137 | <0.05 | EPTW | - |
| Rohrer et al. 2001 | M×WC | 10 | 101 | $3.0×10^{-3}$ | FSH | - |
| Bidanel et al. 2001 | M×LW | 13 | - | <0.01 | TEWT | 3-7% |
| Rohrer et al. 2001 | M×WC | 18 | - | - | FSH | - |
| Bidanel et al. 2001 | M×LW | X | 88 | <0.001 | SVWT | 22% |
| Bidanel et al. 2001 | M×LW | X | 89 | <0.001 | TEWT | - |
| Rohrer et al. 2001 | M×WC | X | 80 | $2.5×10^{-15}$ | FSH | - |
| Rohrer et al. 2001 | M×WC | X | 80 | $2.5×10^{-15}$ | TESZ | 20% |
| Ford et al. 2001 | M×WC | X | - | <0.01 | TEWT | - |
| Ford et al. 2001 | M×WC | X | - | <0.01 | ENPR | - |

[1]M: Meishan, WC: White Composite (equal contributions of Chester White, Landrace, large White and Yorkshire), LW: Large White, Du: Duroc.

[2]SSC: Sus scrofa chromosome.

[3]BUGL: Length of bulbo-urethral gland (mm), ENPR: Endocrine profiles, EPWT: Epididymis weight (g), FSH: Follicle stimulation hormone, SVWT: Seminal vesicles weight (g), TESZ: Testes size, TEWT: Testes weight (g).

2.5 Candidate gene analysis

Candidate genes are loci with a high probability to affect a trait of interest, i.e. this gene is a valuable source for association analyses. In order to investigate the hypothesis that a definite gene may affect a particular trait, analyses are performed where allelic variation detected at the candidate gene locus is used as independent variable enabling to statistically prove the effect of the candidate genes on specific phenotypes (Wimmers et al. 2002a). Candidate genes can be identified based on the knowledge of the physiology and biochemistry of a trait (direct candidate gene approach). The most general approach to identification of genes is that of positional cloning by which the inheritance of a trait is compared with that of large numbers of marker genes to locate the region in which the gene responsible for the trait lie.

Other recent approaches to detect candidate genes are based on the analyses of differences of the expression profile of particular subsets of cells and/or individuals with certain phenotypes. These are functional candidate genes because of their temporal spatial distribution of expression. The identification and mapping of expressed sequence tags, ESTs will deliver potential candidates for a trait of interest within QTL regions and facilitate comparative mapping. The positional candidate approach combines linkage information for a particular trait and mapping information of a (direct or functional) candidate gene within the homologous or heterologous species (comparative mapping).

Gene mutations affect gene expression (Geldermann 1996) or enzyme activity (Milan et al. 2000), i.e. that lead to the protein structure alteration and/or protein expression levels. As molecular genetic marker, one is based on cloning/sequencing including microsatellite (Tautz 1993), single nucleotide polymorphisms (SNPs), sequence tagged site (STS) (Olson et al. 1989) and expressed sequence tag (EST) (Adams et al. 1991); the other is DNA-finger printing based on PCR technique including random amplified polymorphic DNA (RAPD) or AFLP (Williams et al. 1990). SNPs is mainly a diallelic marker, even though until to four alleles (Brookes 1999). Microsatellite markers are multiple tandem repeats of sequence. These markers have become a powerful tool for

chromosome mapping and linkage analysis, the study of molecular evolution and population genetics (Brinkmann et al. 1998).

2.5.1 Biological correlation and/or physiological function of candidate genes

In this study, seventeen candidate genes are chosen based on their biological correlation and/or physiological function in male reproduction and are summarized in Table 2.5.

2.5.1.1 Gonadotropin-releasing hormone receptor (*GnRHR*)

The hypothalamic gonadotropin-releasing hormone (*GnRH*) is a key regulator of the reproductive system, triggering the synthesis and release of LH and FSH in the pituitary. GnRH transmits its signal via two specific serpentine receptors that belong to the large group of G-protein coupled receptors (*GPCRs*) (Kraus et al. 2001).

The gonadotropin-releasing hormone receptor (*GnRHR*) is a guanine nucleotide-binding protein-coupled receptor with a characteristic seven transmembrane domain motif. It transduces the hypothalamic message carried by the decapeptide gonadotropin-releasing hormone. At the gonadotrope cell surface the hormone binds to the receptor, leading to pituitary synthesis and secretion of gonadotropins (Cohen 2000).

GnRH deficiencies and *GnRHR* mutations are associated with idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome in humans (Seminara et al. 1998, Layman et al. 1997, Tamaya 2002, Bo-Abbas et al. 2003). Gonadotropin-releasing hormone receptor (*GnRHR*) gene mutations reduce GnRH binding and/or activation of inositol triphosphate or phospholipase C in several human families. Clinical features range from complete to partial hypogonadotropic hypogonadism (Achermann and Jameson 1999).

2.5.1.2 Follicle-stimulating hormone beta (*FSHB*)

Follicle-stimulating hormone acts on the germinal cells in the seminiferous tubules of the testis and is responsible for spermatogenesis up to the secondary spermatocyte

Table 2.5:    Survey of the biological correlation and/or physiological function of candidate genes with male reproduction

| Gene | Biological correlation /physiological function | Author |
|---|---|---|
| *GnRHR* | idiopathic hypogonadotropic hypogonadism | Layman et al. 1997 |
| *FSHB* | spermatogenesis; | Zanella et al. 1999 |
| | testicular morphology and function | Li MD et al. 1998 |
| *LHB* | testicular morphology ; sperm parameters | Wu et al. 1999 |
| *RLN* | correlated with sperm motility in pig | Sasaki et al. 2001 |
| *PRL* | effect on spermatogenic cells (rat) | Hondo et al. 1995 |
| | accessory sexual glands (pig) | Rothschild et al. 1997 |
| *PRLR* | mRNA expression during spermatogenesis | Hondo et al. 1995 |
| *FST* | coexpressed with *INHBA* | Muttukrishna et al. 2001 |
| | modulating of activin action | Mather et al. 1993 |
| | disorders of spermatogenesis | Kaipia et al. 1992 |
| *INHA* | scrotal circumference; FSH concentration; | McKeown et al. 1997 |
| | LH pulse amplitude; marker spermatogenesis | Matorras et al. 1998 |
| *INHBA* | increasing expression during development of Sertoli cells | Kaipia et al. 1992 |
| *INHBB* | intense expression in spermatogonia, round spermatids, Sertoli cells | Marchetti et al. 2003 |
| *AR* | idiopathic infertility | Hiort et al. 2000 |
| *ACR* | sperm fertilizing capacity | Cechova et al. 1984 |
| | effect of its expression on spermatogenesis | O'Brien et al. 1996 |
| *RBP4* | transporter and buffer of vitamine A in boar | Syntin et al. 1996 |
| *OPN* | sperm motility (rat) | Siitteri et al. 1995 |
| | fertility-associated protein (bull) | Cancel et al. 1999 |
| *ACTG2* | expressed spermatogenesis (mouse) | Waters et al. 1985 |
| | distributed acrosomal/postacrosomal regions | Casale et al. 1988 |
| *ACTN1* | sperm function | Yagi and Paranko 1995 |
| *ACTN4* | architecture of spermway stereocilia | Hofer, Drenckhahn 1996 |

stage; later androgens from the testis support the final stages of spermatogenesis (Hafez and Hafez 2000). Follicle-stimulating hormone is a heterodimer composed of alpha and beta subunits that are coded by two distinct genes. The beta subunit offers specificity. The expression of *FSHB* gene in boar is positively associated with activin beta B-subunit (Li MD et al. 1998). Ellendorf et al. (1970) reported that FSH influences the sexual behaviour and testicular function of the boar. FSH concentration of plasma has been shown to be significantly correlated with testicular morphology and function in boar (Zanella et al. 1999). Otherwise, male homozygous *FSHB* knockout mice had normal levels of serum testosterone but had small testes and oligospermia (Layman 2000).

## 2.5.1.3 Luteinizing hormone beta (*LHB*)

Luteinizing hormone is a glycoprotein composed of an alpha and a beta subunit with a molecular weight of 30,000 daltons and a biologic half life of 30 minutes. LH stimulates the interstitial cells of testis. The interstitial cells (Leydig cells) produce androgens after LH stimulation (Hafez and Hafez 2000). LH also influences the sexual behaviour and testicular function of the boar (Ellendorf et al. 1970). LH beta gene expressed during spermatogenesis and male sexual behavior, and FSH beta may participate in spermatogenesis, whereas LH beta is more involved in spermatogenesis (Degani et al. 2003). A mutation causing inactivation of the LH beta subunit in human leads to absence of Leydig cells, lack of spontaneous puberty and infertility (Huhtaniemi et al. 1999).

## 2.5.1.4 Relaxin (*RLN*)

Relaxin is a polypeptide hormone consisting of alpha and beta subunits that are connected by two disulfide bonds. It has a molecular weight of 5700 daltons. Inhibins and insulin are structurally similar, but their biological actions are different. Relaxin is located in the boar testes, specific relaxin-binding sites were localized in the Leydig cells (Dubois and Dacheux 1978, Min and Sherwood 1998). Relaxin has also been

identified in boar seminal plasma and can maintain or increase sperm motility (Bagnell et al. 1993). Samuel et al. (2003) reported that relaxin is an important factor in the development and function of the male reproductive tract in mice and has an essential role in the growth of the prostate and maintenance of male fertility. Relaxin may mediate its effects on growth and development by serving as an antiapoptotic factor, in vitro experiments revealed that relaxin enhances the fertilization capacity and motility of human spermatozoa (Park et al. 1988, Weiss 1989). Sasaki et al. (2001) reported that relaxin levels in seminal plasma have been shown to be significantly correlated with sperm motility trait. However, association of genomic variation at the *RLN* locus with male reproductive traits have not been shown so far.

2.5.1.5 Prolactin (*PRL*) and prolaction receptor (*PRLR*)

Prolactin is a polypeptide hormone secreted by the adenohypophysis as a progonadal hormone that promotes the function of the testis and the reproductive accessory glands (Hair et al. 2002). The mRNAs coding for the long form of prolactin receptor were expressed in germ cells as well as in Leydig and Sertoli cells that suggested *PRLR* played multiple roles for *PRL* in the human male reproductive tract (Hair et al. 2002). *PRLR* mRNA expression is almost consistent with *PRL* binding sites except for elongated spermatids and spermatozoa suggesting that *PRL* may have direct effects on spermatogenic cells (Hondo et al. 1995). *PRLR-/-* knockout mice showed delayed fertility in males; the effects of *PRL* on testosterone production of Leydig cells and accessory reproductive glands can finally be compensated by other regulatory factors (Bole-Feysot et al. 1998).

2.5.1.6 Follistatin, inhibin alpha, inhibin beta A, and inhibin beta B (*FST, INHA, INHBA*, and *INHBB*)

Follistatin is a protein isolated from follicular fluid (Hafez and Hafez 2000), also located in rat testis that may modulate a range of testicular actions of activin (Meinhardt et al. 1998). It not only inhibits the secretion of FSH similar to that of inhibins but also

binds activin and neutralises its biological activity. Thus it modulates the secretion of FSH.

The gonads are the main source of inhibin and related proteins, which contribute to the endocrine regulation of the reproductive system. Sertoli cells in the male and the granulose cells in the female produce inhibins. Inhibins are not steroids but proteins comprising two disulfide bridges subunits called alpha and beta. In male, inhibins are secreted via the lymph. By inhibiting FSH release without altering LH release, inhibins may be partly responsible for the differential release of LH and FSH from the pituitary. Besides the regulation of pituitary FSH, inhibins related proteins regulate Leydig cell function (Risbridger et al. 1996).

Over-expression of the rat inhibin alpha-subunit gene in mice leads to a disruption of the normal inhibin-to-activin ratio and to reproductive deficiencies, and they support the hypothesis that inhibin and activin act to regulate FSH secretion in vivo and are essential for normal gonadal function (Cho et al. 2001). The expression of inhibin beta A and beta B subunits, follistatin, and activin-A receptor messenger RNA (mRNAs) in different stages of rat seminiferous epithelial cycle was analyzed by in situ hybridization (Kaipia et al. 1992).

Both levels of serum inhibin B and seminal plasma inhibin B could reflect testis spermatogenesis status. Levels of seminal plasma inhibin B could also reflect the function of seminiferous duct (Hu and Huang 2002).


2.5.1.7 Acrosin (*ACR*)


Acrosin was long considered the essential zona lysine for sperm penetration, but the number of enzymes present in or attached to the acrosomal membranes suggests that a combination of enzymes acts synergistically during penetration. This is consistent with the heterogenous glycoprotein structure of the zona pellucida. Enzyme exposed during the acrosome reaction is needed for the passage of sperm through the zona, but sperm motility is also required (Yanagimachi et al. 1981).

Boar proacrosin correlation between total proacrosin content and sperm fertilizing capacity has been detected by Cechova et al. (1984). Glogowski et al. (1998) reported

that acrosin activity can be used for boar spermatozoa to evaluate the quality of boar semen.

Boar proacrosin expressed in spermatids of transgenic mice does not reach the acrosome and disrupts spermatogenesis (O'Brien et al. 1996). The diploid expression of the rat proacrosin gene is in agreement with mouse proacrosin gene expression but in contrast to the apparent haploid expression of proacrosin described for the bull and the boar (Raab et al. 1994).

## 2.5.1.8 Androgen receptor (*AR*)

Androgen action is mediated by androgen receptor (*AR*), which is in the testis expressed by Leydig, peritubular, and Sertoli cells. More than 300 different mutations (commonly missense mutations) in the *AR* gene have been reported to cause androgen insensitivity (Patterson et al. 1994, Layman 2002). Androgen receptor gene mutations in males are significantly associated with idiopathic infertility (Hiort et al. 2000), furthermore men with short androgen receptor CAG repeats have the highest sperm output (Von Eckardstein et al. 2001).

The porcine cDNA sequence of *AR* was analysed and identified a polymorphic pentanuclotide repeat $(CCTTT)_n$ in 5´-untranslated region in addition to the microsatellite already detected by Seifert et al. (1999) and in intron 7 (Trakooljul et al. 2000). Trakkooljul et al. (2000) also reported that five alleles of the 5´-UTR microsatellite marker in three commercial breeds of German Landrace, Large White and Pietrain. Two additional alleles were detected in "DUMI-resource population" based on the reciprocal cross of Duroc and Berlin Miniature pig. Moreover, within "DUMI resource population", recorded the number of follicles and corpora lutea at slaughter (age of 200 days), Androgen receptor genotypes as well as genotypes of 80 microsatellite loci will be evaluated for association and linkage with these reproductive traits. First analyses show evidence for an effect of androgen receptor on mammary gland phenotypes (Wimmers et al. 2002a).

## 2.5.1.9 Retinol binding protein 4 (*RBP4*)

Retinoic acid (vitamin A) is derived from endogenous retinol, which is delivered to target cells by RBP. Vitamin A in testicular tissue of the boar and intersex pig (Booth 1970, 1974) directly affects on boar testis (Makaganchuk and Iakimchuk 1974, Palludan 1966). Vitamin A and beta-carotene affect the vitamin E status, ejaculation parameters and health of boar (Wemheuer et al. 1996).

Retinol binding protein expression has been detected in adult boar epididymis in various regions by Syntin et al. (1996). Transthyretin and receptor-binding properties can be transferred from retinol-binding protein (RBP) and its close structural homologue the epididymal retinoic acid-binding protein (ERABP). RBP exhibits three molecular-recognition properties: it binds to retinol, to transthyretin (TTR) and to a cell-surface receptor (Sundaram et al. 2002). Localization of cellular retinol-binding protein mRNA in rat testis and epididymis and its stage-dependent expression during the cycle of the seminiferous epithelium have been detected by Rajan et al. (1990). Therefore, the transport and buffering ability of RBP makes RBP4 a strong candidate gene for sperm production of boar.

## 2.5.1.10 Osteopontin (*OPN*)

Osteopontin (*OPN*), a multifunctional phosphoprotein, Secreted Ca(2+)-binding phosphor-protein 1 (*Spp-1*) found in both hard and soft tissues (Chen et al. 1992, Luedtke et al. 2002), an extracellular matrix protein that also is located in bull seminal plasma, is a 55,000 Dalton fertility-associated protein in Holstein bull (Cancel et al. 1999).

Osteopontin has been identified in rat epididymis and on rat and bull epididymal sperm (Luedtke et al. 2002, Cancel et al. 1999). It has been reported that the immunoreactivity of osteopontin on the sperm head in rats disappears as sperm transit the epididymis that may serve as a "decapacitation" factor to prevent premature activation of epididymal sperm motility or fertilizing ability (Siitteri et al. 1995).

## 2.5.1.11 Actin gamma (*ACTG2*)

In boar spermatozoa, the localization of actin changes after acrosome reaction (Palecek et al. 1999). Indirect immunofluorescence and immunoelectron microscopy showed that gamma and beta actins are codistributed in the acrosomal and postacrosomal regions. Sperm actin was also identified on two-dimensional gel as two spots in the isoelectric point and molecular weight corresponding to beta and gamma actins. Coelectrophoresis of sperm actin and chicken gizzard actin and immunoblots stained with the specific antibodies confirmed the presence of these two isoforms of actin (Casale et al. 2001). Gamma actin is therefore potentially involved in membrane changes during acrosome-reaction with an important implication on sperm function.

## 2.5.1.12 Actinin alpha 1 and 4 (*ACTN1* and *ACTN4*)

Actin, alpha-actinin are expressed in the testicular, epididymal, and ejaculated spermatozoa and in the epithelium of the bovine epididymis (Yagi and Paranko 1992). Destabilization of membranes with selected extractions induces changes in the acrosomal lamina mimicking acrosomal vesicle formation. The lateral prongs and posterior ring structure, respectively, may serve as anterior and posterior anchors for the extraction-resistant post-acrosomal sheath. The lateral prongs may also be a merger zone for alpha-actinin with important implication on sperm function. The latter two proteins may be involved in acrosomal vesicle formation. It is apparent that extractions have a significant effect on the detectability of sperm cytoskeletal elements (Yagi and Paranko 1995). Since alpha-actinin can form crossbridges between adjacent actin filaments (bundles) at longer distances than the other crosslinker of the stereocilium core bundle, fimbrin, it is assumed that alpha-actinin is essential for both the formation of the stem portions of spermway stereocilia and for the generation of their striking branching pattern (Hofer and Drenckhahn 1996). A developmentally regulated temporal sequence of expression of alpha-actinin might control the unique architecture of spermway stereocilia (Hofer and Drenckhahn 1996).

2.5.2 Locations and genotyping methods

In this experiment, the candidate gene loci located on swine chromosomes and the genotyping methods were reported by different authors or in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query). Polymorphisms of *ACTN1* and *ACTN4* locus were detected by comparative sequencing of animals. Genotyping methods were developed by ourselves. This information is summarized in Table 2.6.

Table 2.6:        Survey of locations and genotyping methods of candidate genes

| Gene | Location | Author | Methods | Author |
|------|----------|--------|---------|--------|
| *GnRHR* | SSC8 | Rohrer 1999a | DEV | - |
| *RLN* | SSC1 | Chowdhary et al. 1994 | B | Wimmers et al. 2002b |
| *FSHB* | SSC2 | Mellink et al. 1995 | A | Rohrer et al. 1994 |
| *ACTG2* | SSC3 | Yerle et al. 1998 | DEV | - |
| *ACR* | SSC5 | Yasue et al. 1999a | DEV | - |
| *LHB* | SSC6 | Mellink et al. 1995 | DEV | - |
| *ACTN4* | SSC6 | Martins-Wess et al. 2003 | DEV | - |
| *ACTN1* | SSC7 | Wimmers et al. 2005 | DEV | - |
| *PRL* | SSC7 | Vincent et al. 1998b | A | Vincent et al. 1998 |
| *OPN* | SSC8 | Alexander et al. 1996 | A | Knoll et al. 2001 |
|       |       |                       | C | Ellegren et al. 1993a |
| *RBP4* | SSC14 | Messer et al. 1996a | A | Rothschild et al. 2000 |
| *INHA* | SSC15 | Rettenberger et al. 1996 | A | Hiendleder et al. 2002 |
| *INHBB* | SSC15 | Nonneman et al. 2002 | C | Nonneman et al. 2003 |
| *PRLR* | SSC16 | Vincent et al. 1997 | A | Vincent et al. 1997 |
| *FST* | SSC16 | Lahbib-Mansais et al. 2000 | C | Ellegren et al. 1993b |
| *INHBA* | SSC18 | Lahbib-Mansais et al. 1996 | DEV | - |
| *AR* | SSCX | Seifert et al. 1999 | C | Trakoojul et al. 2000 |

UK: unknown; A: PCR-RLFP, PCR based restriction fragment length polymorphism technique; B: PCR-SSCP, PCR based single strand conformation polymorphism technique; C: Microsatellite; DEV: developed by ourselves.

2.6 Genotyping techniques

For genetic markers SNPs and microsatellite, which are based on cloning/sequencing (Tautz 1993), the simple genotyping techniques based on PCR technique are often applied in molecular genetics research work. These techniques include PCR-RFLP, PCR-SSCP and microsatellite etc.

2.6.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is in vitro a rapid procedure for the amplification of a specific DNA segment with a thermostable DNA polymerase which is isolated from a thermophilic bacterium-*Thermus aquaticus (Taq)* (Saiki et al. 1985 and 1988, Mullis and Faloona 1987, Innis et al. 1990).

In reaction solution three nucleic acid segments are present, one is double strand genomic DNA which is amplified, the other are two single strands oligonucleotide primers which are capable of hybridizing to a template single-stranded nucleic acid by leaving part of the template to the 3'-end of the primer single-stranded. The primers are surplus compared to the amplified DNA in the solution. Moreover, the reation solution also consists of a protein component, the DNA polymerase, desoxy ribonucleotid triphosphates (dNTPs), buffers as well as salts.

A synthesis cycle consists of denaturation of the template into single strands, annealing of primers to each original strand for new strand synthesis and extension of the new DNA strands from the primers. The template denaturates into single strands as stencil for the following synthesis. The primers hybridize with single DNA strands (Annealing) at their opposite 3´-end, so that the new strands synthesize from 5´ to 3´ with the catalyzer of DNA polymerase, the DNA segment extends between the primers. In the last cycle the new synthsized DNA strands participate as stencils for amplification in the next cycles. The quantity of the amplifications increases exponentially [2×(n-1)] from cycle to cycle. The PCR permits the rapid increasing of short DNA fragments of up to 10,000 base paires. The PCR is accomplished with a Thermocycler.

2.6.2 Single strand conformation polymorphism (SSCP)

Single strand conformation polymorphism (SSCP) has emerged as one of the most popular mutation detection techniques (Masato 1989), which were developed by Orita et al. (1989). The reasons for the popularity of the technique are its technical simplicity and its relatively high sensitivity for the detection of sequence variations (Hayashi 1991).

The process of SSCP involves PCR amplification of the fragment of interest, denaturation of the double stranded PCR product with heat and formamide, and electrophoresis on a non-denaturing polyacrylamide gel. During electrophoresis, single stranded DNA fragments fold into a three-dimensional shape according to their primary sequence. The eletrophoretic mobility of separation then becomes a function of the shape of the folded, single stranded molecules. If the sequence of a wild-type (or reference) sequence differ from that of the fragment being tested, even by only a single nucleotide, it is possible that at least one of the strands, if not both, will adopt different three- dimensional conformations and exhibit an unique eletrophoretic mobility.

Advantages and disadvantages of SSCP analysis were described by Nataraj et al. (1999). Advantages: (1) simplicity of usage; (2) no special equipment necessary; (3) mutant bands separated from wild-type can be isolated for analysis and non-radioactive labelling available. Disadvantages: (1) size of fragment analyzed limited; (2) absence of theory; (3) multiple conditions to detect all mutations; (4) gels sometimes difficult to interpret and less applicable to DNA with unknown sequence.

2.6.3 Restriction fragment length polymorphisms (RFLP)

Restriction fragment length polymorphisms (RFLP) as powerful new technology is to identify variants within a specific region of the genome using digest of total human DNA and applied to constitute a genetic linkage map by Botstein et al. (1980).

DNA restriction enzymes recognize specific sequences in DNA and catalyze endonucleolytic cleavages, yielding fragments of defined lengths. PCR-based RFLP marker, restriction fragments may be displayed by electrophoresis in agarose gels,

separating the fragments according to their molecular size. Difference among individuals in the lengths of a particular restriction fragment could result from many kinds of genotypic difference: one or more individual bases could differ, resulting in loss of a cleavage site or formation of a new one, alternatively, insertion or deletion of blocks of DNA within a fragment could alter its size. These genotypic changes can all be recognized by the altered mobility of restriction fragments on agarose gel eletrophoresis.

On the other hand, fragments encoding specific sequences within a large and complex population of DNA fragments can be detected by hybridization using the method of Southern (Southern 1975). The DNA fragments in agarose gel are transferred onto nitrocellulose paper and hybridized with radioactive probe sequences. The use of probe sequences of very high specific radioactivity has permitted the identification by this method of single-copy sequences in mammalian DNA (Jeffreys and Flavell 1977).

## 2.6.4 Microsatellite

Microsatellites (a variable number of tandem repeats) are short segments of DNA (1-6 base pairs) that have a repeated sequence (Tautz 1993). Polymorphisms are usually due to differences of length in the amplified product and are very variable. This variabilities are defined slipped-strand mispairing (Levinson and Gutman 1987) or irregular crossing over (Krawczak and Schmidtke 1994) and are positively correlated with the number of motif repetitions (Brinkmann et al. 1998). More or less micosatellites distributed frequently on genome (Wintero et al. 1992). The polymorphisms have become a powerful tool for the identification of chromosome mapping and linkage analysis, and the study of molecular evolution and population genetics (Brikmann et al. 1998).

In human, it is estimated that each 6,000 bases pair had a microsatellite marker with a size more than 20 base pairs (Beckmann and Weber 1992). The motif $(A)_n$ and $(CA)_n$ are the most frequent forms in pig. There is $(A)_n$ motif average each 7,000 bp from 400,000 copy number and $(CA)_n$ motify each 300,000 bp from 100,000 copy number (Wintero et al. 1992, Ellegren 1993a).

The polymorphisms of microsatellites are revealed by PCR-based amplification and polyacrylamid gel electrophoresis. The variation of the tandem repeated sequence is visible as different lengthens of amplificated fragments (Weber and May 1989). The efficiency of microsatellite genotyping can be increased using the automatic DNA sequencer, more times loading on the gel and through coamplification of more microsatellite loci with non overlapping fragment-lengthen for each other (Multiplex PCR) (Yue et al. 1999). Microsatellites are codominant marker (Weber and May 1989, Litt and Luty 1989). By evaluation, stammer bands that are shorter than the amplified fragments, are more difficult to distinguish a right band from stammer bands (Walsh et al. 1996). Stammer bands may lead to wrong determination of heterozygote genotype.

## 3 Material and methods

### 3.1 Experimental strategy

356 AI boars composed of Pietrain (PI) and Pietrain × Hampshire (PI×HA) were genotyped by means of PCR-RFLP/SSCP or microsatellite for the chosen candidate genes in order to elucidate their effect on sperm concentration (SCON), semen volume per ejaculate (VOL), sperm motility (MOT), plasma droplets rate (PDR) and abnormal sperm rate (ASR). In addition, these genes were also tested for association with the fertility traits non return rate (NRR) and number of piglets born alive (NBA). Figure 3.1 shows the experimental design.
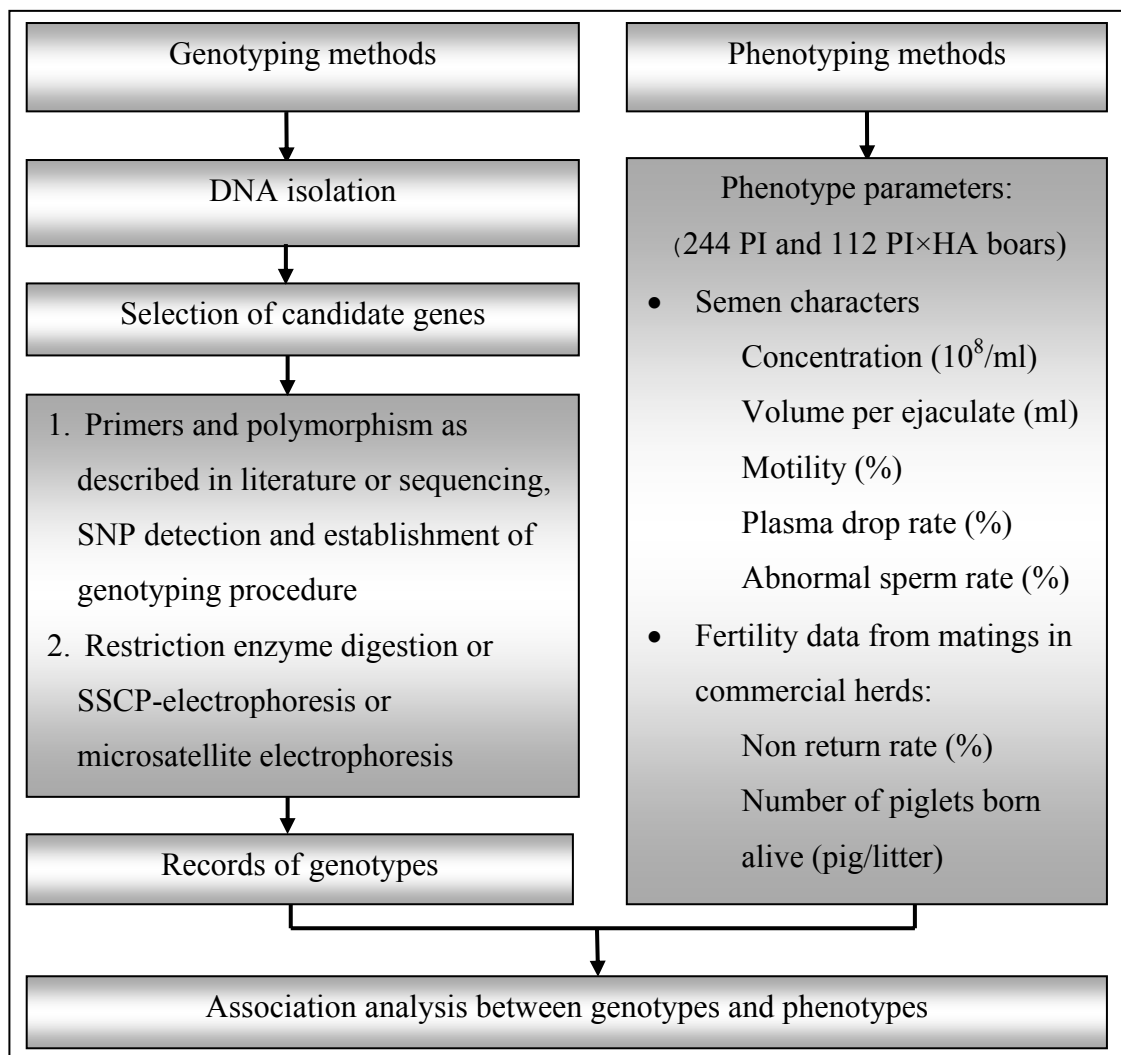


Figure 3.1:    Schematic description of experimental strategy

3.2 Materials

3.2.1 Animals

In total, 356 boars of an AI boar station, born between 1990 and 1999, and used in commercial pig herds mainly of North-Western Germany were included in the study. The boar population was composed of the breed Pietrain (PI; n=244) and the crossbred Pietrain × Hampshire (PI×HA; n=112).

3.2.2 Sperm sample

For this study 356 sperm samples of boars were collected in an AI boar station, which were produced from January 2000 to December 2001. Each sperm sample, 1 ml from an individual boar, was collected and stored at − 20°C for molecular analysis. Sperm quality was evaluated with a standard method according to the guidelines of the World Health Organization.

3.2.3 Phenotype records

Sperm quality traits including sperm concentration [SCON $1(0^8/ml)$], semen volume per ejaculate [VOL (ml)], sperm motility [MOT (%)], plasma droplets rate [PDR (%)] and abnormal spermatozoa rate [ASR (%)] were obtained from each ejaculate with light microscopy according to the guidelines of the World Health Organization. For each boar the repeated measurements of sperm quality traits were available. The semen samples of boars were collected along with date recording.

Fertility trait non return rate [NRR (%)] and number of piglets born alive [NBA (pig/litter)] of each boar was given as the deviation from the population means within breed, parity of sow, farm and season classes. This data was derived from mated commercial pig herd mainly of North-Western Germany.

## 3.2.4 Laboratory materials

The following materials were used for molecular genetics analysis in laboratory. They include equipments, softwares, chemicals and kits, solutions, buffers and primers.

## 3.2.4.1 Equipments

| | | | |
|---|---|---|---|
| Automated sequencer | LI-COR | 4200 | MWG (Ebersberg) |
| Thermocycler | MJ Research | PTC100 | Biozym, Hess Oldendorf |
| Thermocycler | Minicycler | PTC150 | Biozym Hess. Oldendorf |
| Thermocycler | BIO RAD | iCycler | BIO RAD (München) |
| Centrifuge | HERMLE | Z233MK | HERMLE (Wehingen) |
| Centrifuge | HERMLE | Z323K | HERMLE (Wehingen) |
| Thermoshaker | Gerhardt | - | Gerhardt (Bonn) |
| Incubator | Memmert | BB16 | Memmert (Schwabach) |
| Electrophoresis(horizontal) | SUB-cell | GT | BIO RAD (München) |
| Electrophoresis(vertical) | UniEquip | S2S | Uniequip (Martinsried) |
| Electrophoresis(vertical) | Sequi-Gen | GT | BIO RAD (München) |
| Electrophoresis(vertical) | UniEquip | DAIICHI | Uniequip (Martinsried) |
| Gel dryer | BIO RAD | 583 | BIO RAD (München) |
| Power supply | BIO RAD | Pac3000 | BIO RAD (München) |
| Power supply | BIO RAD | Pac300 | BIO RAD (München) |
| Spectrophotometer(UV) | DU$^{®}$-62 | PM2K | Unterschleissheim-Lohhof |
| UV Transilluminator | UniEquip | Uvi-tec | Uniequip (Martinsried) |
| WasserReinigungsAnlagen | Millipore | Milli Q | Millipore (Eschborn) |
| WasserReinigungsAnlagen | Millipore | Milli R | Millipore (Eschborn) |

## 3.2.4.2 Used softwares

| | |
|---|---|
| BLAST program | http://www.ncbi.nlm.nih.gov/blast/ |
| ClustalW Multiple Sequence | http://searchlauncher.bcm.tmc.edu/ |
| Alignment | multialign/Options/clustalw.html |

| | |
|---|---|
| Image Analysis program (Version 4.10) | LI-COR Biotechnology, USA |
| One-Dscan program | Scanalytics Inc., Billerica, MA |
| SAS Version 8.2 | SAS Institute Inc., Cary, NC, USA |
| Compute pI /Mw tool program | http://www.expasy.ch/tools/pi_tool.html |
| Webcutter 2.0 | http://rna.lundberg.gu.se/cutter2/ |
| Genepop 3.4 | http://wbiomed.curtin.edu.au/genepop/index.html |
| Multiple sequence alignment by Florence Corpet | http://prodes.toulouse.inra.fr/multalin/multalin.html |
| Primer design program | Primer express software version 2.0 |

## 3.2.4.3 Chemicals and kits

Biomol (Hamburg): Phenol, Lambda DNA *Eco*91I (BstE II) and Lambda DNA *Hind*III

Biozym Diagnostik (Hessisch-Oldendorf): Sequagel XR sequencing gel (National Diagnostics) and SequiTherm Excel$^{TM}$II DNA sequencing kit (Epicentre Technologies)

GeneCraft (Münster): Taq polymerase

MWG Biotech (Ebersberg): Oligonucleotide primers.

Promega (Mannheim): pGEM$^{®}$-T vector, T4 DNA ligase and 2× rapid ligation buffer, Taq polymerase and 10× reaction buffer, Restriction enzyme: *Hae*III, *Hin*6I, *Bst*EII, *Msp*I, *Alu*I, *Bst*UII, *Apa*I.

Qiagen (Hilden): QIA quick PCR purification Kit

Roche Diagnostics GmbH (Mannheim): Expand$^{TM}$ long Template PCR system.

Roth (Karlsruhe): Acetic acid, Ampicillin, Ammonium peroxydisulphate (APS), Boric acid, Bromophenol blue, Calcium chloride, Chlorofrom, Dimethyl sulfoxide (DMSO), dNTP, Ethylenediaminetetraacetic acid (EDTA), Ethanol, Ethidium bromide, Formadehyde, Formamide, Glycerin, Hydrochloric acid, Hydrogen peroxide (30 %), Isopropyl β-D-thiogalactoside (IPTG), N,N´-dimethylform-amide, Nitric acid, Peptone, Proteinase K, Sodium dodecyl sulphate (SDS), Silver nitrate, Sodium carbonate, Sodium chloride, Sodium hydroxide,

N,N,N′,N′-Tetramethylethylene-diamine (TEMED), Tris, 5-bromo-4-chloro-3-indolyl-β-D-galactopyra-noside (X-gal), Xylencyanol and Yeast extract.

Serva Electrophoresis GmbH (Heidelberg): Acrylamide (molecular biology grade) and Bisacrylamide.

Sigma-Aldrich Chemie GmbH (Taufkirchen): 2, 2′Azino bis (3-ethylbenzthiazo-line-6-sulphonic acid) diammonium salt (ABTS), Agarose, Blue dextran, Calcium chloride, Diethyl barbituric acid, Ethylene glycol-bis (2-amino- ethylether)-N,N,N′,N′-tetraacetic acid (EGTA), Gelatin, Fetal calf serum, Gutamin, Histopaque-1077, Isopropanol, Magnesium chloride, Penicillin, Phytohemagglutinin (PHA), RPMI-1640 medium, Streptomycin G, Sodium barbituric acid, Sodium barbiturate, *Taq* polymerase, VectoretteII system.

## 3.2.4.5 Solutions and buffers

All solutions used in this investigation were prepared with deionized and demineralized (Millipore) water and pH was adjusted with sodium hydroxide or hydrochloric acid.

| APS solution: | Ammoniumpersulfat | 5 g |
| 10% (w/v) | Water (added to) | 50 ml |
| | | |
| Acrylamide (40%): | Acrylamide | 76g (78.4g) |
| 19:1 (49:1) | Bis-acrylamide | 4g (1.6g) |
| | Water added to | 200 ml |
| | | |
| Acrylamide gels (12%): | 40 % Acrylamide | 4.5 ml |
| | TBE (10×) | 750 μl |
| | Formamide: glycerol (1:3 v/v) | 750 μl |
| | Water | 9 ml |
| | APS (100.0 mg/ml) | 130 μl |
| | TEMED | 10 μl |
| | | |
| Blue dextran buffer: | Blue dextran (50.0 mg/ml) | 1 ml |

|                           | EDTA (0.5M) (186.1 mg/ml)          | 50 μl       |
|                           | Formamide                          | 5 ml        |

| IPTG solution:            | IPTG                               | 1.2 g       |
|                           | Water added to                     | 10 ml       |

| LB-agar plate:            | Sodium chloride                    | 8 g         |
|                           | Peptone                            | 8 g         |
|                           | Yeast extract                      | 4 g         |
|                           | Agar-Agar                          | 12 g        |
|                           | Sodium hydroxide (40.0 mg/ml)      | 480 μl      |
|                           | Water added to                     | 800 ml      |

| LB-broth:                 | Sodium chloride                    | 8 g         |
|                           | Peptone                            | 8 g         |
|                           | Yeast extract                      | 4 g         |
|                           | Sodium hydroxide (40.0 mg/ml)      | 480 μl      |
|                           | Water added to                     | 800 ml      |

| Lysis buffer:             | SDS (10%)                          | 200 μl      |
|                           | Tris-HCl (1M) pH 8.0               | 4 ml        |
|                           | EDTA (0.5M) pH 8.0                 | 4 ml        |
|                           | Proteinase K 2% (W/V)              | 4.44 ml     |
|                           | Mercaptoethanol                    | 4 ml        |
|                           | Water added to                     | 200 ml      |

| Natrium acetate solution: | Natrium acetate pH 5.3             | 3 M         |

| PAA loading buffer:       | Formamide                          | 98 % (v/v)  |
|                           | EDTA pH 8.0                        | 10 mM       |
|                           | Bromophenol blue                   | 0.5 mg/ml   |
|                           | Xylenzyanol                        | 0.5 mg/ml   |

| | | |
|---|---|---|
| Proteinase K solution: | Proteinase K in 1× TE-buffer | 2% (w/v) |
| Saline: | $Na_2HPO_32H_2O$ | 6.19 g |
| | $KH_2PO_4$ | 2.54 g |
| | NaCl | 4.14 g |
| | Formaldehyde (38%) | 125 ml |
| | Distilled water | 100 ml |
| SDS solution: | Sodium dodecylsulfat in water | 10% (w/v) |
| Sequence loading buffer: | Formamide | 83% (v/v) |
| | EDTA pH 8.0 | 4 mM |
| | Blue dextran | 10m g/ml |
| Silane solution: | Silane | 3 μl |
| | Ethanol 95% (added) | 1 ml |
| Silver staining solution: | Sodium carbonate | 30 g |
| (Development solution) | Water added to | 1000 ml |
| | Formaldehyde | 650 μl |
| Silbernitrate solution: | Silbernitrate | 5 g |
| (0.2%) | Water added | 2500 ml |
| SSCP loading buffer: | Formamide | 47.5 ml |
| | Sodium hydroxide | 200 mg |
| | Bromophenol blue | 125 mg |
| | Xylenecyanol | 125 mg |
| 50× TAE-buffer, pH 8: | Tris | 242 mg |
| | Acetic acid | 57.1 ml |
| | EDTA (0.5M) (186.1 mg/ml) | 100 ml |
| | Water added to | 1000 ml |

| 10× TBE-buffer: | Tris | 108 g |
|---|---|---|
| | Boric acid | 55 g |
| | EDTA (0.5M) (186.1mg/ml) | 40 ml |
| | Water added to | 1000 ml |
| | | |
| 1× TE-buffer: | Tris (1M) | 10.0 ml |
| | EDTA (0.5M) (186.1 mg/ml) | 2.0 ml |
| | Water added to | 1000.0 ml |
| | | |
| X-gal: | X-gal | 50 mg |
| | N, N´-dimethylformamide | 1 ml |

3.2.4.6 Used primer sequences

In this study all primer sequences were derived from published porcine sequences or were used as described in literature. The list of used primers is given in Table 3.1.

Table 3.1:      List of primer sequence of candidate genes

| Gene | Primer sequence (5´→ 3´) | FL[‡] | Reference |
|---|---|---|---|
| *ACTN1* | FW: GCATTGTCAACTACAAGCCCAA | 933 bp | *BF442592 |
| | RV: TCATCTGCTCCTGGCTGATG | | |
| *ACTN4* | FW: CCGAGTTCAACCGCATCAT | 514 bp | *BI338543 |
| | RV: AAGGAATGCCAGAGTGCTTTG | | |
| *FSHB* | FW: AGTTCTGAAATGATTTTTCGGG | 624 bp | Rohrer et al. |
| | RV: TTTGCCATTGACTGTCTTAAAGG | | 1994 |
| *PRL* | FW: GTGCAGTCATCCTGTCCCAC | 2750 bp | *X14068 |
| | RV: GTTGATGGCCTTGGTAATGAA | | |
| *PRLR* | FW: CCCAAAACAGCAGGAGAACG | 457 bp | Vincent et al. |
| | RV: GGCAAGTGGTTGAAAATGGA | | 1997 |
| *RBP4* | FW: GAGCAAGATGGAATGGGTT | 550 bp | Rothschild et |
| | RV: CTCGGTGTCTGTAAAGGTG | | al. 2000 |

Table 3.1: (continue)

| | | | |
|---|---|---|---|
| *INHA* | FW: CACATATGTATTCCGGCC | 731 bp | Hiendleder et |
| | RV: CCGTCTCGTACTTGAAAG | | al. 2002 |
| *OPNin6* | FW: TCACCGATTTCCCCACCGACAC | 1300 bp | Knoll et al. |
| | RV: TGGCTGCGGGTTTCCACACTG | 1000 bp | 1999 |
| *GnRHR* | FW: GGAATGACAAGCACAAAGCA | 200 bp | *AF227686 |
| | RV: GGAGCTCAGAAAGCACAGAGA | | |
| *LHB* | FW: CCCAGGGACTGCTGTTGT | 240 bp | *D00579 |
| | RV: GAGTAATCCACCCGGATCTGT | | |
| *RLN* | FW: TGAAACGCCTGGAGCAGAAG | 228 bp | Wimmers et |
| | RV: GTTTCCAGCTGAGGCTCTTC | | al. 2002b |
| *AR* | FW: TGCATTCAGAAGGCGGAAG | 800$^\dagger$ | Trakooljul et |
| | RV: GGTTCACAATATGTTCCTG | | al. 2000 |
| *FST* | FW: AGTAGCTTTCTGCCTAGTGGATGC | 700$^\dagger$ | Ellegren et al. |
| | RV: CTACAGAAAGTTAACATGAGGAAT | | 1993 |
| *INHBA* | FW: CTCGTGTTCTCTTACCAGAAGG | 700$^\dagger$ | Campbell et |
| | RV: ACCCAGGTCGTAAGGTATGTC | | al. 2001 |
| *INHBB* | FW: CGCTCCTTTCGAGCACACA | 700$^\dagger$ | *AY116585 |
| | RV: CGTATCTGCCGCTCCTCTTCT | | |
| *ACR* | FW:GAATGATAAGATTGGAGACGAGTTCCC | 800$^\dagger$ | Yasue et al. |
| | RV:CAAATAATTCCTCTTCTTCAACGTGTGTG | | 1999 |
| *ACTG2* | FW: AAACACATCTTCCTCTTCCCTT | 700$^\dagger$ | *U12122 |
| | RV: GGAAGCTCTGCATTGTGGAGTT | | |
| *OPNpro* | FW: CCAATCCTATTCACGAAAAAGCTAG | 800$^\dagger$ | *AF336054 |
| | RV: TCCCACACTTCCCCCTCTG | | |

$^\ddagger$Fragment length, $^\dagger$IDR, *from GenBank: http://www.ncbi.nlm.nih.gov/PubMed/.

## 3.3 Methods

### 3.3.1 Evaluation of sperm quality

Sperm samples were evaluated with standard procedures according to the guidelines of the World Health Organization in the laboratory of an AI Boar station. The method of evaluation was described by Kevin J. Rozeboom in animal science facts: ANS00-812S.

(1) Semen volume and sperm concentration

After collection of boar semen, assessment of ejaculate was performed by visual and olfactory perceptions. Semen volume and sperm concentration were determined with the balance and hemacytometer or photospectrometer.

(2) Sperm motility

Sperm motility was detected in a 1:10 dilution of semen. When the semen extender was prepared it was gently rotated. Then, a small sample (5 to 10 ml) was removed with a small disposable plastic pipette and placed in a clean glass test tube that was warmed to 36 to 37 °C (body temperature) in small water bath. A small drop was placed on a pre-warmed slide and a cover slip was gently placed over the drop. Immediately, the sample was examined in a self illuminating microscopy (capable of 100×, 400×, magnification and glass slides with coverslip). The percentage of sperm that are progressively was estimated in a field, forward moving. The percentage of sperm in 5 fields (in 4 corners and a middle field) had been estimated and an average was established. The estimate in 5% or 10% units was recorded.

(3) Sperm morphology

Evaluation of sperm morphology was carried out after the motility estimate was completed and sperm activity stooped, by switching to the 400× objective and observing individual cells in 5 fields. The percentage of cells was estimated that were "normal" or "abnormal".

(4) Acrosome integrity

A 1:1 dilution of semen and a mixture (1:1) of formal saline and Acrosome stain on a glass slide with the saline (Naphthol yellow or erythrocin stain) were prepared. One or two drops of semen and 1-2 drops of the stain mixture were placed on a glass slide and mixed gently with the tip of the pipette. The edge of a second slide was used to draw the mixture across the flat slide to produce a thin layer. The slide was allowed to air dry. After that, a drop of microscope immersion oil was placed under the slide in self illuminating phase contrast microscope (capable of 100×, 400×, 1000× [oil] magnification) and viewed first at 10× to focus, and then switched to either 40× or 100× to view individual cells. The percentage of cells in 5 fields was estimated that were subjectively scored into classes "normal" or "abnormal".

3.3.2 Molecular genetic methods

The basic molecular genetic methods in this experiment included DNA isolation, detection of polymorphism and genotyping by means of PCR-RFLP, PCR-SSCP, and microsatellite.

3.3.2.1 DNA isolation

In order to extract genomic DNA, sperm samples were used after defrosting from -20 °C. Sperm samples of 0.5 ml were mixed with 4 ml 0.9% sodium chloride solution, centrifuged at 5000× g for 10 minutes. The supernatant was discarded. The pellets were resuspended in 4 ml of digestion buffer. In order to digest protein in the pellet suspension, 4 ml lysis buffer containing proteinase K, SDS and mercaptoethanol were added and the samples were incubated at 37 °C overnight. An equal volume of phenol-chloroform (1:1 v/v) was added and the two phases were mixed until an emulsion was formed. The two phases were separated by centrifugation at 5000× g for 10 minutes. The aqueous supernatant solution was collected in fresh sterilized tubes. Phenol-chloroform extraction was repeated again. One-tenths volume of 3.0 M sodium acetate (pH 5.2) and an equal volume of isopropanol were added and the samples were shaken

gently until precipitation of DNA. The DNA was washed with 70 % ethanol and dried. Finally the DNA was dissolved with 1 ml TE-buffer and kept at 4 $^o$C. After dissolution of DNA (at least overnight), 10 µl was taken and diluted by the addition of 990 µl of TE-buffer. The mixture was swirled gently and the absorbance of this DNA dilution was measured at 260 and 280 nm wavelength in Spectrophotometer UV/visible light (DU$^®$-62). The concentration of the DNA was calculated with the formula:

Concentration of DNA (µg/ml) = $OD_{260nm} \times 50 \times 100$

OD:    optical density

50:     $1 OD_{260} = 50$ µg/ml

100:    diluting factor for measurement is 1:100

Meanwhile, the DNA quality was checked with formula: Ratio = $OD_{260nm}$: $OD_{280nm}$ and agarose gels 0.5% (W/V) containing 0.8 µg/ml ethidium bromide to run electrophoresis in 1× TAE-buffer. Finally, good quality DNA were taken out and diluted in 100 µl (50 ng/µl) with deionized and demineralized (Millipore) water and stored at the temperature of 4 °C.

3.3.2.2 Primer design

A total of 7 pairs of primer were designed from the published sequence (*Sus scrofa*) using Primer Express Soft Ware (version 2.0, ABI), to amplify amplicon length ranging from 220 to 2750 bp with 45 to 70 % GC content and a primer melting temperature ranging from 56 to 65 °C.

3.3.2.3 Polymerase chain reaction (PCR)

PCR amplification was performed in 25 µl reaction volume in 1× PCR-buffer (GeneCraft or Sigma), containing 2 µl of 100 ng genomic DNA templates, 0.5 µl of each primer (10 µM), 0.5 µl of each dNTPs (10 mM), and 0.5 µl of *Taq* polymerase (GeneCraft or Sigma). The PCR reactions were carried out in a Thermocycler. The used thermal cycling programs depend on the primer, length of amplified fragment, 'GC'

content and structure of fragment, etc. For each locus the thermal cycling program was described in Table 3.2. Five microliters of PCR product were electrophoresed in 1% (w/v) agarose gels in 1× TAE containing ethidium bromide and documented.

3.3.2.4 Sequencing of PCR fragments

Sequencing of PCR fragments consisted of PCR product purification, ligation, transformation, screening of transformants, M13 PCR and sequencing.

(1) PCR product purification

PCR reaction (20 μl) was separated by electrophoresis in 1% (w/v) agarose gels in 1× TAE-buffer containing 0.8 μg/ml ethidium bromide. PCR fragments were excised from the gels and homogenized in 600 μl of TE-buffer. Six hundred microliters of phenol-chloroform (1:1 v/v) were added and mixed. After centrifugation at 14,000 g for 10 minutes at 4 $^{o}$C, supernatant aqueous solution was transferred into a new tube, 1:10 volume of sodium acetate (3.0 M, pH 5.2) and equal volume of cooled ethanol were added. The solution was shaken gently and kept at -80 $^{o}$C for 30 minutes. The DNA pellet was precipitated by centrifugation at 14,000 g for 30 min at 4 $^{o}$C, washed with 75% ethanol, dried at room temperature, resuspended in 10 μl of water and kept at −20 $^{o}$C.

(2) Ligation

Ligation of PCR fragments into plasmid vector pGEM$^{®}$–T (Promega) was carried out in 5μl of volume reaction containing 2.5 μl of 2× rapid ligation buffer, 0.5 μl (25 ng) of vector, 0.5 μl of T4 DNA ligase (3 units/μl) and 1.5 μl of PCR product. The reaction was incubated at 4 $^{o}$C overnight.

(3) Transformation

High efficiency competent cells (DH5α *E. coli*) were used for the plasmid transformation. Three µl of the ligation mixture and 60 µl of the thawed competent cells were incubated on ice for 30 min. Thereafter the cells were heat-shocked for 1.5 minutes in a water bath at exactly 42 $^o$C followed by immediate cooling on ice for 2 minutes. Then, 650 µl of LB-broth-ampicillin was added to the mixture and incubated for one and half hour at 37 $^o$C with shaking (~ 110 rpm). After that, the transformation culture was inoculated in duplicate in previous prepared LB/ampicillin/IPTG/X-Gal medium, which contained 20 µl of X-Gal solution and 20 µl of IPTG solution. Then the plates were incubated overnight at 37 $^o$C.

(4) Screening of transformants

Screening of transformants for the insert were done by picking of two white colonies from each plate in 30 µl of 1× PCR-buffer (Sigma) solution for M13 PCR for further confirmation of the transformation success.

(5) M13 PCR

M13 PCR was performed to confirm transformation success and to identify the clones with the insert. Four independent white colonies, expected to contain an insert, for each sample along with two blue colonies as control have been picked up in 30 µl of 1× PCR-buffer (Sigma) solution and left to boil at 95 $^o$C for 15 min. M13 PCR has been carried out in 20 µl reaction containing 1 µl 10× buffer, 0.5 µl dNTP (10 mM), 0.5 µl forward primer (10 µM) (5′-GCTATTACGCCACTGGCGAAAGG-3′), 0.5 µl reverse primer (10µM) (5′-CCCCAGGCTTTACACTTTATGCTTCC-3′), 0.5 U Taq polymerase and 10 µl of the colony lysate. The reaction was carried out at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 sec, 65 °C for 30 sec, 70 °C for 1 minute and a final extension step for 5 minutes at 70 °C. The PCR products were loaded on 1% (w/v) agarose gel with ethidium bromide in 1× TAE-buffer. The gel was photographed

under an UV-transilluminator. White colonies which showed longer fragments than blue colonies, one for each sample were selected for sequencing.

(6) Sequencing

The positive clones of M13-PCR fragments were sequenced according to the dideoxy chain-termination method (Sanger et al. 1977) using a SequiTherm Excel $^{TM}$ II DNA sequencing Kit (Epicentre Technologies, Biozym). Briefly, 4 sequencing reactions of the different termination mixes (ddATP, ddCTP, ddGTP, ddTTP) were setup for each PCR fragment. Each reaction consist of 1 µl of termination mix and 2 µl of the premix solution (3.6 µl of sequencing buffer, 0.8 pM of 700 IRD-labeled SP6 primer (5´-TAAATCCACTGTGATATCTTATG-3´), 0.8 pM of 800 IRD-labeled T7 primer (5´-ATTATGCTGAGTGATATCCCGCT-3´), 1.5 µl of M13 PCR product and 2.5 units *Taq* polymerase) and PCR was performed for 32 cycles at 95 $^{o}$C for 30 sec, 59 $^{o}$C for 30 sec and 70 $^{o}$C for 60 sec. The sequencing reactions ended with 1.5 µl of the stop buffer and denaturation at 85 $^{o}$C for 5 minutes. This mixed solution was loaded onto 41 cm addition of 6% Sequagel-XR (National Diagnostics, Biozym). Electrophoresis was performed on a LI-COR model 4200 automated DNA sequencer in 1× TBE-buffer at 50 $^{o}$C, 50 W and 1500 V. Sequence data were analysed by using Image Analysis Program, version 4.10 (LI-COR Biotechnology).

3.3.2.5 Screening of polymorphism in *ACTN1* and *ACTN4* gene

To identify polymorphisms of the porcine *ACTN1* and *ACTN4* gene, primers for PCR amplification were designed based on porcine EST sequence (BF442592 and BI338543) from GenBank (Table 3.1). Genomic DNA from two pig breeds (Pietrain and Pietrain × Hampshire) was used as DNA templates for PCR amplification. PCR reactions were performed using the standard PCR protocol and the thermal cycling programs as shown in Table 3.2. The amplified fragments length of *ACTN1* and *ACTN4* gene were about 900 bp and 504 bp.

The DNA sequences of pig breeds were analyzed with software BLAST (Altschul et al. 1997) or software Multiple Sequence Alignment to find out the polymorphisms within these sequences.

### 3.3.2.6 Development of genotyping methods

The observed polymorphisms of *ACTN1* and *ACTN4* genes were analyzed with software Webcutter 2.0. In order to genotype for *ACTN1* and *ACTN4* loci, molecular genetic techniques based on PCR were used to establish protocols for large scale genotyping of animal samples in these genes.

### 3.3.2.7 Genotyping by PCR-RFLP

PCR-RFLP was used to identify polymorphisms for the loci *FSHB*, *PRL*, *PRLR*, *INHA*, *RBP4*, *OPNin6*, *ACTN1* and *ACTN4*. Their primers were described in published literature or derived from published porcine gene sequence (Table 3.1).

(1) PCR amplification

PCR amplifications for *FSHB*, *PRL*, *PRLR*, *RBP4*, *INHA*, *OPNin6*, *ACTN1* and *ACTN4* loci were carried out using standard PCR protocols. For PCR reaction condition see Chapter 3.3.2.2. The thermocyler programs are described in Table 3.2. Five microliters of PCR product were electrophoresed in 1% (w/v) agarose gels in 1× TAE containing ethidium bromide and documented.

(2) Restriction enzyme digestion

After checking the PCR products, they were used for RFLP analysis. The restriction enzyme digestion was carried out in 15 μl of reaction mixture of each sample containing 10 μl of PCR product, 1.5 μl of 10× buffer and 0.15 μl of BSA. The reactions were incubated for at least 4 hours to overnight at 30 °C – 60 °C. The information is shown in Table 3.3.

Table 3.2:       Survey of the thermocyler programs for the PCR amplifications

| Locus | Thermocyler program | Author |
|---|---|---|
| *FSHB* | 94 °C 2´, 30 × (94 °C 1´, 58 °C 1´, 72 °C 1´), 72 °C 5´ | Rohrer et al. 1994 |
| *PRLR* | 95 °C 2´, 40 × (93 °C 30´´, 60 °C 1´, 72 °C 1´), 72 °C 3´ | Rohrer et al. 1998 |
| *INHA* | 95 °C 5´, 40 × (94 °C 30´´, 54 °C 90´´, 72 °C 1´), 72 °C 5´ | Hiendleder et al. 2002 |
| *RBP4* | 94 °C 5´, 35 × (94 °C 30´´, 56 °C 45´´, 72 °C 45´´), 72 °C 5´ | Rothschild et al. 2000 |
| *OPNin6* | 95 °C 2´, 35 × (94 °C 45´´, 65 °C 1´, 72 °C 2´´), 72 °C 7´ | Agnieszka et al. 2001 |
| *ACTN1* | 94 °C 5´, 35 × (94 °C 30´´, 63 °C 30´´, 72 °C 1´), 72 °C 5´ | DEV |
| *ACTN4* | 94 °C 5´, 40 × (94 °C 30´´, 63 °C 1´, 72 °C 1.5´), 72 °C 5´ | DEV |
| *PRL* | 94 °C 3´, 35 × (94 °C 30´´, 62 °C 30´´, 72 °C 3´), 72 °C 10´ | DEV |
| *GnRHR* | 95 °C 3´, 40 × (94 °C 30´´, 60 °C 30´´, 72 °C 30´´), 72 °C 3´ | DEV |
| *LHB* | 95 °C 3´, 35×(94 °C 30´´, 62 °C 30´´, 72 °C 30´´), 72 °C 5´ | DEV |
| *RLN* | 95 °C 3´, 8 × (94 °C 30´´, 65 °C 30´´, 72 °C 30´´),* 25 × (94 °C 30´´, 61 °C 30´´, 72 °C 30´´), 72 °C 5´ | Wimmers et al. 2002b |
| *AR* | 95 °C 3´, 40 × (94 °C 30´´, 60 °C 1´, 72 °C 1´), 72 °C 3´ | Trakooljul et al. 2000 |
| *FST* | 95 °C 3´, 10 × (94 °C 30´´, 60 °C 30´´, 72 °C 30´´),* 30 × (94 °C 30´´, 55 °C 30´´, 72 °C 30´´), 72 °C 5´ | DEV |
| *INHBA* | 95 °C 3´, 35 × (94 °C 30´´, 59 °C 30´´, 72 °C 30´´), 72 °C 5´ | DEV |
| *INHBB* | 95 °C 3´, 35 × (94 °C 30´´, 58 °C 30´´, 72 °C 30´´), 72 °C 5´ | DEV |
| *ACR* | 95 °C 3´, 8 × (94 °C 30´´, 66 °C 30´´, 72 °C 30´´),* 30 × (94 °C 30´´, 62 °C 30´´, 72 °C 30´´), 72 °C 5´ | DEV |
| *ACTG2* | 95 °C 5´, 35 × (94 °C 30´´, 56 °C 30´´, 72 °C 1´), 72 °C 5´ | DEV |
| *OPNpro* | 95 °C 5´, 5 × (94 °C 30´´, 60 °C 30´´, 72 °C 1´),* 30 × (94 °C 30´´, 60 °C 30´´, 72 °C 1´), 72 °C 5´ | DEV |

*Decrement of 0.5°C per cycle, DEV: developed in our laboratory.

Table 3.3:  Candidate gene polymorphisms: Restriction enzymes, recognition sequences, incubation temperature and corresponding fragments

| Locus | Rest. Enz. | Reco.Sequ.(5´→3´) | Tm* | Allele and their length (bp) |
|---|---|---|---|---|
| *FSHB* | *Hae*III | GG\|CC | 37 °C | A: 332+208+84<br>B: 208+173+159+84 |
| *PRL* | *Bst*UII | CG\|CG | 37 °C | A: 1294+1020+439<br>B: 2134+439<br>C: 1459+1294<br>D: 2750 |
| *PRLR* | *Alu*I | AG\|CT | 37 °C | A: 125+110+79+77+67<br>B: 125+90+79+77+67+20 |
| *RBP4* | *Msp*I | CC\|GG | 37 °C | A: 190+154+136<br>B: 154+136+125 |
| *INHA* | *Hin*6I | G\|CGC | 37 °C | A: 262+245+139+80<br>B: 245+182+139+80+80 |
| *OPNin6* | - | - | - | A: 1300<br>B: 1000 |
| *ACTN1* | *Bst*EII | G\|GTNACC | 60 °C | A: 744+189<br>B: 933 |
| *ACTN4* | *Apa*I | GGGCC\|C | 30 °C | A: 323+191<br>B: 323+115+76 |

Tm*: incubation temperature

(3) Detection of DNA fragments

Detection of restriction fragment length polymorphisms (RFLPs) of *FSHB*, *PRL*, *OPNin6*, *ACTN1* and *ACTN4* was carried out by electrophoresis in 1-3% (w/v) agarose gels in 1× TAE-buffer containing 0.8 μg/ml ethidium bromide. For *PRLR*, *RBP4* and *INHA*, the restriction fragment length polymorphisms were detected with 12% PAA non-denaturing gels in 1× TBA-buffer running for 1-1.5 hours at 450 Volts. DNA bands were visualized by silver staining. Briefly, DNA in polyacrylamide gel was fixed with 10% (v/v) acetic acid for 10 min, 1% (v/v) nitric acid for 10 min and washed twice with

water. The gel was incubated in 0.2% (v/w) silver nitrate solution for 20 min and washed again with water. The gel was washed 2-3 times with silver staining solution and incubated until DNA bands appeared. The reaction was stopped with 10% acetic acid for 1 minute and washed three times with water. The gel was dried in a gel dryer (BioRad) at 80 $^{\circ}$C for 2 hours.

3.3.2.8 Genotyping by PCR-SSCP

In this study, the PCR-SSCP technique was applied to analyse polymorphisms of *GnRHR*, *RLN*, and *LHB* genes. The polymorphisms of *LHB* and *GnRHR* loci were described by Jiang et al. (1999 and 2001, respectively). Their primer sequences were derived from published pig sequence D00579 and AF227686, repectively. The polymorphism, primer sequences and PCR conditions of *RLN* locus were described by Wimmers et al. (2002b). Primers and fragment lengths of these candidate genes are summarized in Table 3.1.

(1) PCR amplification

PCR amplification for *GnRHR*, *RLN* and *LHB* was performed in 10 µl reaction mixtures containing 1.0 µl 10× PCR-buffer (Sigma), 1 µl of 50 ng genomic DNA templates, 0.2 µl of each primer (10 µM) , 0.2 µl of each dNTPs (10 mM) and 0.2 U of *Taq* polymerase (Sigma). Primers are shown in Table 3.1 and the relevant thermocylcer programs are described in Table 3.2.

(2) Electrophoresis

The PCR products were mixed 1:10 with SSCP-loading buffer and denatured at 95 $^{\circ}$C for 5 min and immediately cooled on ice. The products (6 µl) were loaded on 12 % non-denaturing polyacrylamide gels and electrophoresed in 0.5× TBE, constant power (12 W), for 3-4 hours at room temperature; for the *GnRHR* gene, electrophoreses was for 6-7 hours at 4 °C. DNA bands were visualized by silver staining.

### 3.3.2.9 Genotyping by microsatellte

Microsatellite at *AR, FST, INHBA, INHBB, ACR, ACTG2* and *OPNpro* loci were described in published literature or GenBank. Primer sequences of *ACR, ACTG2, OPNpro* and *INHBB* were derived from published pig sequences in GenBank. Primer sequences of *AR, FST* and *INHBA* were described by Trakooljul et al. (2000), Ellegren (1993b) and Campbell et al. (2001), respectively. Information on these primer sequences are given in Table 3.1.

### (1) PCR amplification

In brief, 18-24 bp primers were used to amplify fragments of 93 – 228 bp. One primer was end-labelled with [γ$^{-32}$P] dATP. PCR reactions were made in 10μl reaction volumes with 1.0 μl 10× PCR-buffer (Sigma), 1 μl of 50 ng genomic DNA templates, 0.5 μl of each primer (10 μM), 0.25 μl of each dNTPs (10 mM) and 0.5 U of *Taq* polymerase (Sigma). The PCR conditions are described in Table 3.2.

### (2) Electrophoresis

Both plates were cleaned twice with water and ethanol (75%) and dried with paper tissue. 100 μl of binding silane were applied onto the area of one glass plate, where the comb is inserted. These plates and spacers were assembled and fixed as sandwich with rails. Gel solution containing Sequa Gel XR (SQG-XR-842 MWG, National Diagnostics) 15ml, Sequa Gel Complete Buffer Reagent (National Diagnostics) 3.75 ml, DMSO (Carl Roth GmbH) 200 μl and APS (10% w/v) 150 μl, was swirled gently to mix and penetrated into the plates. A comb was inserted on the gel sandwich and placed in horizontal position allowing polymerization for half to one hour.

After that, PCR products were diluted 1:10 (or 1:15) with dextranblue-buffer and loaded on 6% polyacrymide gel. At the same time size markers (of 75, 100, 105, 120, 145, 175, 200, 204, 230, 255, 300, 320 bp) were loaded in both side lanes and the middle lane. Electrophoresis was performed in 1× TBE-buffer at 50 $^{o}$C, 50 W, 40 mA and maximal 1500 V in a DNA Sequencer (Li-COR, Model 4200, MWG-BIOTECH). Analysis of the

gel image was carried out with the One-Dscan software (SCanalytics, Division of CSPI, USA). At first the standard markers of the gel were calibrated, then determined all appeared alleles of this candidate gene.

3.4 Statistical analysis

3.4.1 Genotype analysis

3.4.1.1 Calculation of Allele and genotype frequencies

Allele and genotype frequencies of candidate genes *FSH, LHB, INHA, PRL, PRLR, GnRHR, RLN, OPNin6, RPB4, ACTN1, ACTN4, AR* within breed were manually calculated.

The allele and genotype frequencies of microsatellite markers *FST, ACR, ACTG2, INHBA, INHBB* and *OPNpro* within breed were computed with software Genepop 3.3 option 5 described by Raymond and Rousett (1994).

3.4.2.2 Test of Hardy-Weinberg equilibrium

(1) Diallelic markers

For diallele makers *FSHB, LHB, INHA, PRLR, GnRHR, RLN, OPNin6, RBP4, ACTN1* and *ACTN4* in Hardy-Weinberg equilibrium within breed were manually revealed by Chi-square test.

(2) Multiple allelelic markers

In order to test genetic variation at individual loci within breed, heterozygote degree for the multiple allele marker *FST, ACR, ACTG2, INHBA, INHBB* and *PRL* were determined. The observed (*Ho*) and expected heterozygote degree of individual loci were calculated according to the following formula described by Nei (1987).

Observed heterozygote degree ($H_o$):

$$H_o = \frac{1}{N}\left[\sum_{i=1}^{k}\sum_{j=i+1}^{k}A_iA_j\right]$$

$k$:　　number of allele

$A_iA_j$:　number of animals with genotype $A_iA_j$

$N$:　　sum of animals

Expected heterozygote degree ($H_e$):

$$H_e = \frac{2N\left(1 - \sum_{i=1}^{k}P_i^2\right)}{2N - 1}$$

$k$:     number of allele

$P_i$:   fequency of the $i$-th allele

$N$:     sum of animals


Test of Hardy-Weinberg equilibrium for these markers within population was carried out with software GENEPOP Version 3.3 option 1 (Raymond and Rousett 1994).


3.4.2 Association analysis between candidate genes and boar reproductive traits


3.4.2.1 Association analysis for fertility traits


The fertility traits: non return rate (NRR [%]) and number of piglets born alive (NBA [pig/litter]) were only available as an average of the mated sows. The statistical fixed model comprises the effects of breed, boar born year and genotype.


Model 1 for association analysis for boar fertility traits (NRR, NBA)

$$y_{ijkl} = \mu + breed_i + genotype_j + BY_k + \varepsilon_{ijkl}$$


where:

$y_{ij}$:            = boar fertility traits (NRR, NBA)

$\mu$:             = population mean for boar fertility traits

$breed_i$ :        = fixed effect of breed (i= Pi, Pi×Ha)

$genotype_j$:     = fixed effect of genotype (see table 4a, 4b)

$BY_k$:           = fixed effect of boar birth year (k=1-3: <96, 96/97, 98/99)

$\varepsilon_{ijkl}$:           = residual


This analysis was performed with the procedure "PROC GLM" of the SAS software package (SAS System for Windows, release 8.02).

Breed

Breed was classified into PI and PI×HA in the statistical model, the observed sample size 244 of PI boars and 122 of PI×HA boars.

Boar birth year

For boar birth year effect, three classes were established, a class with all boars born before 1996, another class with boars born between 1996 and 1997, and other with boars born between 1998 and 1999. By this organization 356 boars were distributed as population: first class 49, second class 137, third class 170.

Genotype

The effects of single genotypes at the 18 gene loci were separately estimated, these occupied genotypes of gene loci in statistical analysis were summarized.

The genotypes of *FSHB*, *PRLR*, *OPNin6*, *ACTN1* and *ACTN4* were divided into three classes AA, AB and BB (Table 3.4). Genotypes of *LHB* were classified CC, CT and TT. The genotypes of *RBP4* were classified AA and AB, Genotypes of *GnRHR* were classified CC and CG, for *RLN* locus CC and CA genotypes. The genotype BB of *RBP4*, GG of *GnRHR* and AA of *RLN* were excluded due to being very rare or absent in the boar population. Genotypes of *PRL* locus were classified AA AB AC, BC, and CC. Genotype of *AR* locus were divided into five classes `196´, `193´, `186´, `173´, and `172´, genotype `193´ was excluded from the statistical analysis. The used genotypes in statistical analysis are shown in Table 3.4 and Table 3.5.

Table 3.4:     The use of genotypes for the loci *FSHB*, *PRLR*, *RBP4*, *INHA*, *OPNin6*, *ACTN1* and *ACTN4* in the statistical analysis

| Genotype | *FSHB* (n) | *PRLR* (n) | *RBP4* (n) | *INHA* (n) | *OPNin6* (n) | *ACTN1* (n) | *ACTN4* (n) |
|---|---|---|---|---|---|---|---|
| AA | 245 | 237 | 85 | 4 | 80 | 202 | 123 |
| AB | 97 | 81 | 271 | 123 | 118 | 108 | 156 |
| BB | 14 | 38 | - | 229 | 158 | 18 | 49 |

Table 3.5: The use of genotypes for the loci *GnRHR*, *RLN*, *LHB*, *AR* and *PRL* in the statistical analysis

| *GnRHR* | | *RLN* | | *LHB* | | *AR* | | *PRL* | |
|---|---|---|---|---|---|---|---|---|---|
| Genotype | n | Genotype | n | Genotype | n | Genotype | n | Genotype | n |
| CC | 285 | CC | 302 | CC | 133 | *196* bp | 170 | AA | 70 |
| GC | 68 | CA | 53 | CT | 161 | *186* bp | 132 | AB | 61 |
| | | | | TT | 62 | *173* bp | 26 | AC | 117 |
| | | | | | | *172* bp | 26 | BC | 5 |
| | | | | | | | | CC | 53 |

For six microsatellite markers *OPNpro*, *ACTG2, FST, IHBB, INHA,* and *ACR*, when an allelic frequency was less than 5%, the allele was replaced by a pseudo allele 999 in statistical analysis. If a genotype frequency was less than 1% in a population, then genotype was removed from the statistical calculation. The uses of genotypes of these microsatellite markers are shown in Table 3.6 and Table 3.7.

For microsatellite marker at *OPN* gene, the rare allele 126, 130, and 138 were replaced by a pseudo allele 999. The rare genotypes 118/999, 146/999, and 999/999 were excluded from statistical analysis. In PI population, genotype 118/999 and 142/999 were excluded. In PI×HA crossbred population, only allele 130 was less than 5% and automatically replaced by a pseudo allele 999, but genotype 118/126, 118/138, and 138/146 were excluded from statistical analysis.

For the *ACTG2* locus, a pseudo allele 999 replaced allele 95, 97, 105, 113, 115, and 119. In PI×HA population allele 97 was included because the frequency was more than 5%. The rare genotypes 103/117 and 117/117 were removed from statistical analysis. Genotype 117/117 in PI population and genotypes 93/117 93/999, 97/97, 103/117, 103/999, 111/111, 111/117, and 117/117 in PI×HA population were excluded from statistical analysis.

For the microsatellite marker at *ACR* gene, the allele 186, 196, 210, 212, 216, 218, 224, and 226 were replaced by a pseudo allele 999. The allele 228 in PI population and allele 214 in PI×HA population were also replaced by a pseudo allele 999. But allele 226 and 228 in PI×HA population were included in statistical analysis. The genotype 202/228,

214/999 and 999/999 were removed from statistical analysis. Genotype 999/999 in PI population was also excluded from statistical analysis.

For the *FST* locus, the allele 140, 141, 145, 146, and 149 were replaced by a pseudo allele 999, but allele 146 in PI population had a frequency of more than 5% and was included in the statistical analysis. The genotype 142/143 was excluded from the statistical analysis. The rare genotype 142/143, 143/999, 146/148, and 146/999 in PI population, genotype 144/999 and 148/148 in PI×HA population were removed from statistical calculation.

Table 3.6: The use of genotypes for the loci *OPNpro*, *INHBB*, *FST* and *ACTG2* in the statistical analysis

| *OPNpro* | | *INHBB* | | *FST* | | *ACTG2* | |
|---|---|---|---|---|---|---|---|
| Genotype | n | Genotype | n | Genotype | n | Genotype | n |
| 118/118 | 14 | 161/163 | 10 | 142/142 | 40 | 93/93 | 10 |
| 118/142 | 34 | 161/171 | 20 | 142/147 | 18 | 93/103 | 12 |
| 118/146 | 8 | 161/173 | 6 | 142/148 | 12 | 93/109 | 37 |
| 118/148 | 46 | 163/171 | 12 | 142/999 | 8 | 93/111 | 11 |
| 142/142 | 36 | 163/173 | 6 | 143/143 | 65 | 91/117 | 5 |
| 142/146 | 17 | 163/175 | 6 | 143/147 | 18 | 93/999 | 9 |
| 142/148 | 83 | 163/999 | 6 | 143/148 | 50 | 103/103 | 10 |
| 148/999 | 13 | 171/171 | 87 | 143/999 | 11 | 103/109 | 45 |
| 146/146 | 8 | 171/173 | 73 | 144/144 | 15 | 103/111 | 21 |
| 146/148 | 13 | 171/175 | 53 | 143/147 | 9 | 103/999 | 18 |
| 148/148 | 51 | 171/999 | 16 | 143/148 | 13 | 109/109 | 32 |
| 148/999 | 26 | 173/173 | 14 | 143/999 | 6 | 109/111 | 41 |
| | | 173/175 | 24 | 147/147 | 30 | 109/117 | 13 |
| | | 175/175 | 13 | 147/999 | 10 | 109/999 | 20 |
| | | 175/999 | 4 | 148/148 | 22 | 111/111 | 16 |
| | | | | 148/999 | 10 | 111/117 | 9 |
| | | | | 999/999 | 18 | 111/999 | 24 |
| | | | | | | 117/999 | 7 |
| | | | | | | 999/999 | 13 |

Table 3.7:     The use of genotypes for the loci *INHBA* and *ACR* in the statistical
               analysis

| *INHBA* | | | | *ACR* | |
| --- | --- | --- | --- | --- | --- |
| Genotype | n | Genotype | n | Genotype | n |
| 252/252 | 208 | 202/202 | 10 | 208/214 | 23 |
| 252/254 | 83 | 202/208 | 53 | 208/228 | 40 |
| 252/256 | 34 | 202/214 | 15 | 208/999 | 72 |
| 252/999 | 14 | 202/999 | 17 | 214/214 | 6 |
| 254/254 | 9 | 208/208 | 101 | 214/228 | 4 |
| | | | | 228/999 | 8 |

For microsatellite marker at *INHBA* locus, a pseudo allele 999 was used instead of the
allele 248, 258, and 262. Allele 256 in PI population was also replaced by a pseudo
allele 999. The rare genotype 254/256, 254/999, 256/256 and 256/999 were excluded
from statistical analysis. Genotype 999/999 in PI population and genotype 254/256,
256/256, and 256/999 were removed from statistical calculation.

At locus *INHBB*, allele 159 was less than 5% and automatically replaced by a pseudo
allele 999 in statistical calculation. Allele 159, 161, and 163 in PI population and allele
175 in PI×HA population were replaced by a pseudo allele 999. The rare genotype
161/175, 163/163, and 173/999 were removed from statistical analysis. The rare
genotype 999/999 and genotype 161/999 and 163/163 in PI×HA population were also
excluded from statistical calculation.

(1) Estimation of additive and dominance effects on fertility traits

The additive and dominance effects on the fertility traits non return rate and number of
piglets born alive were evaluated if the effect of genotypes of a marker on the traits
were significant. The additive effect was estimated by comparison of the means of the
traits value for homozygote. i.e. $a = ½(BB-AA)$. The dominance effect was expressed as
the deviation of means of heterozygote from the means of both homozygote genotype
means. i.e. $d = AB - ½(AA+BB)$. The estimation of the effect was tested by t-test on
significant deviation from zero. In this study, the additive and dominance effects were

estimated for the PCR-RFLP/SSCP markers except for loci *GnRHR*, *RLN* and *RBP4* because only two genotypes of these markers emerged in the population. For the *PRL* locus additive and dominance effect were evaluated only between allele A and C due to absence of homozygote BB in this study.

(2) Estimation of allele substitution effect on fertility traits

For microsatellite markers *OPNpro*, *FST*, *ACTG2*, *ACR*, *INHBA* and *INHBB,* allele substitution effects of their frequent alleles were evaluated. The following frequent alleles were included in allele substitution effect analysis: allele 118, 142, 146, and 148 at locus *OPNpro*; allele 142, 143 144, 147, and 148 at locus *FST*; allele 93, 103, 109, 111, and 117 at locus *ACTG2*; allele 202, 208, 214, and 228 at locus *ACR*; allele 252, 254, and 256 at locus *INHBA*; allele 161, 163, 171, 173, and 175 at locus *INHBB*. Locus *AR* located on SSCX (Seifert et al., 1999) was excluded in estimates of the allele substitution effect. The allele substitution effects showed whether the breeding value of fertility traits NBA and NRR would change if the frequency of the single allele was increased by selective inseminating. Allele substitution effects were tested by multiple linear regression analysis with different covariables. These analyses revealed whether the relevant allele positively or negatively influenced the fertility traits of boars that was homozygote or heterozygote for this allele. The statistical analysis was carried out using the procedure "PROC GLM" of the SAS software package.

Model 2 for estimation of allele substitution effect on boar fertility traits

$$y_{ijk} = \mu + breed_i + b_j.allele_{ijk} + BY_k + \varepsilon_{ijk}$$

where:

$y_{ijk}$ = boar fertility traits (NRR, NBA)

$\mu$ = population mean for boar fertility traits

$breed_i$ = fixed effect of breed (i= 1-2:PI, PI×HA)

$allele_{ijk}$ = the frequency of allele by the ijk-th boar

$b_j$ = linear regression coefficient for all alleles (j: see Table 3.6)

$BY_k$ = fixed effect of boar birth year (k=1-3: <1996, 96/97, 98/99)

$\varepsilon_{ijk}$ = residual

For the classes of breeds and boar birth year used in this model for statistical analysis see model 1. The used covariables in this model are shown in Table 3.8.

Table 3.8:     The use of covariables for the microsatellite markers *ACR*, *ATG*, *FST*, *INHBA*, *INHBB* and *OPNpro*

| Locus | *ACR* | *ACTG2* | *FST* | *INHBA* | *INHBB* | *OPNpro* |
|-------|-------|---------|-------|---------|---------|----------|
| j     | 4     | 5       | 5     | 3       | 5       | 4        |

   j: covariable

3.4.2.2 Association analysis for sperm quality traits

Analyses of variance were performed with the procedure "PROC MIXED" of the SAS software package to address the effects on sperm quality including sperm concentration (SCON [$\times 10^8$/ml]), sperm motility (MOT [%]), semen volume per ejaculate (VOL [ml]), plasma droplets rate (PDR [%]) and abnormal spermatozoa rate (ASR [%]). Statistical analysis were done with a mixed model with the fixed effects of breed, collected season (eight seasons within two years), age (covariable), genotype of boars and random permanent effect of the boar (repeated measurement).

Model 3 for association analysis for sperm quality traits:

$$y_{ijhlm} = \mu + a_i + b_{ijhlm} + c_h + d_l + animal_{ijhlm} + \varepsilon_{ijhlm}$$

Whereas:

$y_{ijhlm}$:          = sperm quality traits (SCON, VOL, MOT, PDR, ASR)

$\mu$ :               = population mean of boar sperm quality traits

$a_i$ :               = fixed effect of breed (i=Pi; Pi×Ha)

$b_{ijhlm}$:          = fixed effect of boar age (covariable)

$c_h$ :               = fixed effect of season (h=1- 8)

$d_l$ :               = fixed effect of genotype ( table X)

$animal_{ijhlm}$:     = permanent environmental effect of the ijhlm-th boar (random)

$\varepsilon_{ijhlm}$: = residual

Boar age

Boar age was derived from the difference between sperm collection date and boar birth date in days. This factor was covariable in statistical model 3.

Breed

For two classes of breed PI and PI×HA, 40,112 repeated measurements of PI breed and 12,413 repeated measurements of PI×HA breed were used in statistical analysis.

Season

Sperm collection season were quarter years from January 2000 to December 2001, also 8 seasons were used in statistical analysis as described in Table 3.9.

Table 3.9:      The sample numbers for season classes in the statistical analysis

| Classes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---------|------|------|------|------|------|------|------|------|
| A | 7432 | 7788 | 7773 | 7077 | 6425 | 5856 | 5521 | 4653 |

A: observed sample number

Genotype

The candidate genes *FSHB*, *PRL*, *INHA*, *OPNin6*, *ACTN1* and *ACTN4* were classified in three genotypes AA, AB and BB. Locus *RBP4* was classified in AA and AB genotypes. Candidate genes *GnRHR* and *RLN* were divided into CC and GC, CC and CA, respectively. *LHB* was classified in CC, CT, and TT three genotypes. *AR* and *PRL* were classified into four genotypes 196, 186, 173, 172 and five genotypes AA, AB, AC, BC, CC, respectively. The observed numbers for genotypes are shown in Table 3.10 and Table 3.11.

Table 3.10:     The observed numbers for genotypes of the loci *FSHB*, *PRLR*, *RBP4*, *INHA*, *OPNin6*, *ACTN1* and *ACTN4* in the statistical analysis

| Genotype | *FSHB* (N*) | *PRLR* (N*) | *RBP4* (N*) | *INHA* (N*) | *OPN* (N*) | *ACTN1* (N*) | *ACTN4* (N*) |
|----------|------|------|------|------|------|------|------|
| AA | 32999 | 31198 | 12444 | 524 | 11494 | 26361 | 16250 |
| AB | 13077 | 11162 | 35405 | 15807 | 15566 | 15085 | 20823 |
| BB | 1814 | 5590 | - | 31500 | 20931 | 2466 | 6914 |

*N = number of animal genotypes × semen samples

Table 3.11:    The observed numbers for genotypes of the loci *GnRHR*, *RLN*, *LHB*, *AR* and *PRL* in the statistical analysis

| GnRHR | | RLN | | LHB | | AR | | PRL | |
|-------|------|------|------|------|------|--------|------|------|-------|
| Geno[1] | N* | Geno | n | Geno | n | Geno | n | Geno | n |
| CC | 38819 | CC | 40222 | CC | 18004 | *196* bp | 24470 | AA | 8939 |
| GC | 8531 | CA | 7600 | CT | 20895 | *186* bp | 15783 | AB | 8078 |
| | | | | TT | 8950 | *173* bp | 3297 | AC | 16007 |
| | | | | | | *172* bp | 4071 | BC | 803 |
| | | | | | | | | CC | 7830 |

[1]Geno: genotype; *N = number of animal genotypes × semen samples

For microsatellite markers *OPNpro, ACR, FST, ACTG2, INHBA* and *INHBB*, alleles were replaced by a pseudo alllele 999 when the allele frequency was less than 5% within the boar population. For genotypes at these markers, the numbers of observations in statistical calculation are shown in Table 3.12 and 3.13.

Table 3.12:    The number of observations for genotypes of the loci *ACTG2* and *INHBA* in the statistical analysis

| ACTG2 | | | | | | INHBA | |
|----------|------|----------|------|----------|------|----------|-------|
| Genotype | N* | Genotype | N* | genotype | N* | genotype | N* |
| 93/93 | 405 | 97/103 | 912 | 109/109 | 989 | 252/252 | 29001 |
| 93/97 | 497 | 97/109 | 175 | 109/111 | 1112 | 252/254 | 10853 |
| 93/109 | 1662 | 97/111 | 1103 | 109/117 | 490 | 252/256 | 4348 |
| 93/111 | 524 | 97/117 | 203 | 109/999 | 156 | 252/999 | 1428 |
| 93/117 | 205 | 97/999 | 473 | 111/117 | 50 | 254/254 | 1250 |
| 93/999 | 138 | 103/109 | 1582 | 111/999 | 277 | 254/256 | 503 |
| 97/97 | 163 | 103/117 | 178 | 117/999 | 296 | 254/999 | 206 |
| | | 103/999 | 159 | 999/999 | 183 | 256/256 | 282 |
| | | | | | | 256/999 | 121 |

*N = number of animal genotypes × semen samples

Table 3.13:    The number of observations for genotypes of the loci *ACR*, *FST*, *INHBB* and *OPNpro* in the statistical analysis

| *ACR* | | *OPNpro* | | *FST* | | *INHBB* | |
|---|---|---|---|---|---|---|---|
| Genotype | n | Genotype | N* | Genotype | N* | Genotype | N* |
| 202/202 | 1469 | 118/118 | 1749 | 142/142 | 5407 | 161/163 | 1303 |
| 202/208 | 8236 | 118/142 | 4557 | 142/143 | 59 | 161/171 | 2334 |
| 202/214 | 2202 | 118/146 | 1056 | 142/147 | 1877 | 161/173 | 682 |
| 202/228 | 389 | 118/148 | 6469 | 142/148 | 1422 | 161/175 | 128 |
| 202/999 | 2262 | 118/999 | 189 | 142/999 | 1146 | 163/163 | 234 |
| 208/208 | 14008 | 142/142 | 4953 | 143/143 | 8477 | 163/171 | 1287 |
| 208/214 | 3149 | 142/146 | 1811 | 143/147 | 2075 | 163/173 | 740 |
| 208/228 | 4549 | 142/148 | 11647 | 143/148 | 6820 | 163/175 | 831 |
| 208/999 | 8112 | 142/999 | 1365 | 143/999 | 1514 | 163/999 | 750 |
| 214/214 | 1089 | 146/146 | 1104 | 144/144 | 2029 | 171/171 | 11717 |
| 214/228 | 502 | 146/148 | 1968 | 144/147 | 1162 | 171/173 | 10399 |
| 214/999 | 308 | 146/999 | 165 | 144/148 | 2047 | 173/175 | 8162 |
| 228/999 | 1217 | 148/148 | 7618 | 144/999 | 867 | 173/999 | 1642 |
| 999/999 | 500 | 148/999 | 2843 | 147/147 | 3755 | 173/173 | 1605 |
| | | 999/999 | 294 | 147/999 | 1506 | 173/175 | 3548 |
| | | | | 148/148 | 3613 | 173/999 | 126 |
| | | | | 148/999 | 1650 | 175/175 | 2098 |
| | | | | 999/999 | 2556 | 175/999 | 406 |

*N = number of animal genotypes × semen samples

(1) Estimation of additive and dominance effect on sperm quality traits

For the evaluation of genetic effects on sperm quality traits at candidate genes *FSHB*, *PRL*, *PRLR*, *INHA*, *OPNin6*, *LHB*, *ACTN1*, and *ACTN4*, when a marker was significantly associated with any of sperm quality traits (SCON, VOL, MOT; PDR, and ASR), the additive and dominance effects on sperm quality traits were estimated.

(2) Estimation of allele substitution effect on sperm quality traits

In order to elucidate genetic effects of microsatellite markers *FST, ACR, ACTG2, INHBA, INHBB*, and *OPNpro*, the allele substitution effects on sperm quality traits were estimated if any of these six microsatellite markers was significantly associated with any of the sperm quality traits (SCON, VOL, MOT, PDR, and ASR). The frequent alleles at the above mentioned markers are referred in Table 3.8. Allele substitution effects on sperm quality traits were evaluated using multiple linear regression analysis with different allele covariables. Statistical analysis was carried out using the procedure "PROC MIXED" of the SAS software package with the following model.

Model 4 for estimation of allele substitution effect on sperm quality traits

$$y_{ijhlm} = \mu + a_i + b_{ijhlm} + c_h + d_l allelel_{ijhlm} + animal_{ijhlm} + \varepsilon_{ijhlm}$$

Whereas:

| | |
|---|---|
| $y_{ijhlm}$: | = sperm quality traits (SCON, VOL, MOT, PDR, ASR) |
| $\mu$: | = population mean of boar sperm quality traits |
| $a_i$ : | = fixed effect of breed (i=PI; PI×HA) |
| $b_{ijhlm}$: | = fixed effect of boar age (covariable) |
| $c_h$ : | = fixed effect of season (h=1- 8) |
| Allele$_{ijhlm}$ : | = frequency of the relevant allele by the ijhlm-th boar |
| $d_l$ : | = linear regression coefficient for all allele (l: see Table 3.8) |
| animal$_{ijhlm}$: | = permanent environmental effect of the ijhlm-th boar (random) |
| $\varepsilon_{ijhlm}$: | = residual |

Classes of breed, boar age and sperm collection season are shown in the chapter 4.3.2.2.

# 4 Results

## 4.1 Genotyping of candidate genes

### 4.1.1 Detection of polymorphisms in *ACTN1* and *ACTN4* genes

Polymorphisms in *ACTN1* and *ACTN4* were detected in different pig breeds by comparative sequencing. PCR-RFLP technique was established in order to genotype animal samples.
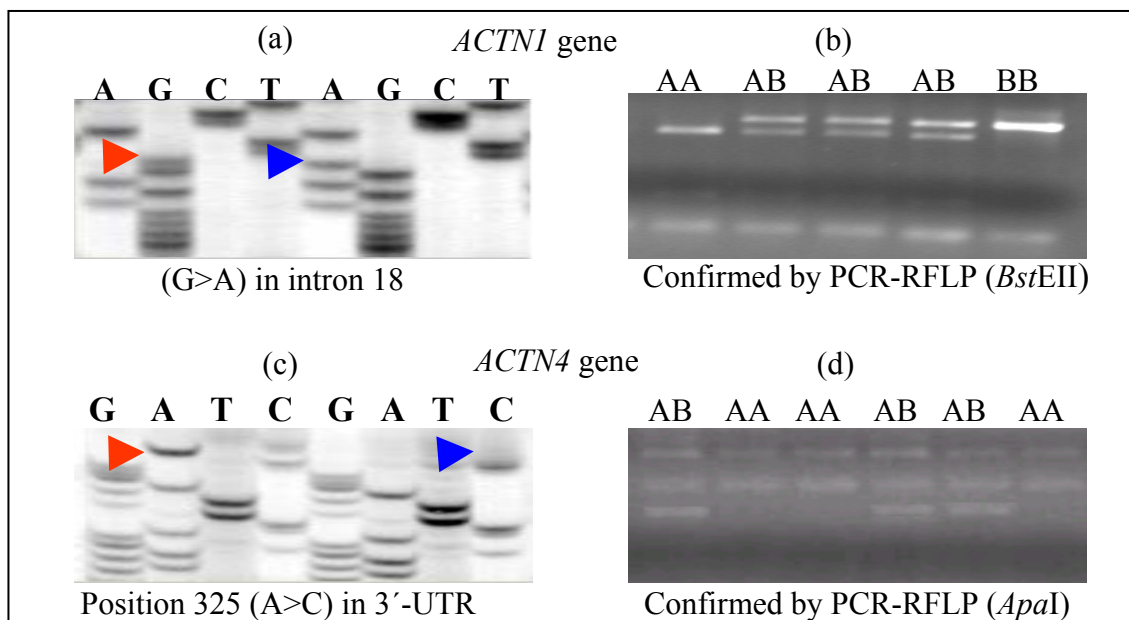


Figure 4.1:    Sequence analysis of *ACTN1* and *ACTN4* gene revealing single nucleotide polymorphisms (SNPs)

All ESTs of the porcine actinin alpha 1 and actinin alpha 4 genes from GenBank (http://pigest.genome.iastate.edu/query) were applied to detect polymorphisms. In accordance with pig sequence BF442592 and BI338543, two fragments were obtained from porcine sperm genomic DNA of several individual boars of breeds Pietrain and Pietrain × Hampshire crossbred. One polymorphism at position 189 in intron 18 of the porcine *ACTN1* gene was detected. The SNP was a transition from guanine (G) to

adenine (A) and confirmed by PCR-RFLP with restriction enzyme *Bst*EII [Figure 4.1 (a, b)]. An other polymorphism at position 396 in the 3´-UTR of the porcine *ACTN4* gene was detected. The SNP was a transition from adenine (A) to cytosine (C) and confirmed by PCR-RFLP with restriction enzyme *Apa*I as illustrated in Figure 4.1 (c, d).

4.1.2 Determination of genotypes

In total, eighteen candidate gene loci were investigated. Eight candidate genes *FSHB*, *PRL*, *PRLR*, *INHA*, *RBP4*, *OPNin6*, *ACTN1*, and *ACTN4* were genotyped by means of PCR-RFLP (Figure 4.2), genotypes of *ACTN1* and *ACTN4* are shown in Figure 4.1 (b, d). Three candidate genes *GnRHR*, *LHB*, and *RLN* were genotyped with PCR-SSCP (Figure 4.3) and in seven microsatellite markers *AR*, *FST*, *INHBA*, *INHBB*, *ACR*, *ACTG2*, *OPNpro*, the length polymorphisms of the repetitive sequences were determined using a *LI-COR* DNA sequencer model 4200 (Figure 4.4).
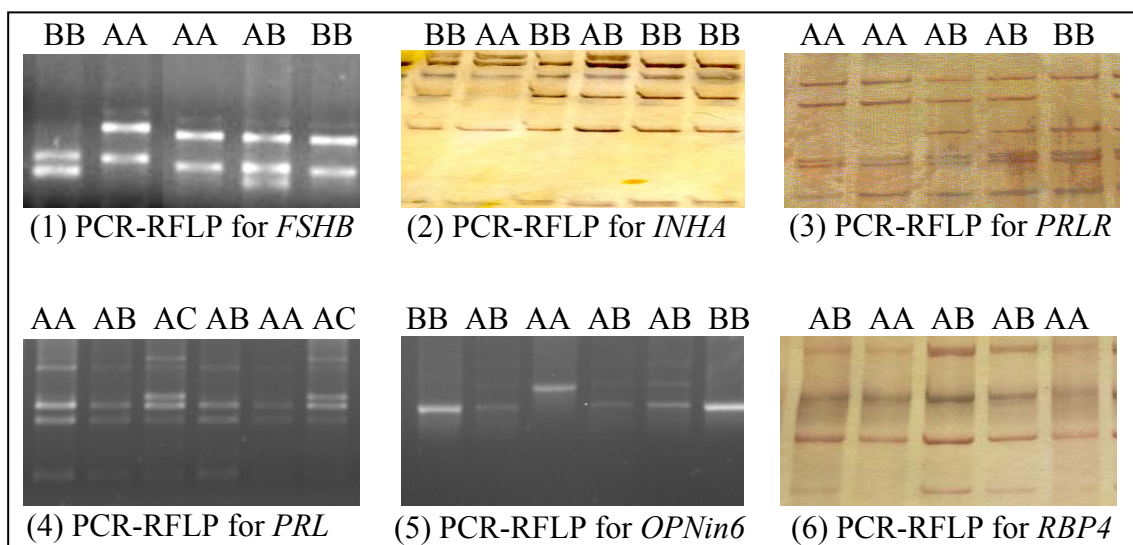


Figure 4.2:    Description of polymorphisms in candidate genes *FSHB*, *INHA*, *PRLR*, *PRL*, *OPNin6*, and *RBP4* tested by means of PCR-RFLP

| GC   CC   CC   GG   GC | AA   CA   CC   CC   CC | CC   CT   TT   CT   CT   CT |
| (1).PCR-SSCP for *GnRHR* | (2). PCR-SSCP for *RLN* | (3). PCR-SSCP for *LHB* |
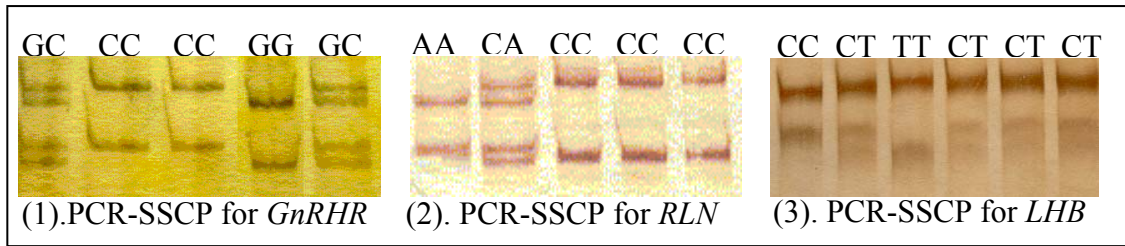
Figure 4.3:    Description of polymorphisms at *GnRHR*, *RLN*, and *LHB* loci tested by
means of PCR-SSCP



|  |  |  |
| (1) *ACR* | (2) *FST* | (3) *INHBA* |
| (4) *INHBB* | (5) *AR* | (6) *ACTG2* |
| (7) *OPNpro* |  |  |
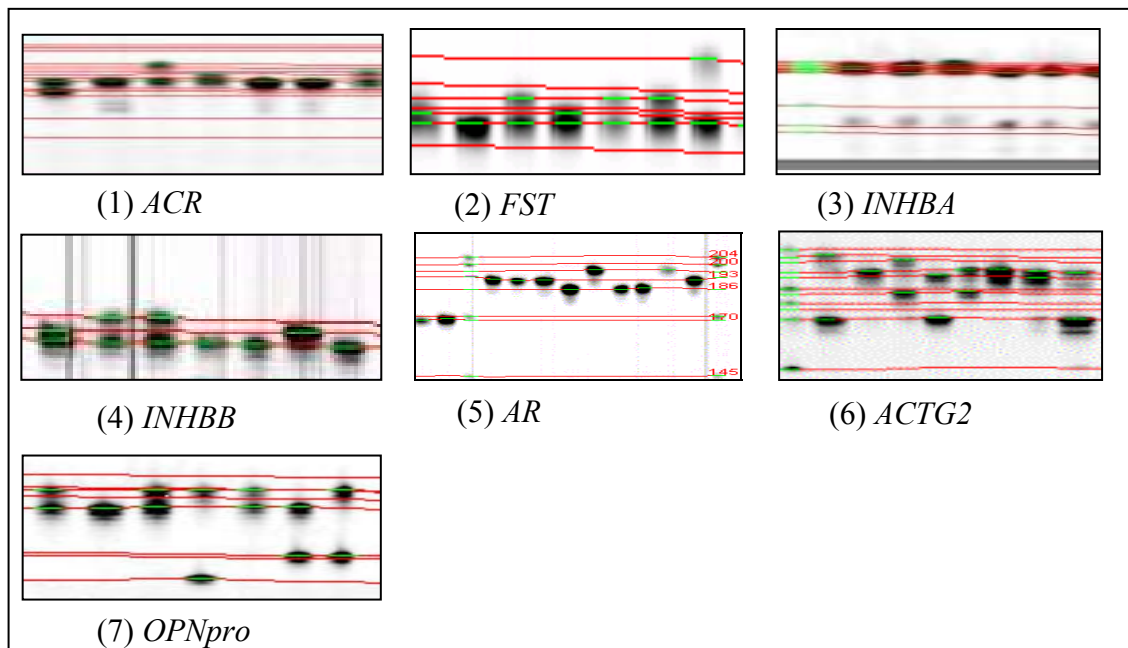
Figure 4.4:    Description of polymorphisms of candidate genes *ACR*, *INHBA*, *INHBB*,
*FST*, *AR*, *ACTG2*, and *OPNpro* genotyped by means of microsatellite.

## 4.2 Analysis of boar reproductive traits

The boar reproductive traits were analysed with procedure MEANS and FREQU of
software SAS V8.02. The traits were compared among the different populations.

### 4.2.1 Comparison of fertility traits among boar populations

Fertility data [no return rate (NRR) and number of piglets born alive (NBA)] of each
boar were given as the deviation from the population means within breed, parity of sow,

farm and season classes. Their sample size (n), means (μ), standard deviation (SD) and ranges dependent on population were compared in Table 4.1.

Table 4.1:      Sample size, means, standard deviations and ranges of fertility traits

| Population | Traits[1] | n | μ | SD | Minimum | Maximum |
|---|---|---|---|---|---|---|
| MIXED | NRR (%) | 356 | 0.554 | 6.217 | -24.07 | 19.62 |
|  | NBA (pig/litter) | 356 | 0.012 | 0.550 | -2.97 | 1.40 |
| PI | NRR (%) | 244 | 0.405 | 6.939 | -24.07 | 19.62 |
|  | NBA (pig/litter) | 244 | 0.031 | 0.562 | -1.69 | 1.37 |
| PI×HA | NRR (%) | 112 | 0.894 | 4.111 | -12.23 | 13.79 |
|  | NBA (pig/litter) | 112 | 0.034 | 0.519 | -2.97 | 1.40 |

[1]NRR: non return rate, NBA: number of piglets born alive. NRR and NBA have been corrected with factors: parity, farm, season and breed of sow.

For NRR trait, the mean of crossbred PI×HA was higher than of PI, but standard deviation turned out contrary to the means. The range values of crossbred PI×HA were not as wide as those of PI. The mean, standard error and ranges of PI were close to those of MIXED population due to bigger sample size than PI×HA population. For NBA trait, mean, standard error and ranges in both populations were very close. These values of fertility traits were in normal distribution and within the usual ranges.

4.2.2 Comparsion of sperm quality traits among boar populations

Sperm quality traits including sperm concentration [SCON ($10^8$/ml)], semen volume per ejaculate [VOL (ml)], sperm motility [MOT (%)], plasma droplets rate [PDR (%)] and abnormal spermatozoa rate [ASR (%)] were obtained from each ejaculate with the evaluation of semen quality, sample size (n), means (μ), standard errors (SE) and ranges of sperm quality traits dependent on population are shown in Table 4.2.

Table 4.2:    Sample size, means, standard errors and ranges of sperm quality traits

| Population | Traits[1] | n | μ | SE | Minimum | Maximum |
|---|---|---|---|---|---|---|
| MIXED | SCON ($10^8$/ml) | 49416 | 2.987 | 0.979 | 1.0 | 6.0 |
| | VOL (ml) | 52221 | 250.19 | 68.44 | 41 | 499 |
| | MOT (%) | 49671 | 85.43 | 3.49 | 75 | 92 |
| | PDR (%) | 41591 | 6.59 | 2.37 | 1 | 15 |
| | ASR (%) | 43011 | 6.64 | 2.55 | 3 | 18 |
| PI | SCON ($10^8$/ml) | 37813 | 2.962 | 0.968 | 1.0 | 6.0 |
| | VOL (ml) | 39918 | 244.31 | 64.66 | 41 | 499 |
| | MOT (%) | 38140 | 85.44 | 3.52 | 75 | 92 |
| | PDR (%) | 31259 | 6.56 | 2.36 | 1 | 15 |
| | ASR (%) | 33523 | 6.58 | 2.51 | 5 | 18 |
| PI×HA | SCON ($10^8$/ml) | 11603 | 3.067 | 1.008 | 1.0 | 6.0 |
| | VOL (ml) | 12303 | 269.29 | 76.43 | 41 | 499 |
| | MOT (%) | 11531 | 85.09 | 3.39 | 75 | 92 |
| | PDR (%) | 10332 | 6.68 | 2.39 | 1 | 15 |
| | ASR (%) | 9488 | 6.85 | 2.68 | 3 | 15 |

[1]SCON: sperm concentration, VOL: semen volume per ejaculate, MOT: sperm motility, PDR: rate of sperm with proximal plasma droplets, ASR: abnormal spermatozoa rate.

Means and standard deviations of SCON and VOL trait in the crossbred PI×HA were larger than these in the PI population, but their ranges were similar. Because sample size of the PI population was double as of the PI×HA population, therefore, means and standard errors of SCON and VOL trait in the PI population were close to these in the MIXED population. Means, standard errors and ranges of MOT, PDR and ASR trait in every population were very close.

All observed values of sperm quality traits were in normal distribution within the usual ranges in corresponding populations.

4.3 Analysis of genotypes

Analysis of genotype was composed of calculation of allele and genotype frequencies and test of Hardy-Weinberg equilibrium for single locus within population.

4.3.1 Frequencies of alleles and genotypes

4.3.1.1 Diallelic loci

The genotype and allele frequencies of the diallelic loci within the PI and the PI×HA boars were calculated. The results are shown in Table 4.3 and Table 4.4, respectively.

The frequencies of homozygote AA and BB of the *FSHB* gene were very close in the PI population and the PI×HA population. Homozygote AA was most frequent and more than 2/3 in both populations, and homozygote BB was rare, 3.28% in the PI population and 5.36% in the PI×HA population. The heterozygote genotype was 27.87% in the PI population and 25.89% in the PI×HA population. The allele frequency of *FSHB* gene in the PI population and the PI×HA population was almost similar. Allele A was very frequent, more than 0.80 in both populations.

Homozygote AA of the *PRLR* locus in the PI×HA population was more than in the PI population, the frequency of homozygote AA was 83.04% and 58.61%, respectively. The heterozygote AB and homozygote BB in the PI population were more than those in the PI×HA population. The frequencies were 27.05% and 14.34%; 14.29% and 2.68%, respectively. The frequency of allele A was 0.90 in the PI×HA population and 0.72 in the PI population.

The frequency of homozygote AA of the *RBP4* gene was 33% in the PI population, and 2.68% in the PI×HA population. Heterozygote genotype was 66.39% in the PI population and 97.34% in the PI×HA population. Homozygote BB was not observed in both populations in this experiment. The frequency of allele A of *RBP4* locus in the PI (0.67) population was more than in the PI×HA population (0.51).

Table 4.3: The genotype frequencies and sample size (n) for the loci *FSHB*, *PRLR*, *RBP4*, *INHA*, *OPNin6*, *ACTN1*, *ACTN4*, *GnRHR*, *RLN*, and *LHB*

| Locus | Genotype | PI | | PI×HA | | Sum |
| --- | --- | --- | --- | --- | --- | --- |
| | | n | Frequency (%) | n | Frequency (%) | (n) |
| *FSHB* | AA | 168 | 68.85 | 77 | 68.75 | 245 |
| | AB | 68 | 27.87 | 29 | 25.89 | 97 |
| | BB | 8 | 3.28 | 6 | 5.36 | 14 |
| *PRLR* | AA | 143 | 58.61 | 93 | 83.04 | 236 |
| | AB | 66 | 27.05 | 16 | 14.29 | 82 |
| | BB | 35 | 14.34 | 3 | 2.68 | 38 |
| *RBP4* | AA | 82 | 33.61 | 3 | 2.68 | 85 |
| | AB | 162 | 66.39 | 109 | 97.34 | 271 |
| | BB | 0 | 0 | 0 | 0 | 0 |
| *INHA* | AA | 2 | 0.82 | 2 | 1.79 | 4 |
| | AB | 51 | 20.90 | 72 | 64.29 | 123 |
| | BB | 191 | 78.28 | 38 | 33.93 | 229 |
| *OPNin6* | AA | 71 | 29.10 | 9 | 8.04 | 80 |
| | AB | 81 | 33.20 | 37 | 33.04 | 118 |
| | BB | 92 | 37.70 | 66 | 58.93 | 158 |
| *ACTN1* | AA | 125 | 55.80 | 77 | 74.04 | 202 |
| | AB | 84 | 37.50 | 24 | 23.08 | 108 |
| | BB | 15 | 6.70 | 3 | 2.88 | 18 |
| *ACTN4* | AA | 83 | 37.05 | 40 | 38.46 | 123 |
| | AB | 110 | 49.11 | 46 | 44.23 | 156 |
| | BB | 31 | 13.84 | 18 | 17.31 | 49 |
| *GnRHR* | CC | 212 | 86.88 | 74 | 66.07 | 286 |
| | GC | 30 | 12.30 | 38 | 33.93 | 68 |
| | GG | 2 | 0.82 | 0 | 0 | 2 |
| *RLN* | CC | 201 | 82.38 | 101 | 90.18 | 302 |
| | CA | 42 | 17.21 | 11 | 9.82 | 53 |
| | AA | 1 | 0.41 | 0 | 0 | 1 |
| *LHB* | CC | 86 | 35.25 | 47 | 41.96 | 133 |
| | CT | 114 | 46.72 | 47 | 41.96 | 161 |
| | TT | 44 | 18.03 | 18 | 16.07 | 62 |

Table 4.4: Allele frequencies of the loci *FSHB, PRLR, RBP4, INHA, OPNin6, ACTN1, ACTN4, GnRHR, RLN,* and *LHB*

| Locus | Allele | PI (n=244) | PI×HA (n=112) | Locus | Allele | PI (n=244) | PI×HA (n=112) |
|-------|--------|------------|---------------|-------|--------|------------|---------------|
| *FSHB* | A | 0.83 | 0.82 | *ACTN1**  | A | 0.75 | 0.86 |
|        | B | 0.17 | 0.18 |            | B | 0.25 | 0.14 |
| *PRLR* | A | 0.72 | 0.90 | *ACTN4**  | A | 0.62 | 0.61 |
|        | B | 0.28 | 0.10 |            | B | 0.38 | 0.39 |
| *INHA* | A | 0.11 | 0.34 | *GnRHR*   | C | 0.93 | 0.83 |
|        | B | 0.89 | 0.66 |            | G | 0.07 | 0.17 |
| *RBP4* | A | 0.67 | 0.51 | *RLN*     | C | 0.91 | 0.95 |
|        | B | 0.33 | 0.49 |            | A | 0.09 | 0.05 |
| *OPN*  | A | 0.46 | 0.25 | *LHB*     | C | 0.59 | 0.63 |
|        | B | 0.54 | 0.75 |            | T | 0.41 | 0.37 |

*ACTN1* and *ACTN4* locus: n = 224 in PI, n = 104 in PI×HA

The frequency of homozygote AA of *INHA* gene was very low, only 0.82% in the PI population and 1.79% in the PI×HA population. Heterozygote genotype was 20.90 % in the PI population, and 64.29% in the PI×HA population. Allele B of *INHA* was frequent in the PI population and the PI×HA population. The frequencies were 0.89 in the PI population and 0.66 in the PI×HA population.

The frequency of homozygote BB of the *OPNin6* locus was 37.70 % in the PI population and 58.93% in the PI×HA population. The heterozygote genotype was almost similar, 33.20% in the PI population and 33.04% in the PI×HA population. The allele A of the *OPNin6* gene had a frequency of 0.40 in the PI population and of 0.20 in the PI×HA population.

The frequency of homozygote AA of the *ACTN1* locus was 55.80% in the PI population, 74.04% in the PI×HA population. The heterozygote genotype was 37.50% and 23.08%, respectively. The frequency of allele A of this gene was 0.75 in the PI

population and 0.86 in the PI×HA population. Three genotypes in both populations were observed in this experiment.

The heterozygote of the *ACTN4* gene was 49.11% in the PI population and 44.23% in the PI×HA population. Three genotypes appeared within both populations. The frequency of allele A of *ACTN4* gene was almost similar in both populations, 0.62 in the PI population and 0.61 in the PI×HA population.

The homozygote CC of the *GnRHR* gene was 86.88% in the PI population and 66.07% the PI×HA population. The heterozygote genotype was 12.30% and 33.93%, respectively. The homozygote GG was observed only in the PI, with a frequency of 0.82%. This genotype did not appear in the PI×HA population. Allele C of the *GnRHR* locus was frequent in the PI population (0.93) and the PI×HA population (0.83).

The frequency of homozygote CC of the *RLN* gene was 82.38% in the PI population and 90.18% in the PI×HA population. The heterozygote genotype was 17.21% and 9.82%, respectively. Homozygote AA with a frequency of 0.41% appeared only in the PI population in this experiment. Allele C of the *RLN* locus was frequent and almost similar in the PI and the PI×HA, with frequencies of 0.91 and 0.95, respectively.

For the *LHB* locus, 35.25% of homozygote CC in the PI population and 41.96% of homozygote CC in the PI×HA population were observed in this experiment. Heterozygote genotype was 46.72% in the PI population and 41.96% in the PI×HA population. Three genotypes appeared in the genotyped boars. Allele C of the *LHB* gene was frequent, the frequency was 0.59 in the PI population and 0.63 in the PI×HA population.

4.3.1.2 Multiple allelic loci

(1) *PRL* locus

In this experiment, five genotypes of the *PRL* locus were observed in the PI population

and the PI×HA population as shown in Table 4.5. Frequencies of homozygote AA and CC were 20.85% and 21.34% in the PI population, 27.37% and 8.42% in the PI×HA population, respectively. Frequencies of heterozygote AB, AC were 13.74% and 41.71% in the PI population, 33.68% and 30.53% in the PI×HA population, respectively. Heterozygote BC was observed only in the PI population with the frequency of 2.37%. Homozygote BB was not observed in both populations.

Table 4.5:     The genotype frequencies of the *PRL* locus and sample size (n)

| Genotype | PI | | PI×HA | | SUM |
|---|---|---|---|---|---|
| | n | Frequency (%) | n | Frequency (%) | n |
| AA | 44 | 20.85 | 26 | 27.37 | 70 |
| AB | 29 | 13.74 | 32 | 33.68 | 61 |
| AC | 88 | 41.71 | 29 | 30.53 | 117 |
| BC | 5 | 2.37 | 0 | 0 | 5 |
| CC | 45 | 21.34 | 8 | 8.42 | 53 |

The frequencies of allele A, B, C were 0.49, 0.08, and 0.43 in the PI population; 0.59, 0.17, and 0.24 in the PI×HA population. The allele frequencies of the *PRL* gene locus within both populations are shown in Table 4.6.

Table 4.6:     Allele frequencies of the *PRL* gene

| Allele | A | B | C |
|---|---|---|---|
| PI(n = 211) | 0.49 | 0.08 | 0.43 |
| PI×HA (n = 95) | 0.59 | 0.17 | 0.24 |

(2) *AR* locus

Table 4.7 shows the genotype frequencies of the *AR* gene. It was observed that five genotypes were in the PI population and three genotypes in the PI×HA population. Allele `*196* bp´ was very frequent in the PI population. In the PI×HA population the most frequent allele was the `*186* bp´ with frequency of 0.795. In the PI population, the

observed frequencies of the five alleles varied from 0.008 to 0.623. Three alleles appeared in the PI×HA population with frequencies ranged from 0.045 to 0.795.

Table 4.7: Allele frequencies of the *AR* locus and sample size (n)

| Allele | | *196* bp | *193* bp | *186* bp | *173* bp | *172* bp |
|---|---|---|---|---|---|---|
| PI | n = 244 | 152 | 2 | 43 | 21 | 26 |
| | Frequency | 0.623 | 0.008 | 0.176 | 0.086 | 0.107 |
| PI×HA | n = 112 | 18 | 0 | 89 | 5 | 0 |
| | Frequency | 0.161 | 0 | 0.795 | 0.045 | 0 |
| SUM | n = 356 | 170 | 2 | 132 | 26 | 26 |

(3) *FST* locus

Table 4.8: The genotype frequencies of the *FST* locus

| Genotype | PI (n=244) (%) | PI×HA (n=112) (%) | Genotype | PI (n=244) (%) | PI×HA (n=112) (%) |
|---|---|---|---|---|---|
| 140/147 | 0.4 | 0 | 143/148 | 16.0 | 9.8 |
| 141/141 | 0.4 | 0.9 | 143/149 | 0.4 | 0 |
| 141/144 | 0.4 | 0 | 144/144 | 4.1 | 4.5 |
| 141/146 | 0.4 | 3.6 | 144/147 | 2.5 | 2.7 |
| 141/147 | 0 | 1.8 | 144/148 | 5.3 | 0 |
| 141/148 | 1.6 | 0 | 144/149 | 1.6 | 0.9 |
| 142/142 | 7.4 | 19.7 | 145/145 | 0.8 | 4.5 |
| 142/143 | 0.4 | 0 | 145/147 | 2.9 | 0 |
| 142/145 | 0 | 0.9 | 145/148 | 2.0 | 0 |
| 142/146 | 1.3 | 3.6 | 145/149 | 0.4 | 0 |
| 142/147 | 2.6 | 10.7 | 146/146 | 2.5 | 0 |
| 142/148 | 2.9 | 4.5 | 146/148 | 0.4 | 0 |
| 143/143 | 17.6 | 19.6 | 147/147 | 9.8 | 5.4 |
| 143/146 | 3.3 | 1.8 | 148/148 | 8.6 | 0.9 |
| 143/147 | 4.1 | 7.1 | | | |

The genotype frequencies of the *FST* locus are shown in Table 4.8. In this experiment, 27 genotypes in the PI population were observed. The frequent genotypes homozygote `*142/142*´, `*143/143*´, `*147/147*´, `*148/148*´ and heterozygote `*143/148*´ were with frequencies of 7.4%, 17.6%, 9.8%, 8.6%, and 16%, respectively. The frequencies of other rare genotypes varied from 0.4% to 5.3%. Eighteen genotypes appeared in the PI×HA population. The genotypes `*142/142*´, `*143/143*´, `*143/147*´, and `*143/148*´ were very frequent, their frequencies were 19.7%, 19.6%, 7.1%, and 9.8%, respectively. The other rare genotypes varied from 0.9% to 5.4%.

Table 4.9 shows the allele frequencies of the highly polymorphic marker *FST* locus with ten alleles in the PI population and nine alleles in the PI×HA population. In the PI population, allele `*142* bp´, `*143* bp´, `*147* bp´, and `*148* bp´ were frequent, 0.10, 0.297, 0.174, and 0.227, respectively. The frequencies of the other rare alleles were less than 0.10, varying from 0.002 to 0.09. In contrast, within the PI×HA population, the frequent alleles `*142* bp´, `*143* bp´, and `*147* bp´ had frequencies of 0.295, 0.29, and 0.165, respectively. The other alleles had frequencies ranging from 0.004 to 0.08.

Table 4.9:        Allele frequencies of the *FST* locus

| Allele | *140* bp | *141* bp | *142* bp | *143* bp | *144* bp | *145* bp | *146* bp | *147* bp | *148* bp | *149* bp |
|---|---|---|---|---|---|---|---|---|---|---|
| PI (n=244) | 0.002 | 0.016 | 0.109 | 0.297 | 0.090 | 0.035 | 0.051 | 0.160 | 0.227 | 0.012 |
| PI×HA (n=112) | 0 | 0.036 | 0.295 | 0.290 | 0.062 | 0.022 | 0.054 | 0.165 | 0.080 | 0.004 |

(4) *INHBA* locus

Table 4.10 shows the genotype frequencies of the *INHBA* gene. Ten genotypes in the PI population were observed. The frequent genotypes were homozygote `*252/252*´ and heterozygote `*252/254*´. Their frequencies were 64.3% and 22.1%. The frequencies of the other genotypes were less than 10%, ranging from 0.4% to 8% in the PI population. In this study, eight genotypes appeared in the PI×HA population. The genotypes `*252/252*´, `*252/254*´, and `*252/256*´ were frequent, their frequencies were 45.5%,

25.9%, and 16.1%, respectively. The frequencies of other genotypes varied from 0.9% to 8%.

Table 4.10: The genotype frequencies of the *INHBA* locus

| Genotype | PI (n=244) (%) | PI×HA (n=112) (%) | Genotype | PI (n=244) (%) | PI×HA (n=112) (%) |
|---|---|---|---|---|---|
| 248/252 | 0.4 | 0 | 252/262 | 1.2 | 0 |
| 248/254 | 0.8 | 0 | 254/254 | 2.9 | 1.8 |
| 252/252 | 64.3 | 45.5 | 254/256 | 0.8 | 0.9 |
| 252/254 | 22.1 | 25.9 | 256/256 | 0.4 | 0.9 |
| 252/256 | 6.6 | 16.1 | 256/258 | 0 | 0.9 |
| 252/258 | 0 | 8.0 | 256/262 | 0.4 | 0 |

The allele frequencies of the *INHBA* locus are shown in Table 4.11. The most frequent allele was `*252 bp´* in the PI population. The frequency was 0.797. The frequencies of other alleles varied from 0.006 to 0.145. Four alleles `*252* bp´, `*254* bp´, `*256* bp´, and `*258* bp´ appeared in the PI×HA population, their frequencies were 0.705, 0.152, 0.098, and 0.045, respectively. Obviously, allele `*252* bp´ was the most frequent in the PI×HA population.

Table 4.11: Allele frequencies of the *INHBA* locus

| Allele | *248* bp | *252* bp | *254* bp | *256* bp | *258* bp | *262* bp |
|---|---|---|---|---|---|---|
| PI (n=244) | 0.006 | 0.797 | 0.145 | 0.043 | 0 | 0.008 |
| PI×HA (n=112) | 0 | 0.705 | 0.152 | 0.098 | 0.045 | 0 |

(5) *INHBB* locus

The genotype frequencies of the *INHBB* locus are shown in Table 4.12. Within 244 the genotyped PI boars, thirteen genotypes of *INHBB* locus were observed. Their frequencies varied from 0.4% to 27%. Frequent genotypes were homozygote `*171/171´*, heterozygote `*171/173´*, `*171/175´*, and `*173/175´*. Their frequencies were 27%, 23%,

20.05%, and 9.8%, respectively. In total, fourteen genotypes of the *INHBB* locus in the PI×HA population were observed, their frequencies varied from 0.9% to 18.8%. Obviously, the frequent genotypes were `*161/163′*, `*161/171′*, `*171/171′*, and `*171/173′*. Their frequencies were 8.9%, 17%, 18.8%, and 15.2%, respectively.

Table 4.12:     The genotype frequencies of the *INHBB* locus

| Genotype | PI (n=244) (%) | PI×HA (n=112) (%) | Genotype | PI (n=244) (%) | PI×HA (n=112) (%) |
|---|---|---|---|---|---|
| 159/163 | 0 | 5.4 | 163/171 | 2.9 | 4.5 |
| 159/171 | 0.4 | 13.4 | 163/173 | 2.5 | 0 |
| 159/173 | 0 | 2.7 | 163/175 | 2.5 | 0 |
| 159/175 | 0 | 3.6 | 171/171 | 27.0 | 18.8 |
| 161/163 | 0.4 | 8.9 | 171/173 | 23.0 | 15.2 |
| 161/171 | 0 | 17.0 | 171/175 | 20.5 | 2.7 |
| 161/173 | 0.4 | 4.5 | 173/173 | 4.9 | 1.8 |
| 161/175 | 0 | 0.9 | 173/175 | 9.8 | 0 |
| 163/163 | 0.4 | 0.9 | 175/175 | 5.3 | 0 |

Table 4.13:     Allele frequencies of the *INHBB* locus

| Allele | *159* bp | *161* bp | *163* bp | *171* bp | *173* bp | *175* bp |
|---|---|---|---|---|---|---|
| PI (n=244) | 0.002 | 0.004 | 0.043 | 0.506 | 0.227 | 0.217 |
| PI×HA (n=112) | 0.125 | 0.156 | 0.103 | 0.451 | 0.129 | 0.036 |

The results in Table 4.13 show that six alleles of the *INHBB* distributed differently within both populations. Allele `*171* bp′*, `*173* bp′*, and `*175* bp′* were more frequent in the PI population. Their frequencies were 0.506, 0.227, and 0.217, respectively. The frequencies of the other alleles in this population were less than 0.05, ranging from 0.002 to 0.043. But in the PI×HA population the frequencies of alleles were more than 0.10 except for allele `*175* bp′* (0.036). The frequent allele was `*171* bp′*, the frequency of this allele was 0.451 in the PI×HA population. Six alleles were observed in both populations.

(6) *ACR* locus

The genotype frequency of the *ACR* locus within population is shown in Table 4.14. In this experiment, twenty five genotypes in the PI population were observed. The frequencies of the genotype homozygote `*208/208´* and heterozygote `*202/208´* were more than 50%, equal to 33.6% and 20.9%, respectively. The frequencies of other genotypes ranged from 0.4% to 8.9%. In total, twenty genotypes appeared in the PI×HA population, more than the half were homozygote `*208/208´* and heterozygote `*208/226´*, `*208/228´*, their frequency were 17.0%, 17.0%, and 32.1%, respectively. The other genotypes varied with frequencies ranging from 0.9% to 4.5% in the PI×HA population.

Table 4.14:     The genotype frequencies of the *ACR* locus

| Genotype | PI (n=244) (%) | PI×HA (n=112) (%) | Genotype | PI (n=244) (%) | PI×HA (n=112) (%) |
|---|---|---|---|---|---|
| 186/202 | 0.8 | 0 | 208/216 | 0.8 | 3.6 |
| 186/208 | 5.3 | 0.9 | 208/218 | 4.9 | 4.5 |
| 196/202 | 0.4 | 0 | 208/224 | 0 | 1.8 |
| 196/208 | 0.4 | 0 | 208/226 | 0.4 | 17.0 |
| 202/202 | 3.6 | 0.9 | 208/228 | 1.6 | 32.1 |
| 202/208 | 20.9 | 1.8 | 212/218 | 0 | 1.8 |
| 202/210 | 1.6 | 0 | 212/226 | 0 | 0.9 |
| 202/212 | 5.3 | 1.8 | 212/228 | 0 | 1.8 |
| 202/214 | 0.4 | 0 | 214/214 | 2.0 | 0.9 |
| 202/216 | 2.5 | 0.9 | 214/218 | 0.4 | 0 |
| 202/218 | 0.4 | 0 | 214/226 | 0.4 | 0 |
| 202/224 | 0.8 | 0 | 214/228 | 0.8 | 1.8 |
| 208/208 | 33.6 | 17.0 | 218/228 | 0.4 | 3.6 |
| 208/210 | 0 | 1.8 | 226/228 | 0.4 | 0 |
| 208/212 | 2.9 | 2.7 | 208/214 | 8.6 | 1.8 |

Table 4.15 shows the allele frequencies of the highly polymorphic marker *ACR* locus. Twelve alleles of *ACR* locus were observed in the PI population, allele `*202* bp´ and

`208 bp´` were frequent, their frequencies were 0.203 and 0.57. The frequencies of the other alleles were less than 0.10, ranged from 0.004 to 0.074. In the PI×HA population, eleven alleles were observed. The allele `208` bp´, and `228` bp´ were frequent, their frequencies were 0.509 and 0.196. The other alleles varied between 0.004 and 0.089.

Table 4.15:     Allele frequencies of the *ACR* locus

| Allele | *186* bp | *196* bp | *202* bp | *208* bp | *210* bp | *212* bp | *214* bp | *216* bp | *218* bp | *224* bp | *226* bp | *228* bp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PI (n=244) | .031 | .004 | .203 | .578 | .008 | .041 | .074 | .004 | .031 | .004 | .008 | .014 |
| PI×HA (n=112) | .004 | 0 | .036 | .513 | .009 | .045 | .027 | .018 | .054 | .009 | .089 | .196 |

(7) *ACTG2* locus

The genotype frequencies of the *ACTG2* locus are shown in Table 4.16. In this experiment, thirty-eight genotypes in the PI population were observed. The genotype homozygote `109/109´`, `111/111´` and heterozygote `93/109´`, `103/109´`, `103/111´`, `109/111´` were frequent. Their frequencies were 9.8%, 6.1% and 8.6%, 11.9%, 8.6%, 11.9%, respectively. The frequency of other genotypes varied from 0.4% to 4.1%. In total, twenty-nine genotypes of *ACTG2* locus appeared in the PI×HA population, more than half of the genotypes were heterozygote `93/109´`, `97/103´`, `97/111´`, `103/109´`, `109/111´`. Their frequencies were 14.3%, 7.1%, 8.9%, 14.3%, and 10.7%, respectively. The other genotypes had frequencies ranging from 0.9% to 4.5% in the PI×HA population.

Table 4.17 shows the allele frequency of the highly polymorphic marker *ACTG2* locus. In total, 12 alleles were observed in both boar populations. In the PI population, allele `93` bp´, `103` bp´, `109` bp´, and `111` bp´ were more frequent, their frequencies were 0.125, 0.186, 0.307, and 0.217, respectively. The frequencies of the other alleles were less than 0.06, ranging from 0.002 to 0.051 in the PI population. In the PI×HA population the allele `93` bp´, `97` bp´, `103` bp´, `109` bp´, and `111` bp´ were frequent. Their frequencies were 0.147, 0.147, 0.116, 0.313, and 0.143, respectively. The other alleles had frequencies ranging from 0.004 to 0.063 in the PI×HA population.

Table 4.16:     The genotype frequencies of the *ACTG2* locus

| Genotype | PI (n=244) (%) | PI×HA (n=112) (%) | Genotype | PI (n=244) (%) | PI×HA (n=112) (%) |
|---|---|---|---|---|---|
| 93/93 | 2.9 | 2.7 | 103/105 | 0 | 0.9 |
| 93/97 | 0 | 4.5 | 103/109 | 11.9 | 14.3 |
| 93/103 | 4.9 | 0 | 103/111 | 8.6 | 0 |
| 93/105 | 0.4 | 0.9 | 103/115 | 2.5 | 0 |
| 93/109 | 8.6 | 14.3 | 103/117 | 0 | 0.9 |
| 93/111 | 2.9 | 3.6 | 105/105 | 0.4 | 0 |
| 93/115 | 0.8 | 0 | 105/111 | 0.8 | 1.8 |
| 93/117 | 1.6 | 0 | 105/117 | 0 | 0.9 |
| 95/97 | 0 | 0.9 | 109/109 | 9.8 | 7.1 |
| 95/103 | 0.8 | 0 | 109/111 | 11.9 | 10.7 |
| 95/105 | 0.4 | 0 | 109/115 | 2.9 | 0 |
| 95/109 | 2.0 | 0.9 | 109/117 | 3.3 | 4.5 |
| 95/111 | 1.6 | 0 | 109/119 | 0.8 | 0.9 |
| 95/113 | 0.4 | 0 | 111/111 | 6.1 | 0.9 |
| 97/97 | 0 | 0.9 | 111/113 | 0.4 | 0 |
| 97/103 | 0.4 | 7.1 | 111/115 | 1.2 | 0.9 |
| 97/109 | 0.4 | 2.7 | 111/117 | 3.3 | 0.9 |
| 97/111 | 0 | 8.9 | 111/119 | 0.4 | 0 |
| 97/113 | 0 | 0.9 | 113/113 | 0.8 | 0 |
| 97/117 | 0 | 1.8 | 113/117 | 0.4 | 0 |
| 97/119 | 0 | 0.9 | 113/119 | 0.4 | 0 |
| 99/105 | 0.4 | 0 | 115/117 | 0.4 | 0 |
| 99/113 | 0 | 0.9 | 117/117 | 0.4 | 0.9 |
| 99/117 | 0 | 0.9 | 117/119 | 0.4 | 0 |
| 103/103 | 4.1 | 0 | | | |

Table 4.17:    Allele frequencies of the *ACTG2* locus

| Allele | 93 bp | 95 bp | 97 bp | 99 bp | 103 bp | 105 bp | 109 bp | 111 bp | 113 bp | 115 bp | 117 bp | 119 bp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PI (n=244) | .127 | .027 | .006 | .002 | .186 | .012 | .307 | .217 | .016 | .041 | .049 | .010 |
| PI×HA (n=112) | .147 | .027 | .143 | .004 | .116 | .022 | .308 | .143 | .013 | .004 | .062 | .013 |

(8) *OPNpro* locus

Table 4.18 shows the genotype frequencies of the microsatellite marker in the promoter region of the *OPN* gene named *OPNpro* locus. Within the PI population, thirteen genotypes for *OPNpro* locus were observed. The genotype homozygote `*142/142´*, `*148/148´* and heterozygote `*118/142´*, `*118/148´*, `*142/148´* were more frequent, their frequencies were 14.8%, 14.8% and 10.7%, 14.4%, 26.7%, respectively. The other genotypes frequencies of this locus ranged from 0.4% to 5.8% in the PI population.

Table 4.18:    The genotype frequencies of the *OPNpro* locus

| Genotype | PI (n=243) (%) | PI×HA (n=112) (%) | Genotype | PI (n=243) (%) | PI×HA (n=112) (%) |
|---|---|---|---|---|---|
| 118/118 | 5.8 | 0 | 130/148 | 0 | 2.7 |
| 118/126 | 0.4 | 0.9 | 138/142 | 0 | 6.3 |
| 118/138 | 0 | 0.9 | 138/146 | 0 | 0.9 |
| 118/142 | 10.7 | 7.1 | 138/148 | 0 | 7.1 |
| 118/146 | 1.6 | 3.6 | 142/142 | 14.8 | 0 |
| 118/148 | 14.4 | 9.8 | 142/146 | 3.3 | 8.0 |
| 126/130 | 0 | 1.8 | 142/148 | 26.7 | 16.1 |
| 126/142 | 0.4 | 2.7 | 146/146 | 2.1 | 2.7 |
| 126/148 | 1.6 | 9.8 | 146/148 | 3.3 | 4.5 |
| 130/142 | 0 | 1.8 | 148/148 | 14.8 | 13.4 |

In total, eighteen genotypes appeared in the 112 genotyped PI×HA boars. The genotypes homozygote `*148/148´* and heterozygote `*118/148´*, `*126/148´*, `*142/148´*

were frequent. Their frequencies were 13.4% and 9.8%, 9.8%, 16.1%, respectively. The other genotype frequencies of *OPNpro* ranged from 0.9% to 7.1% in the PI×HA population.

The results of allele frequencies of the *OPNpro* locus are shown in Table 4.19. Seven alleles distributed differently between the PI and the PI×HA population. Five alleles `*118* bp´, `*126* bp´, `*142* bp´, `*146* bp´, and `*148* bp´ were observed in the PI population. Their frequencies were 0.193, 0.012, 0.354, 0.062, and 0.379, respectively. Allele `*118* bp´, `*142* bp´, and `*148* bp´ were obviously more frequent in this population. Seven alleles appeared within the PI×HA population. The frequent alleles were `*118* bp´, `*142* bp´, `*146* bp´, and `*148* bp´, their frequencies were 0.112, 0.210, 0.115, and 0.384, respectively. The other allele frequencies ranged from 0.031 to 0.076 in this population.

Table 4.19:     Allele frequencies of the *OPNpro* locus

| Allele | *118* bp | *126* bp | *130* bp | *138* bp | *142* bp | *146* bp | *148* bp |
|---|---|---|---|---|---|---|---|
| PI (n=243) | 0.193 | 0.012 | 0 | 0 | 0.354 | 0.062 | 0.379 |
| PI×HA (n=112) | 0.112 | 0.076 | 0.031 | 0.076 | 0.210 | 0.115 | 0.384 |

4.3.2 Test of Hardy-Weinberg equilibrium

4.3.2.1 Diallelic loci

The chi-square test revealed (Table 4.20) that the diallelic loci *FSHB, ACTN1, ACTN4, GnRHR, RLN*, and *LHB* loci were in Hardy-Weinberg equilibrium in both populations. Loci *PRLR* and *RBP4* were not in Hardy-Weinberg equilibrium in both populations. Locus *INHA* was in Hardy-Weinberg equilibrium only in PI population, and *OPN*in6 locus was in Hardy-Weinberg equilibrium only in PI×HA population.

Table 4.20:    Chi-square test revealing genotypes of the diallelic loci in Hardy-Weinberg equilibrium

| Locus | PI | | PI×HA | |
|---|---|---|---|---|
| | $\chi^2$ | p-value | $\chi^2$ | p-value |
| *FSHB* | 0.13 | 0.72 | 2.18 | 0.14 |
| *PRLR* | 26.05 | 3.33E-7 | 4.18 | 0.04 |
| *INHA* | 0.56 | 0.45 | 20.34 | 6.48E-6 |
| *RBP4* | 60.24 | 8.39E-15 | 100.61 | 1.12E-23 |
| *OPNin6* | 26.75 | 2.32E-7 | 1.31 | 0.25 |
| *ACTN1* | 0.02 | 0.89 | 0.44 | 0.51 |
| *ACTN4* | 0.33 | 0.57 | 0.59 | 0.44 |
| *GnRHR* | 1.13 | 0.29 | 2.24 | 0.13 |
| *RLN* | 0.65 | 0.42 | 0.30 | 0.58 |
| *LHB* | 0.34 | 0.56 | 1.12 | 0.29 |

4.3.2.2 Multiple allelic loci

(1) Heterozygote degree

Heterozygote degree is powerful for discrimination of a molecular genetic marker. The heterozygote value of single locus in the PI and the PI×HA population was estimated with programme GENEPOP Version 3.3. The results are shown in Table 4.21. The heterozygote degree was moderate to high with the value of observed heterozygote ranging from 0.32 to 0.75 in the PI population, and from 0.47 to 0.88 in the PI×HA population. The value of expected heterozygote varied from 0.34 to 0.81 in PI population, and from 0.47 to 0.83 in the PI×HA population

Table 4.21:     The observed and expected heterozygote degrees of the multiple allelic loci

| Locus | Obs[1]. Heterozygote | | Exp[2]. Heterozygote | |
|---|---|---|---|---|
| | PI | PI×HA | PI | PI×HA |
| *PRL* | 0.58 | 0.64 | 0.57 | 0.56 |
| *FST* | 0.49 | 0.47 | 0.81 | 0.79 |
| *INHBA* | 0.32 | 0.52 | 0.34 | 0.47 |
| *INHBB* | 0.62 | 0.79 | 0.64 | 0.73 |
| *ACR* | 0.60 | 0.81 | 0.62 | 0.69 |
| *ACTG2* | 0.75 | 0.88 | 0.80 | 0.83 |
| *OPNpro* | 0.62 | 0.84 | 0.69 | 0.77 |

[1]Obs: observed, [2]Exp: expected

(2) Estimation of *Fis*-value

In order to determine significant deviation from Hardy-Weinberg equilibrium for 8 multiple allelic loci within population, the test of each locus for Hardy-Weinberg equilibrium was carried out with software GENEPOP Version 3.3.

The estimates of *Fis*-value for 8 loci in the PI population and the PI×HA population according to Cockerham and Weir (1984) (W&C) and Robertson and Hill (1984) (R&H) are shown in Table 4.22. Loci *INHBA* and *INHBB* were in Hardy-Weinberg equilibrium only in the PI population, and locus *INHBA* was in Hardy-Weinberg equilibrium only in the PI×HA population. For other loci within both populations, the deviations from Hardy-Weinberg equilibrium were significant with p-values of $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$.

Table 4.22:     Estimates of *Fis*-value of multiple allelic loci within population

| Locus | PI | | | PI×HA | | |
|---|---|---|---|---|---|---|
| | W&C[1] | R&H[2] | P-value[3] | W&C | R&H | P-value |
| *PRL* | -0.013 | -0.037 | 0.0003*** | -0.138 | -0.087 | 0.0000[***] |
| *FST* | 0.400 | 0.301 | 0.0000*** | 0.403 | 0.324 | 0.0000*** |
| *INHBA* | 0.054 | 0.027 | 0.2693[n.s] | -0.103 | -0.044 | 0.4575[n.s] |
| *INHBB* | 0.033 | 0.020 | 0.7893[n.s] | -0.075 | -0.068 | 0.0002*** |
| *ACR* | 0.023 | 0.051 | 0.0000*** | -0.186 | -0.004 | 0.0023** |
| *ACTG2* | 0.062 | 0.085 | 0.0000*** | -0.068 | -0.038 | 0.0000*** |
| *OPNpro* | 0.096 | 0.113 | 0.0133** | -0.084 | -0.064 | 0.0235[*] |

[1]W&C: estimated method according to Cockerham and Weir (1984)

[2]R&H: estimated method according to Robertson and Hill (1984)

[3]Significant deviation of Hardy-Weinberg equilibrium

*$P \leq 0.05$      **$P \leq 0.01$      ***$P \leq 0.001$  n.s = no significant

4.4 Association analysis between genotypes and phenotypes

4.4.1 Boar fertility traits

The fertility traits including non return rate [NRR (%)] and number of piglets born alive [NBA (pig/litter)] were only available as an average of the inseminated commercial sows. The statistical fixed model comprised the effects of boar birth year, breed and genotype. The fertility data were analysed for each boar population separately, but also for both boar populations together, named MIXED population. All genotyped eighteen marker loci were applied for the statistical analysis and rare genotypes (frequency is less than 1%) were excluded from the statistical analysis.

4.4.1.1 Non return rate (NRR)

Association between genotypes of all genotyped loci and NRR were analysed using model 1 (association analysis for boar fertility traits). The statistical results are given as least square means and their standard errors. Only two loci *ACTN1* and *ACR* were significantly associated with NRR, no significant effects of the other loci (*FSHB*, *PRL*, *PRLR*, *FST*, *INHA*, *INHBA*, *INHBB*, *ACTG2*, *ACTN4*, *OPNin6*, *OPNpro*, *LHB*, *GnRHR*, *RLN*, *RBP4*, and *AR*) on NRR were observed. For no significant effect relevant loci, the results are shown in Appendix 9.1.

Table 4.23:   Estimates of least square mean for the *ACTN1* genotype effects on NRR[1] (%)

| Genotype | MIXED[2] | PI[2] | PI×HA |
|----------|----------|-------|-------|
| AA | 0.660±0.482[a] | 0.476±0.653[a] | 0.588±0.580 |
| AB | 0.901±0.655[a] | 0.703±0.770[a] | 0.780±0.985 |
| BB | -3.313±1.545[b] | -4.919±1.900[b] | 2.475±2.511 |

[1]Least square means differ significantly for genotypes of the same gene with different subscripts (a, b: $P \leq 0.01$), [2]significant effect ($P < 0.05$) by *F*-test.

Table 4.23 shows estimates of NRR for boars in dependence of the *ACTN1* locus. The candidate gene *ACTN1* was significantly linked or associated with NRR in the MIXED

population ($P < 0.05$) and in the PI population ($P < 0.05$). Locus *ACTN1* had no significant effect on NRR in the PI×HA population. Boars with genotype AA and AB had a significantly higher NRR than those with genotype BB in the MIXED population and the PI population.

In the present experiment, a significant effect of the microsatellite marker *ACR* locus on NRR in the MIXED population ($P < 0.05$) and in the PI population ($P < 0.05$) was observed. Locus *ACR* had no significant effect on NRR in the PI×HA population. The results of the statistical analysis are shown in Table 4.24.

Table 4.24:    Estimates of least square means for the *ACR* genotype effects on NRR (%)

| MIXED[A] | | PI[B] | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 202/202 | 3.481±2.054 | 202/202 | 3.248±2.414 | 208/208 | 0.567±1.046 |
| 202/208 | 0.110±0.956 | 202/208 | 0.189±0.993 | 208/218 | 0.741±2.008 |
| 202/214 | -1.154±1.598 | 202/214 | -1.522±1.899 | 208/226 | 0.505±1.023 |
| 202/999 | 3.325±1.569 | 202/999 | 4.334±1.731 | 208/228 | 1.045±0.822 |
| 208/208 | -0.505±0.689 | 208/208 | -0.839±0.778 | 208/999 | -0.466±1.146 |
| 208/214 | 2.681±1.335 | 208/214 | 2.867±1.511 | 218/228 | -0.690±2.171 |
| 208/228 | 1.427±1.054 | 208/999 | 0.787±1.131 | 218/999 | 3.224±2.492 |
| 208/999 | 0.445±0.749 | 214/214 | -2.308±3.078 | 226/999 | 1.831±3.059 |
| 214/214 | -2.617±2.521 | 214/999 | 5.127±3.455 | 228/999 | 2.871±2.192 |
| 214/228 | 6.974±3.057 | | | 999/999 | 0.151±2.225 |
| 228/999 | 0.029±2.148 | | | | |

[A, B]Significant effect ($P < 0.05$) by *F*-test

In total, 11 genotypes of the *ACR* locus in the MIXED population were used in the statistical analysis. Estimates of NRR for boars in dependence of the *ACR* ranged from -2.617 to 6.947. Boars with genotype `*214/214*´ had the least NRR, and boars with genotype `*214/228*´ had the highest NRR. The estimated differences between genotypes

of *ACR* were checked with multiple pairwise comparisons using Tukey-Kramer adjustment. No significant difference was observed.

Nine genotypes of the *ACR* locus in the PI population were used in the statistical analysis. Estimates of NRR for boar independence of the *ACR* locus varied from -2.308 to 5.127. Boars with genotype `*214/214´* had the least NRR, and boars with genotype `*214/999´* had the highest NRR in this boar population. The difference of least square means between genotypes of the *ACR* was tested by multiple pairwise comparisons using Tukey-Kramer adjustment. No significant difference ($P > 0.05$) was observed.

In the PI×HA boar population, 10 genotypes of the *ACR* locus were used in the statistical analysis. Table 4.24 shows that estimates of NRR for boars in dependence of the *ACR* were various from -0.690 to 3.224. The highest NRR was genotype `*218/999´* and the least NRR was genotype `*218/228´* in this boar population.

(1) Additive and dominance effects on NRR

For the diallelic marker *ACTN1*, significantly associated with NRR, the additive and dominance effects were estimated to be significant in the boars as shown in Table 4.25.

Table 4.25:    Additive (a) and dominance (d) effects of the *ACTN1* gene alleles on NRR (%)

| Effect | MIXED | | PI | |
|---|---|---|---|---|
| | LSM ± SE | p | LSM ± SE | p |
| Additive | -1.987±0.792 | 0.01 | -2.698±0.985 | 0.01 |
| Dominance | 2.227±0.985 | 0.02 | 2.925±1.234 | 0.02 |

The statistical analyses revealed that allele A of the *ACTN1* locus had a positive effect on NRR and the estimates of the heterozygotes were more than the means of estimates of the both homozygotes in the MIXED and the PI population.

(2) Allele substitution effect on NRR

For the microsatellite marker *ACR,* significantly affecting NRR, the allele substitution effect was estimated according to the model 2 (for estimation of allele substitution effect on boar fertility traits) using multiple linear regression analysis with covariables of alleles (four frequent alleles `*202* bp´, `*208* bp´, `*214* bp´, and `*228* bp´ in the MIXED population, three frequent alleles `*202* bp´, `*208* bp´, and `*214* bp´ in PI population). The results show that allele `*202* bp´, `*208* bp´, and `*214* bp´ had negative effects on NRR, allele `*228* bp´ positively affected NRR in the MIXED population and the PI population. A significant effect ($P < 0.05$) of allele `*214* bp´ was observed only in the PI population and the allele substitution effect was -2.179. The allele substitution effects of the other alleles of *ACR* locus on NRR were not significant (Table 4.26).

Table 4.26:     Allele substitution effects for the locus *ACR* on NRR (%)

| Allele | MIXED | | PI | |
|---|---|---|---|---|
| | LSM ± SE | p-value | LSM ± SE | p-value |
| *202* | -0.103±0.943 | 0.91 | -0.957±1.182 | 0.42 |
| *208* | -1.233±0.761 | 0.11 | -2.179±0.996 | 0.03 |
| *214* | -0.658±1.002 | 0.51 | -1.371±1.273 | 0.28 |
| *228* | 1.104±1.124 | 0.33 | - | - |

4.4.1.2 Number of born piglet alive (NBA)

Association analysis between genotypes and NBA was carried out with model 1 (association analysis for boar fertility traits). The statistical results show that the locus *OPNin6* significantly affected NBA in the MIXED population ($P < 0.05$) and in the PI population ($P < 0.05$), the *ACTN1* locus was significantly associated with NBA in the MIXED population ($P < 0.01$) and in the PI×HA population ($P < 0.01$). The results are shown in Table 4.27. The results revealed that the other loci (*FSHB*, *PRL*, *PRLR*, *INHA*, *INHBA*, *INHBB*, *FST*, *RLN*, *ACTN4*, *ACTG2*, *OPNpro*, *LHB*, *GnRHR*, *ACR*, *RBP4*, and *AR*) had no significant effect on NBA in these populations. For the loci, that had no significant effect on NBA, their estimates are shown in Appendix 9.2.

Table 4.27:    Estimates of least square means for the loci *OPNin6* and *ACTN1* genotype effects on NBA[1] (pig/litter)

| Locus | Genotype | MIXED | PI | PI×HA |
|-------|----------|-------|-----|-------|
|       |          | LSM ± SE | LSM ± SE | LSM ± SE |
| *OPNin6* | AA | $0.176\pm0.068^x$ | $0.209\pm0.069^x$ | $-0.016\pm0.175$ |
|       | AB | $-0.005\pm0.054^y$ | $0.001\pm0.066^y$ | $0.008\pm0.091$ |
|       | BB | $0.04\pm0.047^y$ | $-0.009\pm0.060^y$ | $0.026\pm0.075$ |
| *ACTN1* | AA | $0.045\pm0.042^a$ | $0.065\pm0.053$ | $0.070\pm0.067^a$ |
|       | AB | $0.038\pm0.057^a$ | $0.040\pm0.062$ | $0.133\pm0.114^a$ |
|       | BB | $-0.428\pm1.135^b$ | $-0.283\pm0.154$ | $-1.010\pm0.290^b$ |

[1]Least square means differ significantly for genotypes of the same gene with different subscripts (a, b: $P \leq 0.01$; x, y: $P \leq 0.05$).

Homozygote AA of the *OPNin6* locus had more NBA than homozygote BB and heterozygote AB in the MIXED population and the PI population. No significant effect of the *OPNin6* locus on NBA in the PI×HA population was observed.

Boars with genotype BB of the candidate gene *ACTN1* had less NBA than those with genotype AA and AB in the MIXED population and the PI×HA population. Genotype AA and AB of the *ACTN1* locus had a positive effect on NBA. Genotype BB negatively affected NBA in these populations. The *ACTN1* locus had no significant effect on NBA in the PI population.

(1) Additive and dominance effects on NBA

The candidate genes *OPNin6* and *ACTN1* were significantly associated with NBA. Therefore, additive (a) and dominance (d) effects were estimated for these markers. The results are shown as least square means (LSM) and standard error (SE) in Table 4.28.

For the *ACTN1* locus additive and dominance effects on NBA in the MIXED population and the PI×HA population were highly significant ($P \leq 0.01$). The allele A of the

*ACTN1* had a positive effect and the estimated effect of heterozygote was more than the means of both homozygotes in the MIXED population and the PI×HA population.

For the candidate gene *OPNin6*, only additive effects on NBA in the MIXED population and the PI population were significant. No significant dominance effect was observed in these populations. Negative dominance effect in these populations showed that the estimate of NBA in dependence of the *OPNin6* for heterozygote is less than the means of estimates for both homozygotes.

Table 4.28:     Additive (a) and dominance (d) effects of the alleles of the loci *OPNin6* and *ACTN1* on NBA (pig/litter)

| Locus | Effect | MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|---|---|
| | | Estimate | p | Estimate | p | Estimate | p |
| *OPNin6* | a | 0.086±0.038 | 0.03 | 0.109±0.044 | 0.01 | - | - |
| | d | -0.094±0.063 | 0.13 | -0.099±0.077 | 0.20 | - | - |
| *ACTN1* | a | 0.236±0.069 | <0.01 | - | - | 0.540±0.151 | <0.01 |
| | d | 0.230±0.086 | 0.01 | - | - | 0.603±0.188 | <0.01 |

(2) Allele substitution effects on NBA

In this experiment, all six microsatellite markers *FST*, *INHBA*, *INHBB*, *ACR*, *ACTG2*, and *OPNpro* loci were not significantly associated with the NBA. Therefore, for these loci allele substitution effect on NBA was not estimated.

4.4.2 Sperm quality traits

Sperm quality traits included sperm concentration [SCON ($10^8$/ml)], semen volume per ejaculate [VOL (ml)], sperm motility [MOT (%)], plasma droplets rate [PDR (%)] and abnormal spermatozoa rate [ASR (%)]. Statistical analyses were performed using a mixed model 3 (association analysis for sperm quality traits) with the fixed effects of breed, collected season (eight seasons within two years), age (covariable), genotype of boars and random permanent effect of the boar (repeated measurement). The sperm

quality data were also analysed for PI and PI×HA separately as well as for both together named MIXED population. Results of the statistical analysis are presented as least square means (LSM) and standard errors (SE) of least square means. Rare genotypes of candidate genes were excluded from the statistical analysis.

4.4.2.1 Sperm concentration (SCON)

The effects of candidate gene genotypes on SCON were estimated according to model 3 (association analysis for sperm quality traits). The results of the statistical analysis revealed that four loci *FSHB*, *PRL*, *ACR*, and *INHBB* were significantly associated with SCON. No significant effects of the other loci (*FST*, *RLN*, *PRLR*, *INHA*, *INHBA*, *ACTG2*, *ACTN1*, *ACTN4*, *OPNin6*, *OPNpro*, *LHB*, *GnRHR*, *RBP4*, and *AR*) on SCON were observed, their estimated effects of genotypes are shown in Appendix 9.3.

Table 4.29:    Estimates of least square means for the loci *FSHB* and *PRL* genotype effects on SCON[1] ($10^8$/ml)

| Locus | Genotype | MIXED | PI | PI×HA |
|---|---|---|---|---|
| | | LSM ± SE | LSM ± SE | LSM ± SE |
| *FSHB* | AA | 2.967±0.041 | 2.878±0.045$^y$ | 3.136±0.069 |
| | AB | 3.073±0.063 | 3.081±0.070$^x$ | 2.970±0.116 |
| | BB | 3.041±0.154 | 3.299±0.203$^x$ | 2.700±0.227 |
| *PRL* | AA | 3.049±0.073 | 2.879±0.089 | 3.331±0.120$^{ax}$ |
| | AB | 2.885±0.076 | 2.817±0.105 | 2.965±0.107$^y$ |
| | AC | 2.917±0.057 | 2.905±0.061 | 2.819±0.105$^b$ |
| | BC | 3.258±0.251 | 3.196±0.250 | - |
| | CC | 3.119±0.083 | 3.047±0.083 | 3.239±0.219$^{ax}$ |

[1]Least square means differ significantly for genotypes of the same locus with different subscripts (a, b: $P \leq 0.01$; x, y: $P \leq 0.05$).

Table 4.29 shows estimates of SCON for the boars in dependence of the loci *FSHB* and *PRL*. Significant effects ($P \leq 0.01$) of genotypes of the *FSHB* gene on SCON only in the PI population were observed. Boars with genotype AA had lower SCON than those

with AB and BB genotype. Estimates of effects at this locus ranged from 2.700 $\times 10^8$/ml to 3.299 $\times 10^8$/ml in these populations. The candidate gene *PRL* significantly ($P < 0.01$) associated with sperm concentration in the PI×HA population. Homozygote AA and CC had higher SCON than heterozygote AB ($P \leq 0.05$) and heterozygote AC ($P \leq 0.01$). The estimates of SCON in dependence of *PRL* in this population were from 2.819 to 3.331. This locus had no significant effect on SCON in the other population.

Table 4.30:    Estimates of least square means for the locus *ACR* genotype effects on SCON ($10^8$/ml)

| MIXED | | PI | | PI×HA[A] | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 202/202 | 2.972±0.192 | 202/202 | 3.109±0.200 | 208/208 | 3.129±0.133 |
| 202/208 | 3.067±0.088 | 202/208 | 3.014±0.081 | 208/218 | 3.334±0.308 |
| 202/214 | 2.912±0.150 | 202/214 | 3.017±0.158 | 208/226 | 3.004±0.148 |
| 202/214 | 2.687±0.402 | 202/999 | 2.582±0.142 | 208/228 | 3.124±0.095 |
| 202/999 | 2.668±0.146 | 208/208 | 2.912±0.063 | 208/999 | 3.131±0.142 |
| 208/208 | 2.969±0.065 | 208/214 | 3.015±0.127 | 218/228 | 3.153±0.276 |
| 208/214 | 3.083±0.130 | 208/999 | 2.985±0.091 | 218/999 | 3.405±0.307 |
| 208/228 | 3.034±0.101 | 214/214 | 2.723±0.253 | 226/999 | 3.060±0.376 |
| 208/999 | 3.022±0.073 | 214/999 | 3.050±0.192 | 228/999 | 3.034±0.306 |
| 214/214 | 2.618±0.233 | 999/999 | 2.846±0.400 | 999/999 | 1.911±0.268 |
| 214/228 | 3.180±0.328 | | | | |
| 214/999 | 2.935±0.403 | | | | |
| 228/999 | 2.993±0.202 | | | | |
| 999/999 | 3.295±0.330 | | | | |

[A]Significant effect ($P < 0.01$) by *F*-test

The microsatellite marker *ACR* locus significantly ($P \leq 0.01$) affected SCON in the PI×HA population (Table 4.30). Genotype `*218/999*´ of *ACR* locus had the highest SCON (3.405 $\times 10^8$/ml) and genotype `*999/999*´ had the lowest SCON (1.911 $\times 10^8$/ml) in this population. The differences of least square means between genotypes of *ACR* were tested by multiple pairwise comparisons using Tukey-Kramer adjustment. The

significant differences of genotypes are shown in Table 4.31 and they ranged from $1.092 \times 10^8$/ml to $1.439 \times 10^8$/ml. The significantly biggest difference was between genotype `*218/999*´ and `*999/999*´ with adjusted p-value of 0.0096. The smallest difference was between genotype `*208/226*´ and `*999/999*´ (adjusted p-value of 0.0140).

Table 4.31:    Significant differences of least square means between the *ACR* genotypes for SCON ($10^8$/ml)

| Population | Genotype | Genotype | Estimate | Adjust p-value |
|---|---|---|---|---|
| | 208/208 | 999/999 | 1.127±0.301 | 0.0021 |
| | 208/218 | 999/999 | 1.423±0.409 | 0.0183 |
| | 208/226 | 999/999 | 1.092±0.307 | 0.0140 |
| PI×HA | 208/228 | 999/999 | 1.121±0.283 | 0.0008 |
| | 208/999 | 999/999 | 1.220±0.305 | 0.0026 |
| | 218/228 | 999/999 | 1.241±0.392 | 0.0503 |
| | 218/999 | 999/999 | 1.493±0.408 | 0.0096 |

Estimates of sperm concentration for the boars in dependence of the microsatellite marker *INHBB* locus are shown in Table 4.32. This locus was significantly associated with sperm concentration in the MIXED population ($P < 0.05$) and the PI population ($P < 0.01$). No significant relationship between genotypes of *INHBB* locus and SCON in the PI×HA population was observed.

The estimated value of SCON in dependence of the *INHBB* in the MIXED population ranged from $2.689 \times 10^8$/ml to $3.663 \times 10^8$/ml. Boars with the genotype `*161/173*´ had the highest SCON, and boars with genotype `*161/163*´ had the lowest SCON in the MIXED population. The difference between genotypes of the *INHBB* locus was tested using Tukey-Kramer adjustment. No significant differences ($P > 0.05$) were observed.

In the PI population, the estimates of sperm concentration in dependence of the *INHBB* locus ranged from $2.711 \times 10^8$/ml to $3.919 \times 10^8$/ml. Boars with genotype `*999/999*´ had the highest sperm concentration and boars with genotype `*175/999*´ had the lowest SCON in the PI population. The difference of estimates between genotypes was

examined with multiple pairwise comparisons by Tukey-Kramer adjustment. No significant differences were observed.

Table 4.32:    Estimates of least square means for the locus *INHBB* genotype effects on SCON ($10^8$/ml)

| MIXED[A] | | PI[B] | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 161/163 | 2.689±0.190 | 171/171 | 2.866±0.069 | 159/163 | 3.137±0.256 |
| 161/171 | 2.908±0.134 | 171/173 | 2.841±0.075 | 159/171 | 3.175±0.174 |
| 161/173 | 3.663±0.249 | 171/175 | 3.150±0.079 | 159/173 | 3.163±0.573 |
| 161/175 | 3.048±0.556 | 171/999 | 2.981±0.183 | 159/999 | 3.313±0.332 |
| 163/163 | 3.215±0.392 | 173/173 | 2.843±0.159 | 161/163 | 2.771±0.190 |
| 163/171 | 3.099±0.187 | 173/175 | 2.722±0.114 | 161/171 | 2.990±0.135 |
| 163/173 | 3.241±0.232 | 173/999 | 3.290±0.209 | 161/173 | 3.654±0.286 |
| 163/175 | 2.774±0.232 | 175/175 | 3.225±0.152 | 161/999 | 3.115±0.574 |
| 163/999 | 3.057±0.252 | 175/999 | 2.711±0.226 | 163/163 | 2.505±0.574 |
| 171/171 | 2.963±0.067 | 999/999 | 3.919±0.549 | 163/171 | 3.033±0.407 |
| 171/173 | 2.927±0.070 | | | 171/171 | 3.168±0.140 |
| 163/175 | 3.187±0.087 | | | 171/173 | 3.065±0.139 |
| 163/999 | 3.026±0.165 | | | 171/999 | 2.787±0.335 |
| 173/173 | 2.869±0.152 | | | 173/173 | 2.709±0.405 |
| 173/175 | 2.791±0.125 | | | | |
| 173/999 | 3.063±0.556 | | | | |
| 175/175 | 3.293±0.161 | | | | |
| 175/999 | 3.217±0.324 | | | | |

Significant effect by *F*-test: [A] ($P < 0.05$) and [B] ($P < 0.01$)

(1) Additive and dominance effects on SCON

For the significant markers *FSHB* and *PRL*, additive and dominance effects on SCON were estimated in the different populations and the results are shown in Table 4.33. The additive effect was significant but the dominance effect was not significant. The result

shows that the allele A of *FSHB* locus is linked or associated with the lower SCON in the PI population. There was no significant difference between the effect of heterozygote AB on SCON and the mean effect for both homozygotes AA and BB.

The additive and dominance effects between allele A and C of the *PRL* locus on SCON were estimated and both effects were negative. The dominance effect was highly significant but the additive effect was not significant. There was no significant difference between the effects of homozygote AA and CC on SCON in the PI×HA population, and the effect of heterozygote AC was significantly smaller than the mean effect for both homozygote AA and AC. The effects between allele A and B, B and C of *PRL* gene on SCON were not estimated because homozygote BB was absent in the PI×HA population.

Table 4.33:     Additive (a) and dominance (d) effects of alleles of the loci *FSHB* and *PRL* on SCON ($10^8$/ml)

| Locus | Effect | PI | | PI×HA | |
|-------|--------|------------|------|------------|------|
| | | Estimate + SE | p | Estimate + SE | p |
| *FSHB* | a | 0.211±0.101 | 0.04 | - | - |
| | d | 0.007±0.123 | 0.96 | - | - |
| *PRL* | $a_{(A/C)}$ | - | - | 0.044±0.115 | 0.70 |
| | $d_{(A/C)}$ | - | - | -0.472±0.146 | <0.01 |

(2) Allele substitution effects on SCON

Allele substitution effects at the microsatellite marker loci *ACR* and *INHBB*, that were significantly linked or associated with SCON, were estimated using a multiple linear regression analysis with alleles covariable according to model 4 (for estimation of allele substitution effect on sperm quality traits). The statistical results are shown in Table 4.34. All tested alleles of the *INHBB* locus affected SCON negatively excepting allele `*175* bp´ in the MIXED population. The estimates of allele substitution effects ranged from -0.236 to 0.030 in the MIXED population and the PI population. Effects of allele `*171* bp´, `*173* bp´, and `*175* bp´ of the *INHBB* locus in the MIXED population were

stronger than in the PI population. All allele substitution effects in both populations were not significant. Allele `*161* bp´ and `*163* bp´ of the *INHBB* locus in the PI population had very low frequencies and were replaced by a pseudo allele *999* in the statistical analysis, therefore, there were no estimates of allele substitution effects for these two alleles in this population.

For allele `*208* bp´, `*218* bp´, `*226* bp´, and `*228* bp´ of the locus *ACR*, allele substitution effects in the PI×HA population were estimated. The results show that these four alleles affected positively SCON in the PI×HA population. Estimates of allele substitution effects ranged from 0.151 to 0.501. Allele `*208* bp´ and `*218* bp´ of the *ACR* significantly affected SCON. No significant allele substitution effects of allele `*226* bp´ and `*228* bp´ on SCON in the PI×HA population were observed.

Table 4.34:     Allele substitution effects on SCON ($10^8$/ml) for the microsatellite loci *INHBB* and *ACR*

| Locus | Allele | MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|---|---|
| | | LSM ± SE | p | LSM ± SE | p | LSM ± SE | p |
| *INHBB* | *161* | -0.080±0.157 | 0.61 | - | - | - | - |
| | *163* | -0.090±0.181 | 0.62 | - | - | - | - |
| | *171* | -0.073±0.149 | 0.63 | -0.153±0.117 | 0.19 | - | - |
| | *173* | -0.133±0.152 | 0.38 | -0.236±0.127 | 0.06 | - | - |
| | *175* | 0.030±0.160 | 0.85 | -0.035±0.126 | 0.78 | - | - |
| *ACR* | *208* | - | - | - | - | 0.304±0.119 | 0.01 |
| | *218* | - | - | - | - | 0.501±0.219 | 0.02 |
| | *226* | - | - | - | - | 0.151±0.180 | 0.40 |
| | *228* | - | - | - | - | 0.224±0.098 | 0.10 |

4.4.2.2 Semen volume (VOL)

Association between genotypes of candidate genes and boar semen volume per ejaculate was analysed using model 3 (association analysis for sperm quality traits). The statistical results show clearly that candidate gene *RLN*, *FST*, and *ACTG2* significantly

affected boar VOL in the different populations. No significant effects of the other loci (*FSHB, PRL, PRLR, INHA, INHBA, INHBB, ACTN1, ACTN4, OPNin6, OPNpro, LHB, GnRHR, ACR, RBP4,* and *AR*) on VOL in these populations were observed. Those non significant effects of genotypes at candidate gene loci are shown in Appendix 9.4.

Table 4.35 shows the estimates of semen volume per ejaculate for boars in dependence of the *RLN* locus. This locus significantly affected VOL in PI×HA population ($P \leq 0.01$), homozygote CC of the *RLN* locus had significantly more VOL than heterozygote CA ($P < 0.01$). There was no estimate of effect of the genotype AA because the frequency of homozygote AA was very low in the PI×HA population. No significant effect of genotypes of the *RLN* locus on VOL in the MIXED population and the PI population was observed.

Table 4.35:      Estimates of VOL (ml) for the boars in dependence of the *RLN* gene

| Genotype | MIXED | PI | PI×HA[1] |
|---|---|---|---|
| | LSM ± SE | LSM ± SE | LSM ± SE |
| CC | 260.60±2.92 | 246.98±3.00 | 275.93±5.65[a] |
| CA | 251.87±6.51 | 247.87±6.63 | 234.02±15.51[b] |
| AA | - | - | - |

[1]Least square means differ significantly for genotypes of the same gene with
  different superscripts (a, b: $P \leq 0.01$)

The statistical results show that the microsatellite marker locus *FST* was significantly linked or associated with boar semen volume per ejaculate in the PI population ($P < 0.05$). No significant effect of genotypes of the *FST* on VOL in the MIXED population and the PI×HA population were observed (Table 4.36).

Eighteen genotypes of the *FST* in the MIXED population were used in the statistical analysis. The estimates of VOL for boar in dependence of the *FST* ranged from 229.67 to 292.25 ml/ejaculate. Boars with genotype `*142/999´* of the *FST* had the largest VOL, and boars with genotype `*144/147´* had the smallest VOL.

Table 4.36: Estimates of least square means for the *FST* genotype effects on VOL (ml)

| MIXED | | PI[A] | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 142/142 | 266.45±7.26 | 142/142 | 264.74±9.33 | 142/142 | 268.29±12.12 |
| 142/143 | 252.22±45.01 | 142/143 | 244.62±39.86 | 142/147 | 288.28±19.26 |
| 142/147 | 270.72±12.03 | 142/146 | 277.12±22.82 | 142/148 | 268.70±24.95 |
| 142/148 | 279.22±13.01 | 142/147 | 250.51±16.34 | 142/999 | 304.40±25.04 |
| 142/999 | 292.25±15.09 | 142/148 | 284.61±15.05 | 143/143 | 260.54±12.79 |
| 143/143 | 260.17±5.90 | 143/143 | 255.20±6.15 | 143/147 | 275.22±22.11 |
| 143/147 | 267.73±11.56 | 143/146 | 236.63±14.01 | 143/148 | 264.22±19.29 |
| 143/148 | 251.49±6.87 | 143/147 | 259.69±13.22 | 143/999 | 253.89±38.83 |
| 143/999 | 252.33±13.65 | 143/148 | 242.25±6.47 | 144/144 | 264.38±24.20 |
| 144/144 | 258.46±11.61 | 143/999 | 320.04±39.90 | 144/147 | 241.07±38.49 |
| 144/147 | 229.67±15.99 | 144/144 | 250.96±12.57 | 144/999 | 200.12±54.23 |
| 144/148 | 251.13±12.73 | 144/147 | 219.45±16.28 | 147/147 | 253.68±24.57 |
| 144/999 | 272.75±18.36 | 144/148 | 242.72±10.98 | 147/999 | 300.59±38.37 |
| 147/147 | 257.09±8.81 | 144/999 | 280.14±17.72 | 148/148 | 293.60±54.15 |
| 147/999 | 238.71±14.24 | 146/146 | 213.96±17.68 | 999/999 | 303.24±22.12 |
| 148/148 | 241.17±9.90 | 146/148 | 299.46±39.51 | | |
| 148/999 | 240.71±14.42 | 146/999 | 186.08±39.53 | | |
| 999/999 | 257.65±11.22 | 147/147 | 251.26±8.45 | | |
| | | 147/999 | 216.93±13.99 | | |
| | | 148/148 | 230.45±8.63 | | |
| | | 148/999 | 224.89±13.19 | | |
| | | 999/999 | 252.20±19.87 | | |

[A]Significant effect ($P < 0.01$) by *F*-test

Twenty four genotypes of the *FST* locus in the PI population were used in the statistical analysis, the estimated VOL for boar in dependence of *FST* ranged from 219.45 to 320.04 ml/ejaculate. Boars with genotype `*143/999*´ of *FST* had the largest VOL, and boars with genotype `*147/999*´ had the smallest VOL. The differences of LSM for VOL

between different genotypes were tested by Tukey-Kramer adjustment. No significant difference ($P > 0.05$) was observed.

Fifteen genotypes of the *FST* locus in the PI×HA population were used in the statistical analysis. Estimates of semen volume per ejaculate for boars in dependence from the *FST* locus ranged from 200.12 to 304.40 ml/ejaculate with the largest VOL for genotype `*142/999´* and the smallest VOL for genotype `*144/999´*.

Results in Table 4.37 show significant effects ($P \leq 0.01$) of genotypes of *ACTG2* locus on boar semen volume per ejaculate in the PI boars. No significant effects in the MIXED and the PI×HA population were observed.

In total, 21 genotypes of *ACTG2* locus in the MIXED population were used in the statistical analysis. Estimates of semen volume for boar in dependence of *ACTG2* ranged from 196.44 to 321.85 ml/ejaculate. The largest VOL was attributed to genotype `*117/117´*. The smallest VOL in this population belonged to genotype `*103/117´*.

Twenty genotypes of the *ACTG2* locus in the PI population were used in the statistical analysis. Estimates of semen volume for boars in dependence of the *ACTG2* in the PI population varied from 205.99 to 308.03 ml/ejaculate. Boars with genotype `*93/117´* had the smallest VOL and boars with genotypes `*117/117´* had the largest VOL. The differences of least square means between genotypes of *ACTG2* locus in the PI population were tested with multiple pairwise comparisons by Tukey-Kramer adjustment. No significant difference ($P > 0.05$) was observed.

Twenty three genotypes of the *ACTG2* gene in the PI×HA population were used in the statistical analysis. Estimated VOL for boars in dependence of the *ACTG2* locus in this population varied from 164.20 to 341.66 ml/ejaculate. Boars with the genotype `*97/97´* had the smallest VOL, and boars with genotype `*999/999´* had the largest VOL.

Table 4.37:     Estimates of least square means for the locus *ACTG2* genotype effects on
                VOL (ml)

| MIXED | | PI[A] | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 93/93 | 239.64±14.20 | 93/93 | 225.17±15.07 | 93/93 | 256.53±32.11 |
| 93/103 | 250.57±13.24 | 93/103 | 237.15±11.52 | 93/97 | 249.79±32.05 |
| 93/109 | 257.87±7.71 | 93/109 | 235.39±8.70 | 93/109 | 285.54±15.42 |
| 93/111 | 270.91±13.56 | 93/111 | 269.44±15.11 | 93/111 | 262.76±27.82 |
| 93/117 | 230.20±20.09 | 93/117 | 205.99±19.20 | 93/117 | 282.29±55.33 |
| 93/999 | 254.00±16.95 | 93/999 | 243.24±23.24 | 93/999 | 316.18±55.50 |
| 103/103 | 262.76±15.19 | 103/103 | 249.29±13.30 | 97/97 | 164.20±57.22 |
| 103/109 | 249.33±7.14 | 103/109 | 233.11±7.67 | 97/103 | 268.35±22.62 |
| 103/111 | 275.40±10.18 | 103/111 | 261.56±8.70 | 97/109 | 272.17±39.45 |
| 103/117 | 196.44±44.72 | 103/999 | 247.54±13.50 | 97/111 | 268.32±18.48 |
| 103/999 | 261.69±11.24 | 109/109 | 238.08±8.51 | 97/117 | 286.34±55.31 |
| 109/109 | 254.54±8.28 | 109/111 | 240.55±7.39 | 97/999 | 233.15±31.98 |
| 109/111 | 256.64±7.41 | 109/117 | 246.37±14.07 | 103/109 | 266.10±15.37 |
| 109/117 | 259.52±12.41 | 109/999 | 274.27±10.68 | 103/117 | 207.52±55.37 |
| 109/999 | 282.49±10.67 | 111/111 | 265.89±10.36 | 103/999 | 336.82±55.53 |
| 111/111 | 278.97±11.94 | 111/117 | 267.82±14.27 | 109/109 | 274.65±19.66 |
| 111/117 | 276.37±15.20 | 111/999 | 273.28±12.04 | 109/111 | 276.21±18.48 |
| 111/999 | 269.56±9.32 | 117/117 | 308.03±39.82 | 109/117 | 269.46±24.87 |
| 117/117 | 321.85±14.76 | 117/999 | 226.94±23.00 | 109/999 | 287.22±39.27 |
| 117/999 | 248.22±18.23 | 999/999 | 240.94±15.07 | 111/117 | 256.97±55.73 |
| 999/999 | 251.77±12.43 | | | 111/999 | 267.20±32.05 |
| | | | | 117/999 | 258.57±39.12 |
| | | | | 999/999 | 341.66±39.53 |

[A]Significant effect ($P \leq 0.01$) by *F*-test

(1) Additive and dominance effects on VOL

In this experiment, the candidate gene *RLN* was significantly linked or associated with semen volume trait, but the homozygote AA of *RLN* locus was very rare or absent in the different populations, therefore, the additive and dominance effects on VOL were not estimated for this diallelic locus.

(2) Allele substitution effects on VOL

The statistical results showed that the microsatellite marker loci *FST* and *ACTG2* were significantly linked or associated with boar semen volume in the MIXED population and the PI population. For locus *FST* and *ACTG2* the allele substitution effects on VOL were estimated using multiple linear regression analysis with allele covariable according to model 4 (for estimation of allele substitution effect on sperm quality traits). The results are shown in Table 4.38.

Table 4.38:     Allele substitution effects on VOL (ml) in the PI population for the loci *FST* and *ACTG2*

| Locus | Allele | Estimate ± SE | p | Locus | Allele | Estimate ± SE | p |
|-------|--------|---------------|------|-------|--------|---------------|------|
|       | *142*  | 16.11±7.82    | 0.04 |       | *93*   | -16.01±6.99   | 0.02 |
|       | *143*  | 7.89±7.10     | 0.27 |       | *103*  | -9.26±6.70    | 0.17 |
| *FST* | *144*  | 6.16±8.68     | 0.49 | *ACTG2* | *109* | -12.85±6.14   | 0.04 |
|       | *147*  | 2.16±7.97     | 0.79 |       | *111*  | 3.25±6.39     | 0.61 |
|       | *148*  | -3.20±7.74    | 0.68 |       | *117*  | -6.41±9.59    | 0.50 |

Estimated values of allele substitution effect on VOL for the *FST* locus in the MIXED population ranged from -6.10 ml/ejaculate to 7.90 ml/ejaculate. Allele `*142* bp´ and `*143* bp´ had positive effects, whereas allele `*144* bp´, `*147* bp´, and `*148* bp´ had negative effects. The strongest effect was allele `*142* bp´. Allele `*144* bp´ had a weak effect on VOL in this population. All five allele substitution effects on VOL in the MIXED population were not significant.

In the PI population, allele `*142* bp´ of the *FST* locus significantly affected VOL. Allele substitution effect was estimated to be 16.11±7.82 ($P < 0.05$). The other alleles of the *FST* locus also positively affected VOL excepting allele `*148* bp´. But no significant allele substitution effects of these alleles on VOL were observed.

Alleles `*93* bp´, `*103* bp´, `*109* bp´, `*111* bp´, and `*117* bp´ of the *ACTG2* locus were used in the statistical analysis for estimating their allele substitution effects. Allele `*111* bp´ of the *ACTG2* locus positively affected boar semen volume per ejaculate, the other allele substitution effects were negative. All allele substitution effects varied from -16.01 ml/ejaculate to 3.25 ml/ejaculate. The strongest effect on VOL in the PI population was allele `*93* bp´. Allele `*93* bp´ and `*103* bp´ significantly affected VOL in this population. No significant effect on VOL of the other alleles of the *ACTG2* locus was observed.

4.4.2.3 Sperm motility (MOT)

Association study between genotypes of candidate genes and boar sperm motility trait was performed with model 3 (association analysis for sperm quality traits) using the procedure "PROC MIXED" of the SAS software package, followed by multiple pairwise comparisons using Tukey-Kramer adjustment.

Results showed that candidate genes *OPNin6, RBP4, GnRHR, ACR*, and *ACTG2* were significantly associated with sperm motility trait. No significant effects of the loci *FSHB, LHB, RLN, PRL, PRLR, FST, INHA, INHBA, INHBB, AR, OPNpro, ACTN1*, and *ACTN4* were observed in this experiment. The no significant effects on MOT were given as least square means and standard error in Appendix 9.5.

The *OPNin6* locus had a weakly significant effect ($P \leq 0.05$) on MOT in the MIXED population. Homozygote AA of the *OPNin6* locus had lower sperm motility than homozygote BB and heterozygote AB as shown in Table 4.39. No significant effects of genotypes of the locus *OPNin6* in the PI population and the PI×HA were observed.

Significant effects of genotypes of the *RBP4* locus on sperm motility were detected only in the PI population ($P < 0.05$) as shown in Table 4.39. It shows clearly that boars with genotype AA had higher MOT than genotype AB in the PI population. This result in the MIXED population and the PI×HA population was not confirmed. The estimated effects of genotypes of the *RBP4* locus were not significant in the MIXED population and the PI×HA population. The genotype BB was absent in this experiment.

Table 4.39:    Estimates of least square means for the loci *OPNin6*, *RBP4*, and *GnRHR* genotype effects on MOT[1] (%)

| Locus | Genotype | MIXED | PI | PI×HA |
|---|---|---|---|---|
| | | LSM ± SE | LSM ± SE | LSM ± SE |
| *OPNin6* | AA | 84.43±0.26$^y$ | 84.74±0.25 | 84.32±0.82 |
| | AB | 85.07±0.21$^x$ | 85.49±0.24 | 84.52±0.36 |
| | BB | 85.11±0.11$^x$ | 85.36±0.22 | 84.93±0.27 |
| *RBP4* | AA | 85.42±0.26 | 85.65±0.24$^x$ | 84.31±1.17 |
| | AB | 84.89±0.14 | 85.06±0.17$^y$ | 84.77±0.21 |
| | BB | - | – | – |
| *GnRHR* | CC | 84.80±0.15$^y$ | 85.05±0.15$^b$ | 84.77±0.25 |
| | CG | 85.56±0.28$^x$ | 86.48±0.40$^a$ | 84.71±0.37 |
| | GG | - | – | |

[1]Least square means differ significantly for genotypes of the same gene with different superscripts (a, b: $P \leq 0.01$, x, y: $P \leq 0.05$).

Estimates of sperm motility for boars in dependence of the *GnRHR* locus (Table 4.39) show that locus *GnRHR* significantly affected MOT in the MIXED population ($P < 0.05$) and the PI population ($P < 0.01$). The heterozygote GC had higher sperm motility than homozygote CC, this result was similar in both populations. In the PI×HA population, no significant effect of genotypes of the *GnRHR* locus was observed. Genotype GG was very rare or absent in the different populations, therefore, there were no estimates for genotype GG of *GnRHR* locus.

Effects of genotypes of the *ACR* locus on sperm motility were estimated as shown in Table 4.40. The locus *ACR* was significantly associated with sperm motility in the MIXED population ($P < 0.05$) and the PI population ($P \leq 0.01$). No significant effect on MOT for genotypes of *ACR* locus in the PI×HA population was observed.

In total, 14 genotypes of the *ACR* locus in the MIXED population were used in the statistical analysis. Estimates of sperm motility for boar in dependence of the *ACR* ranged from 82.34% to 86.37 %. The highest motility was genotype `*214/214´* and the lowest MOT was genotype `*214/999´*. The estimated differences between genotypes of the *ACR* were tested with multiple pairwise comparisons using Tukey-Kramer adjustment. No significant difference ($P > 0.05$) was observed.

Ten genotypes of the *ACR* locus in the PI population were used in the statistical analysis. Estimates of sperm motility for boars in dependence of the *ACR* locus varied from 83.26% to 87.83%. Boars with genotype `*214/214´* had the highest MOT but boars with genotype `*999/999´* had the lowest MOT. The difference of least square means between genotypes of the *ACR* locus was examined with multiple pairwise comparisons using Tukey-Kramer adjustment. No significant effect ($P > 0.05$) was observed.

In the PI×HA population, 10 genotypes of the *ACR* locus were used in the statistical analysis. Table 4.40 showed that estimates of sperm motility for boar in dependence of *ACR* varied from 83.30% to 86.02%. The highest MOT was genotype `*228/999´* and the lowest MOT was genotype `*226/999´* in this population.

Table 4.40:    Estimates of least square means for the *ACR* genotype effects on MOT (%)

| MIXED[A] | | PI[B] | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 202/202 | 84.45±0.69 | 202/202 | 84.66±0.73 | 208/208 | 84.93±0.52 |
| 202/208 | 85.35±0.32 | 202/208 | 85.54±0.29 | 208/218 | 84.48±1.20 |
| 202/214 | 84.72±0.54 | 202/214 | 84.75±0.57 | 208/226 | 84.90±0.58 |
| 202/214 | 86.31±1.46 | 202/999 | 86.61±0.52 | 208/228 | 84.35±0.37 |
| 202/999 | 86.37±0.53 | 208/208 | 84.90±0.23 | 208/999 | 84.89±0.56 |
| 208/208 | 84.83±0.23 | 208/214 | 85.80±0.46 | 218/228 | 85.70±1.07 |
| 208/214 | 85.51±0.47 | 208/999 | 84.75±0.33 | 218/999 | 85.26±1.20 |
| 208/228 | 84.71±0.36 | 214/214 | 87.83±0.92 | 226/999 | 83.30±1.47 |
| 208/999 | 84.75±0.26 | 214/999 | 83.77±1.03 | 228/999 | 86.02±1.19 |
| 214/214 | 87.65±0.84 | 999/999 | 83.26±1.45 | 999/999 | 85.03±1.05 |
| 214/228 | 85.85±1.19 | | | | |
| 214/999 | 82.34±1.46 | | | | |
| 228/999 | 84.86±0.73 | | | | |
| 999/999 | 84.12±1.19 | | | | |

Significant effect by *F*-test: [A] ($P < 0.05$) and [B] ($P < 0.01$)

Effects of genotypes of the *ACTG2* on sperm motility were estimated as shown in Table 4.41. The locus *ACTG2* was significantly linked or associated with sperm motility in the PI×HA population ($P < 0.05$). No significant associations between genotypes of the *ACTG2* and MOT in the MIXED population and the PI population were observed.

In total, 21 genotypes of the *ACTG2* locus in the MIXED population were used in the statistical analysis. Estimates of sperm motility for boars in dependence of the *ACTG2* locus ranged from 83.29% to 88.50%. The highest MOT was genotype `*117/117*´, and boars with genotype `*93/111*´ had the lowest sperm motility in this population.

Table 4.41: Estimates of least square means for the locus *ACTG2* genotype effects on MOT (%)

| MIXED | | PI | | PI×HA[A] | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 93/93 | 84.45±0.66 | 93/93 | 84.32±0.80 | 93/93 | 85.01±1.11 |
| 93/103 | 85.18±0.62 | 93/103 | 85.45±0.61 | 93/97 | 85.88±1.10 |
| 93/109 | 84.78±0.36 | 93/109 | 85.35±0.46 | 93/109 | 84.00±0.53 |
| 93/111 | 83.29±0.63 | 93/111 | 82.74±0.80 | 93/111 | 84.42±0.96 |
| 93/117 | 86.08±0.94 | 93/117 | 86.06±1.05 | 93/117 | 87.07±1.90 |
| 93/999 | 85.01±0.79 | 93/999 | 83.92±1.23 | 93/999 | 85.64±1.91 |
| 103/103 | 83.70±0.71 | 103/103 | 83.96±0.70 | 97/97 | 89.95±1.98 |
| 103/109 | 85.24±0.33 | 103/109 | 85.30±0.40 | 97/103 | 84.67±0.78 |
| 103/111 | 84.93±0.47 | 103/111 | 85.21±0.46 | 97/109 | 83.26±1.36 |
| 103/117 | 86.75±2.08 | 103/999 | 85.27±0.71 | 97/111 | 84.71±0.64 |
| 103/999 | 85.14±0.52 | 109/109 | 85.43±0.45 | 97/117 | 88.91±1.90 |
| 109/109 | 84.98±0.39 | 109/111 | 85.49±0.39 | 97/999 | 84.69±1.10 |
| 109/111 | 85.10±0.34 | 109/117 | 86.05±0.74 | 103/109 | 85.47±0.53 |
| 109/117 | 85.83±0.58 | 109/999 | 85.38±0.56 | 103/117 | 86.67±1.90 |
| 109/999 | 84.67±0.50 | 111/111 | 84.73±0.54 | 103/999 | 87.38±1.91 |
| 111/111 | 84.47±0.56 | 111/117 | 86.60±0.76 | 109/109 | 84.32±0.68 |
| 111/117 | 85.92±0.71 | 111/999 | 84.93±0.64 | 109/111 | 84.48±0.64 |
| 111/999 | 84.53±0.43 | 117/117 | 88.76±2.10 | 109/117 | 85.70±0.86 |
| 117/117 | 88.50±2.08 | 117/999 | 84.50±1.21 | 109/999 | 82.11±1.35 |
| 117/999 | 85.05±0.85 | 999/999 | 86.07±0.80 | 111/117 | 82.16±1.94 |
| 999/999 | 85.94±0.58 | | | 111/999 | 82.25±1.11 |
| | | | | 117/999 | 84.09±1.34 |
| | | | | 999/999 | 86.32±1.37 |

[A]Significant effect ($P < 0.05$) by *F*-test

In the PI population, 20 genotypes of the *ACTG2* locus were used in the statistical analysis. Estimates of sperm motility for boar in dependence of the *ACTG2* locus varied

from 82.74% to 88.76%. Boars with genotype `*117/117*´ had the highest MOT, and boars with genotype `*93/111*´ had the lowest MOT.

In total, 23 genotypes of the *ACTG2* locus in the PI×HA population were used in the statistical analysis. Estimates of MOT for boars in dependence of the *ACTG2* ranged from 82.11% to 89.95%. Boars with genotype `*97/97*´ had the highest MOT, and boars with genotype `*111/117*´ had the lowest MOT in the PI×HA population. Differences of least square means between the genotypes were checked with multiple pairwise comparisons using Tukey-Kramer adjustment. No significant ($P > 0.05$) difference of estimates between genotypes of the *ACTG2* locus was observed.

(1) Additive and dominance effects on MOT

Candidate genes *OPNin6*, *RBP4*, and *GnRHR* were significantly linked or associated with sperm motility. Homozygote BB of the locus *RBP4* was absent, homozygote GG at the *GnRHR* was very rare or absent in the different populations. Therefore, only for the locus *OPNin6* the additive and dominance effects on MOT were estimated as shown in Table 4.42, the additive effect on MOT was significant. This result showed that allele B of the *OPNin6* was the favourable allele and estimates of MOT for heterozygote were not significant different from means of estimates for both homozygotes.

Table 4.42:     Additive (a) and dominance (d) effects of alleles of the *OPNin6* gene on MOT (%)

| Locus | Effect | MIXED | |
|-------|--------|-------|---|
|       |        | LSM ± SE | p |
| *OPNin6* | a | -0.34±0.15 | 0.02 |
|          | d | 0.30±0.25 | 0.23 |

(2) Allele substitution effects on MOT

Microsatellite marker loci *ACR* and *ACTG2* significantly affected boar sperm quality in
the different populations. Allele substitution effects for loci *ACR* and *ACTG2* in relevant
populations were estimated using multiple linear regression analysis with allele
covariable according to model 4 (for estimation of allele substitution effect on sperm
quality traits). The results are shown in Table 4.43.

For allele `202 bp´, `208 bp´, `214 bp´, and `228 bp´ of the *ACR* locus, estimates of
allele substitution effects on sperm motility ranged from 0.08 to 0.61. In the MIXED
population allele `202 bp´ and `214 bp´ positively affected on MOT, whereas allele `208
bp´ and `228 bp´ had negative effects on MOT. All allele substitution effects were not
significant on MOT. In the PI population, allele `228 bp´ was very rare and replaced by
a pseudo allele *999* in the statistical analysis, no allele substitution effect was estimated
for it. The other 3 frequent alleles positively affected MOT in the PI population, but no
significant allele substitution effect of these alleles was observed.

Table 4.43:     Allele substitution effects on MOT (%) for the loci *ACR* and *ACTG2*

| Locus | Allele | MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|---|---|
| | | LSM ± SE | p | LSM ± SE | p | LSM ± SE | p |
| *ACR* | *202* | 0.19±0.33 | 0.55 | 0.40±0.37 | 0.28 | - | - |
| | *208* | -0.08±0.27 | 0.76 | 0.03±0.31 | 0.93 | - | - |
| | *214* | 0.52±0.35 | 0.14 | 0.61±0.40 | 0.13 | - | - |
| | *228* | -0.02±0.41 | 0.96 | - | - | - | - |
| *ACTG2* | *93* | - | - | - | - | 0.39±0.58 | 0.50 |
| | *103* | - | - | - | - | 0.94±0.67 | 0.16 |
| | *109* | - | - | - | - | 0.03±0.52 | 0.96 |
| | *111* | - | - | - | - | -0.31±0.65 | 0.64 |
| | *117* | - | - | - | - | 1.27±0.81 | 0.11 |

In total, for 5 alleles `93 bp´, `103 bp´, `109 bp´, `111 bp´, and `117 bp´ of the *ACTG2*
locus in the PI×HA population, allele substitution effects on MOT were estimated as

shown in Table 4.43. All alleles positively affected MOT excepting allele `*111* bp´. Estimates of allele substitution effects varied from -0.31 to 1.27. Allele `*117* bp´ had the strongest effect on MOT in the PI×HA population. However, no significant allele substitution effect was observed.

## 4.4.2.4 Sperm plasma droplet rate (PDR)

Association between genotypes of candidate genes and boar sperm plasma droplet rate trait was estimated using model 3 (association analysis for sperm quality traits). The statistical analysis revealed that the loci *GnRHR* and *INHBA* significantly affected PDR. No significant effects of the other candidate genes (*FSHB*, *LHB*, *RLN*, *RLN*, *PRL*, *PRLR*, *INHA*, *INHBB*, *RBP4*, *ACR*, *OPNin6*, *OPNpro*, *ACTG2*, *ACTN1*, and *ACTN4*) on PDR were observed. For no significant effect relevant loci, estimates of PDR are shown as least square means and standard error in Appendix 9.6.

Table 4.44:     Estimates of least square means for the locus *GnRHR* genotype effects on PDR[1] (%)

| Genotype | MIXED[2] | PI[2] | PI×HA |
|---|---|---|---|
| | LSM ± SE | LSM ± SE | LSM ± SE |
| CC | $6.80\pm0.07^a$ | $6.69\pm0.07^a$ | $6.83\pm0.12$ |
| CG | $6.39\pm0.13^b$ | $6.06\pm0.19^b$ | $6.69\pm0.18$ |
| GG | - | - | - |

[1]Least square means differ significantly for genotypes of the same gene with different superscripts (a, b: $P \leq 0.01$); [2]Significant effect ($P < 0.01$) by *F*-test.

In Table 4.44, the estimates of PDR for boars in dependence of the *GnRHR* locus are shown. The results demonstrated that the gene *GnRHR* significantly affected PDR in the MIXED population ($P < 0.01$) and the PI population ($P < 0.01$), but this locus had no significant effect on PDR in the PI×HA population.

Boars with genotype CG of the *GnRHR* locus had higher PDR than those with genotype CC in the MIXED and the PI population. Homozygote GG was very rare or absent, therefore, there was no estimate for this genotype of *GnRHR* locus to show in this table.

The statistical analysis revealed that the gene *INHBA* was significantly associated with boar sperm plasma droplet rate in the MIXED population ($P < 0.05$), but no significant effects on PDR in the other populations were observed (Table 4.45).

In total, 9 genotypes of the *INHBA* locus in the MIXED population were used in the statistical analysis. Estimates of PDR for boars in dependence of the *INHBA* locus ranged from 5.70% to 8.76%. The highest PDR was genotype `*256/999*´ and boar with genotype `*254/256*´ had the lowest PDR in the MIXED population (Table 4.45). Differences of least square means between the genotypes were examined using Tukey-Kramer adjustment. No significant ($P > 0.05$) difference of estimates between genotypes was observed.

In the PI population, 6 frequent genotypes of the *INHBA* locus were used in the statistical analysis. Estimates of PDR for boars in dependence of the *INHBA* locus varied from 5.45% to 7.25%. Homozygote `*254/254*´ animals had the highest PDR and boars with genotype `*252/999*´ had the lowest PDR.

In the PI×HA population, eight frequent genotypes of the *INHBA* locus were used in the statistical analysis. Estimated effects of genotypes of the *INHBA* locus ranged from 6.04% to 8.30%. Boars with genotype `*256/999*´ had the highest PDR and boars with genotype `*254/254*´ had the lowest PDR in this population.

Table 4.45:     Estimates of least square means for the locus *INHBA* genotype effects on PDR (%)

| MIXED[A] | | PI | | PI×HA | |
| --- | --- | --- | --- | --- | --- |
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 252/252 | 6.65±0.08 | 252/252 | 6.61±0.08 | 252/252 | 6.55±0.14 |
| 252/254 | 6.72±0.11 | 252/254 | 6.62±0.13 | 252/254 | 6.82±0.18 |
| 252/256 | 6.86±0.17 | 252/999 | 6.38±0.22 | 252/256 | 7.06±0.25 |
| 252/999 | 6.43±0.29 | 254/254 | 7.25±0.37 | 252/999 | 7.00±0.36 |
| 254/254 | 7.48±0.35 | 252/999 | 5.45±0.56 | 254/254 | 8.70±0.98 |
| 254/256 | 5.70±0.56 | 999/999 | 6.91±0.70 | 254/256 | 6.04±0.92 |
| 254/999 | 5.57±0.96 | | | 256/256 | 7.95±0.93 |
| 256/256 | 6.67±0.08 | | | 256/999 | 8.30±0.97 |
| 256/999 | 8.26±0.71 | | | | |

[A]Significant effect ($P < 0.05$) by *F*-test

(1) Additive and dominance effects on PDR

The candidate gene *GnRHR* significantly affected sperm plasma droplet rate, but homozygote GG was very rare or absent in the different populations, therefore, there were no additive and dominance effects on PDR to be estimated.

(2) Allele substitution effects on PDR

The microsatellite marker *INHBA* locus had a significant effect on sperm plasma droplet rate in this experiment. For this locus allele substitution effect was estimated with multiple linear regression analysis with allele covariable according to model 4 (for estimation of allele substitution effect on sperm quality traits). The results are shown in Table 4.46.

Table 4.46:    Allele substitution effects on PDR (%) for the locus *INHBA*

| Locus | Allele | MIXED | |
| --- | --- | --- | --- |
| | | LSM ± SE | p |
| | *252* | 0.06±0.27 | 0.82 |
| *INHBA* | *254* | 0.19±0.28 | 0.49 |
| | *256* | 0.21±0.31 | 0.49 |

For three frequent alleles `*252* bp´, `*254* bp´, and `*256* bp´, the allele substitution effects were positively on PDR in the MIXED population. Estimates of allele substitution effects on PDR ranged from 0.06 to 0.21. The strongest effect was allele `*256* bp´, and allele `*252* bp´ had a weak effect on PDR. For locus *INHBA*, non significant allele substitution effects on PDR were observed.

4.4.2.5 Abnormal sperm rate (ASR)

Association between genotypes of candidate genes and abnormal sperm rate trait was analysed with model 3 (association analysis for sperm quality traits). The statistical analysis results showed that candidate genes *INHA, GnRHR, AR, INHBA*, and *OPNpro* significantly affected ASR. No significant effects of the other loci *(FSHB, LHB, RLN, PRL, PRLR, FST, INHBB, RBP4, ACR, OPNin6, ACTG2, ACTN1*, and *ACTN4)* on ASR were observed. For the no significant effect relevant loci, estimates of their effects on ASR are shown as least square means and standard errors in Appendix 9.7.

Table 4.47 shows estimates of abnormal sperm rate for boars in dependence of the *INHA, GnRHR*, and *AR* loci.

The candidate gene *INHA* was significantly associated with ASR in the PI ($P \leq 0.05$). Homozygote BB animals had significantly higher ($P < 0.05$) ASR than heterozygote AB in the PI population. No significant effects of the INHA genotypes on ASR in the MIXED population and the PI×HA population were observed.

The candidate gene *GnRHR* was significantly associated with ASR in the MIXED population ($P < 0.05$) and the PI population ($P \leq 0.01$), but no significant effect on ASR in the PI×HA population was observed. Homozygote CC of the *GnRHR* locus had significantly higher ASR than heterozygote CG ($P < 0.05$) in the MIXED population and the PI population. Homozygote GG genotypes were rare or absent in the different populations, therefore, the genotype GG was excluded from statistical analysis.

Significant effect ($P < 0.05$) of the *AR* on abnormal sperm rate in the MIXED population was observed. Boars with genotype `*173* bp´ had a significantly higher ASR than those with genotype `*172* bp´ ($P < 0.01$) as well as genotype `*196* bp´ and `*186* bp´ ($P < 0.05$). No significant effect of the *AR* on ASR in the PI and the PI×HA population was observed.

Table 4.47:     Estimates of least square means for the loci *INHA, GnRHR*, and *AR* genotype effects on ASR (%)

| Locus | Genotype | MIXED | PI | PI×HA |
|-------|----------|-------|-----|-------|
| | | LSM ± SE | LSM ± SE | LSM ± SE |
| *INHA* | AA | 6.78±0.64 | 6.61±0.89$^{xy}$ | 6.93±0.91 |
| | AB | 6.80±0.12 | 6.40±0.18$^{y}$ | 7.14±0.17 |
| | BB | 6.89±0.10 | 6.87±0.09$^{x}$ | 6.53±0.22 |
| *GnRHR* | CC | 6.95±0.09$^{x}$ | 6.84±0.09$^{x}$ | 6.99±0.16 |
| | CG | 6.51±0.17$^{y}$ | 6.23±0.24$^{y}$ | 6.77±0.23 |
| | GG | - | - | - |
| *AR* | *196* bp | 6.83±0.13$^{y}$ | 6.75±0.10 | 6.80±0.33 |
| | *186* bp | 6.82±0.12$^{y}$ | 6.72±0.20 | 6.89±0.15 |
| | *173* bp | 7.53±0.27$^{ax}$ | 7.36±0.28 | 8.11±0.75 |
| | *172* bp | 6.57±0.27$^{b}$ | 6.47±0.25 | - |

[a, b, x, y]Least square means differ significantly for genotypes of the same gene with different superscripts (a, b: $P \leq 0.01$, x, y: $P \leq 0.05$)

Results show that the microsatellite marker *INHBA* locus significantly affected ASR in the MIXED ($P < 0.01$) in the PI ($P < 0.05$) and in the PI×HA population ($P < 0.05$) as shown in Table 4.48.

Table 4.48:     Estimates of least square means for the locus *INHBA* genotype effects on ASR (%)

| MIXED[A] | | PI[B] | | PI×HA[C] | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 252/252 | 6.70±0.10 | 252/252 | 6.72±0.10 | 252/252 | 6.47±0.19 |
| 252/254 | 7.11±0.14 | 252/254 | 6.94±0.17 | 252/254 | 7.39±0.24 |
| 252/256 | 6.77±0.22 | 252/999 | 6.51±0.27 | 252/256 | 6.98±0.33 |
| 252/999 | 6.74±0.36 | 254/254 | 7.43±0.47 | 252/999 | 7.06±0.47 |
| 254/254 | 7.69±0.45 | 252/999 | 5.99±0.72 | 254/254 | 9.24±1.27 |
| 254/256 | 6.27±0.72 | 999/999 | 9.05±0.90 | 254/256 | 7.51±1.22 |
| 254/999 | 6.78±1.24 | | | 256/256 | 7.12±1.23 |
| 256/256 | 7.11±0.88 | | | 256/999 | 8.38±1.26 |
| 256/999 | 9.77±0.91 | | | | |

Significant effect by *F*-test: [A] ($P < 0.01$), [B] ($P < 0.05$), [C] ($P < 0.05$).

In total, 9 genotypes of the *INHBA* locus in the MIXED population were used in the statistical analysis. Estimates of ASR for boars in dependence of *INHBA* locus ranged from 6.27% to 9.77%. Boars with genotype `*256/999*´ had the highest ASR and boars with genotype `*254/256*´ had the lowest ASR in the MIXED population. The differences of LSMs between the genotypes were checked with multiple pairwise comparisons using Tukey-Kramer adjustment. Significant differences of LSM between the genotypes varied from 3.00 % to 3.07% and they are shown in Table 4.49. The biggest difference was between genotype `*252/252*´ and `*256/999*´ with adjust p-value of 0.0215. The significantly smallest difference was between genotype `*252/256*´ and `*256/999*´ (adjust p-value of 0.0358).

In the PI population, six genotypes of the *INHBA* locus were used in the statistical analysis. Estimates of ASR in dependence of the *INHBA* locus ranged from 5.99% to

9.05%. Boars with genotype `*999/999*´ had the highest ASR, and boars with genotypes `*252/999*´ the lowest ASR in the PI population. Differences of LSM between genotypes of *INHBA* locus were tested with Tukey-Kramer adjustment. No significant ($P > 0.05$) difference of LSM between the genotypes in the PI population was observed.

In the PI×HA population eight frequent genotypes of the *INHBA* locus were used in the statistical analysis. Estimates of ASR for boars in dependence of the *INHBA* locus ranged from 6.40 % to 9.24% in the PI×HA population. Boars with homozygote `*252/252*´ genotype had the lowest ASR, and boars with genotype `*254/254*´ had the highest ASR. The difference of LSM between the genotypes in the PI×HA population was examined with multiple pairwise comparisons using Tukey-Kramer adjustment. No significant ($P > 0.05$) difference between the genotypes in the PI×HA population was observed.

Table 4.49:     Significant difference of least square means for ASR (%) between the *INHBA* genotypes

| Population | Genotype | Genotype | Estimate | Adjustment p-value |
|-----------|----------|----------|----------|--------------------|
|           | 252/252  | 256/999  | -3.07±0.91 | 0.0008           |
| MIXED     | 252/256  | 256/999  | -3.00±0.93 | 0.0013           |
|           | 252/999  | 256/999  | -3.03±0.98 | 0.0019           |

Results show clearly that the candidate gene *OPNpro* was significantly associated with the trait ASR ($P < 0.05$) in the PI population. No significant effects of this locus on ASR in the MIXED population and the PI×HA population were observed (Table 4.50).

In total, fifteen genotypes of the *OPNpro* locus in the MIXED population were used in the statistical analysis. Estimates of ASR for boars in dependence of the *OPNpro* locus ranged from 5.36% to 9.12%. Boars with genotype `*118/999*´ had the highest ASR, and boars with genotype `*146/999*´ had the lowest ASR in the MIXED population. No significant difference between the genotypes was observed.

Table 4.50:     Estimates of least square means for the locus *OPNpro* genotype effects on ASR (%)

| MIXED | | PI[A] | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 118/118 | 6.98±0.35 | 118/118 | 6.94±0.34 | 118/138 | 7.06±1.32 |
| 118/142 | 7.05±0.23 | 118/142 | 6.96±0.25 | 118/142 | 7.19±0.50 |
| 118/146 | 7.01±0.45 | 118/146 | 7.83±0.63 | 118/146 | 6.25±0.66 |
| 118/148 | 6.82±0.20 | 118/148 | 6.79±0.21 | 118/148 | 6.71±0.47 |
| 118/999 | 9.12±0.90 | 118/999 | 11.34±1.26 | 126/142 | 7.06±0.77 |
| 142/142 | 6.83±0.23 | 142/142 | 6.78±0.21 | 126/148 | 7.55±0.44 |
| 142/146 | 7.05±0.34 | 142/146 | 6.58±0.51 | 126/999 | 6.03±0.93 |
| 142/148 | 6.85±0.15 | 142/148 | 6.86±0.15 | 138/142 | 7.31±0.60 |
| 142/999 | 7.28±0.39 | 142/999 | 7.98±1.24 | 138/146 | 5.50±1.33 |
| 146/146 | 6.66±0.45 | 146/146 | 6.18±0.56 | 138/148 | 6.71±0.60 |
| 146/148 | 6.07±0.37 | 146/148 | 6.02±0.47 | 142/146 | 7.46±0.47 |
| 146/999 | 5.36±1.28 | 148/148 | 6.51±0.21 | 142/148 | 6.61±0.34 |
| 148/148 | 6.68±0.19 | 148/999 | 6.44±0.62 | 142/999 | 7.41±0.93 |
| 148/999 | 6.98±0.28 | | | 146/146 | 7.43±0.76 |
| 999/999 | 5.97±0.91 | | | 146/148 | 6.00±0.60 |
| | | | | 148/148 | 7.09±0.37 |
| | | | | 148/999 | 6.48±0.77 |

[A]Significant effect ($P < 0.05$) by *F*-test

In the PI population, 13 genotypes of the *OPNpro* locus were used in the statistical analysis. Estimates of ASR for boars in dependence of the *OPNpro* locus varied from 6.02% to 11.34%. Boars with genotype `*118/999*´ had the highest ASR, and boars with genotype `146/148´ had the lowest ASR in the PI population. Differences of least

square means between the genotypes were checked with multiple pairwise comparisons using Tukey-Kramer adjustment. Significant differences are shown in Table 4.51. Significant estimates of difference between the genotypes ranged from 4.38% to 5.32%. The significantly biggest difference was between genotype `*118/999*´ and `*146/148*´ with adjust p-value of 0.0051. The significantly smallest difference was between genotypes `*118/142*´ and `*148/999*´ (adjust p-value 0.0365).

In the PI×HA population, 17 genotypes of the *OPNpro* locus were used in the statistical analysis. Estimates of ASR independence of the *OPNpro* locus ranged from 5.50% to 7.55%. Boars with genotype `*126/148*´ had the highest ASR, and boars with genotype `*168/148*´ had the lowest ASR in this population. No significant difference between genotypes was observed.

Table 4.51:    Significant differences of least square means for ASR (%) between the
                    *OPNpro* genotypes

| Population | Genotype | Genotype | Estimate | Adjust p-value |
|---|---|---|---|---|
|  | 118/118 | 118/999 | -4.40±1.30 | 0.0407 |
|  | 118/142 | 118/999 | -4.38±1.28 | 0.0365 |
|  | 118/148 | 118/999 | -4.55±1.28 | 0.0219 |
|  | 118/999 | 142/142 | 4.56±1.28 | 0.0213 |
|  | 118/999 | 142/146 | 4.76±1.36 | 0.0271 |
| PI | 118/999 | 142/148 | 4.48±1.27 | 0.0242 |
|  | 118/999 | 146/146 | 5.16±1.38 | 0.0114 |
|  | 118/999 | 146/148 | 5.32±1.34 | 0.0051 |
|  | 118/999 | 148/148 | 4.83±1.28 | 0.0098 |
|  | 118/999 | 148/999 | 4.90±1.40 | 0.0282 |

(1) Additive and dominance effects on ASR

The candidate genes *INHA* and *GnRHR* were significantly linked or associated with the trait sperm abnormal rate, therefore, the additive and dominance effects for the locus *INHA* on ASR were estimated as shown in Table 4.52. Homozygote GG of the *GnRHR*

locus was very rare or absent in the different populations, so for the *GnRHR* locus no additive and dominance effects on ASR were estimated.

For the locus *INHA*, the additive and dominance effects on ASR in the PI population were weak. Allele A of the *INHA* locus had greater effect on ASR than allele B. The negative dominance effect revealed that heterozygote was smaller than the means of both homozygotes. Here additive and dominance effects on ASR were not significant in the PI population.

Table 4.52:    Additive (a) and dominance (d) effects of alleles of the *INHA* gene on ASR (%)

| Locus | Effect | PI | |
|---|---|---|---|
| | | LSM ± SE | p |
| *INHA* | a | 0.13±0.45 | 0.76 |
| | d | -0.35±0.48 | 0.48 |

(2) Allele substitution effects on ASR

Results show that microsatellite markers *INHBA, OPNpro,* and *AR* significantly affected ASR in the different populations. For loci *INHBA* and *OPNpro*, allele substitution effects on ASR were estimated using multiple linear regression analysis with allele covariable according to model 4 (estimation of allele substitution effect on sperm quality traits). The *AR* gene is located on SSCX (Seifert et al. 1999), the estimated effects of genotypes of the *AR* locus in boar are genetic effects. Therefore, the *AR* locus was excluded from the statistical analysis for allele substitution effect.

Table 4.53 shows that allele `*254* bp´ of the *INHBA* locus had positive effect on ASR in the populations, the allele substitution effects ranged from 0.01 to 0.27. In contrast, allele `*252* bp´ and `*256* bp´ negatively affected ASR. For the three frequent alleles, no significant allele substitution effect on ASR was observed in these populations. Allele

`256 bp´ was very rare in the PI population and replaced by a pseudo allele 999. Therefore, this allele was excluded from the analysis for allele substitution effect.

In the PI population, in total 4 frequent alleles `118 bp´, `142 bp´ `146 bp´, and `148 bp´ of the *OPNpro* locus were used in the statistical analysis for allele substitution effects on ASR. These four alleles negatively affected ASR and their allele substitution effects ranged from -1.00 to -0.59, and were not significant on ASR in the PI population.

Table 4.53:    Allele substitution effects on ASR (%) for the loci *INHBA* and *OPNpro*

| Locus | Allele | MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|---|---|
| | | LSM ± SE | p | LSM ± SE | p | LSM ± SE | p |
| | *252* | -0.40±0.34 | 0.24 | -0.07±0.24 | 0.77 | -0.71±0.46 | 0.13 |
| *INHBA* | *254* | 0.01±0.35 | 0.97 | 0.17±0.27 | 0.52 | 0.27±0.49 | 0.58 |
| | *256* | -0.24±0.39 | 0.54 | - | - | -0.21±0.53 | 0.69 |
| | *118* | - | - | -0.59±0.54 | 0.27 | - | - |
| | *142* | - | - | -0.70±0.53 | 0.18 | - | - |
| *OPNpro* | *146* | - | - | -1.00±0.56 | 0.07 | - | - |
| | *148* | - | - | -0.89±0.53 | 0.10 | - | - |

5 Discussion

In the present study, AI boars analysed are those that have been selected for artificial insemination, i.e. they represent animals with high breeding values including good reproductive performance. The use of these animals in the analyses does therefore not cover the negative spectrum of trait inheritance.

Boar fertility traits are of low heritability ($h^2 \approx 0.01 - 0.06$; See 2000) and are strongly affected by environmental and genetic effects of the boar itself, the dam and the offspring. Therefore, effects of single loci are expected to be low and require a higher number of animals to be analysed (Long and Langley 1999). In contrast, sperm quality traits have moderate to medium heritability ($h^2 \approx 0.19 - 0.37$; See 2000), but they are sex limited and laborious to measure.

MAS may be a method of selecting for components of litter size in both sexes of swine at very young age and improving accuracy of selection (Cassady et al. 2001). Stimulation studies have demonstrated potential benefits of MAS (Zhang and Smith 1992, 1993, Edwards and Page 1994). Efficiency of MAS relative to phenotypic selection is greatest for lowly heritable and sex-limited traits (Lande and Thompson 1990). Simulation of response to MAS has been shown to be greater than response to selection on either phenotypes or markers alone (Zhang and Smith 1992).

Candidate gene analysis for boar fertility were reported by Steinheuer et al. (2002, 2003) and for sperm quality traits was performed by Hardge et al. (1995), Huang et al. (2001, 2002), Urban and Kuciel (2001), Kemic et al. (2002) and Mackowski et al. (2004). QTL regions mainly for boar sexual development and FSH concentration in plasma as well as sperm daily production have been identified (Bidanel et al. 2001, Rohrer et al. 2001, Ford et al. 2001, Sato et al. 2004).

In order to apply the candidate gene approach to identify loci associated with reproductive performance of boar, the prior task is to select candidate genes based on the known function (biological function and physiological function) and/or based on

information from gene maps [linkage map, physical map, comparative map, QTL-linkage map or expressed sequence tags (ESTs)].

In the present study, seventeen candidate genes (*GnRHR*, *FSHB*, *LHB*, *RLN*, *PRL*, *PRLR*, *FST*, *INHA*, *INHBA*, *INHBB*, *AR*, *ACR*, *RBP4*, *OPN*, *ACTG2*, *ACTN1* and *ACTN4*) were chosen based on their known biological correlations and/or physiological functions in male reproduction. Gene *AR* is also a positional candidate gene for boar reproduction traits (Bidanel et al., 2001). These direct functional candidate genes were assigned to pig chromosomes (Figure 5.1) by different authors (Table 5.1).

Table 5.1:      Survey of candidate genes assigned to pig chromosomes

| Gene | Location | author |
|------|----------|--------|
| *FSHB* | 2p1.6-2p1.2 | Mellink et al. 1995 |
| *LHB* | 6q2.1 | Mellink et al. 1995 |
| *GnRHR* | 8q1.1-1.2 | Rohrer 1999a |
| *RLN* | 1q2.8-2.9 | Chowdhary et al. 1994 |
| *PRL* | 7p1.1-1.2 | Vincent et al. 1998b |
| *PRLR* | 16q1.4, 16q2.2-2.3 | Vincent et al. 1997 |
| *FST* | 16q1.4 | Lahbib-Mansais et al. 2000 |
| *AR* | Xp1.1-q1.1 | Rohrer 1999b |
| | Xq1.3 | Seifert et al. 1999 |
| *INHA* | SSC15 | Retenberger et al. 1996 |
| *INHBA* | 18q2.4 | Lahbib-Mansais et al. 1996 |
| *ACR* | 5p1.5 | Rettenberger et al 1995 |
| *OPN* | 8q2.7 | Alexander et al. 1996 |
| *ACTN4* | 6q1.2 | Martins-Wess et al. 2003 |
| *RBP4* | SS14 | Messer et al. 1996a |
| *INHBB* | SSC15 | Nonneman et al. 2002 |
| *ACTG2* | 3q11-14 | Yerle et al. 1998 |
| *ACTN1* | SSC7 | Wimmers et al. 2005 |
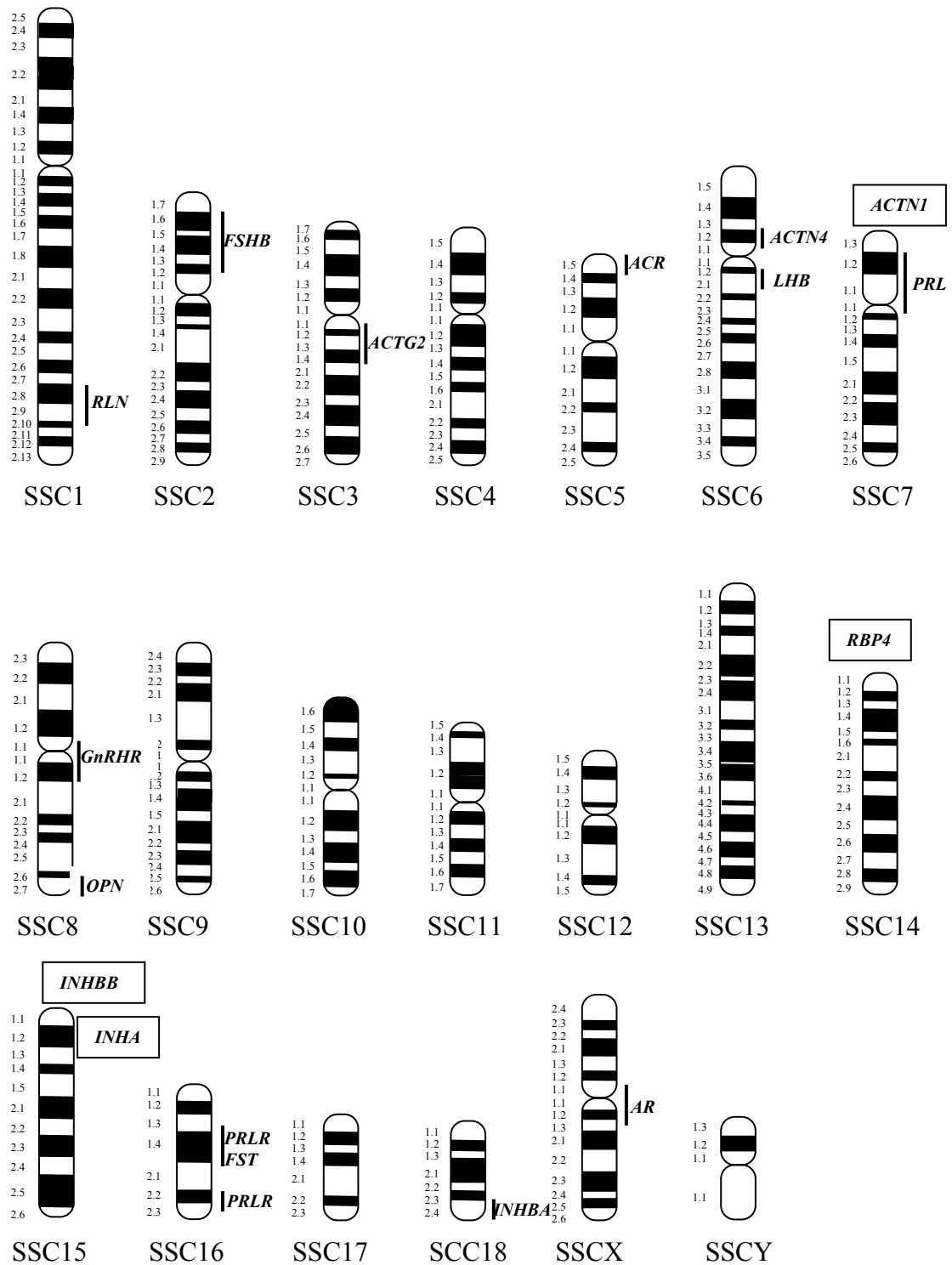
Figure 5.1: An ideogram showing the locations of physiological functional candidate genes for boar reproduction in accordance with published literature.

□: Description of the gene having an unknown position on the chromosome

In molecular approaches, several methodologies (linkage mapping/QTL-mapping, expression analysis and EST-mapping, comparative mapping, direct biological candidate gene approach) have provided a way to find nucleotide polymorphisms that cause phenotypic variation.

The application of MAS is very promising (Plastow 1998, Linville et al. 2001). QTL-studies in swine can provide knowledge of the localisation of genes affecting traits of interest. For identifying the QTL for reproductive traits itself in defined porcine genome segments, the syntonical human chromosome should be viewed based on the comparative gene mapping between pig and human (Goureau et al. 1996). So unknown candidate genes can be found in pig based on the position of the human gene in the homologous segments. Beside these so-called positional candidate genes, some important physiological candidate genes can also be searched in the selected chromosome segment in human based on their association to reproductive parameters. After identification of these new candidate genes, the mutation analysis is primarily performed within the coding segments of the gene. The aim of this analyses is to state phenotype variation associated with the polymorphism.

Otherwise, genes expression analysis is a tool in order to functionally identify genes (Cogburn et al. 2003) contributing to reproductive processes. Gene expression profiles in target tissues can be built (e.g. with cDNA arrays) using the isolated RNA. These gene profiles provide quantitative evidence for the implication of single genes. The identified candidate genes must initially be characterized again in pigs and subsequently checked for polymorphisms in the framework of a mutation analysis.

## 5.1 Detection of polymorphisms for *ACTN1* and *ACTN4* gene

The polymorphism within a gene can successfully be applied to elaborate on the candidate gene genetically affecting phenotypic variation (Rothschild et al. 1996; Hugo 1998; Chardon et al. 1999; Wimmers et al. 2002a). In this study, polymorphisms within the *ACTN1* and *ACTN4* gene were detected using genomic DNA by comparative sequencing of two breeds (Pietrain, Hampshire). One polymorphism at position 189 in intron 18 of the porcine *ACTN1* gene was a transition from guanine (G) to adenine (A) (accession: AY837722). Another polymorphism at position 396 in the 3´-UTR of the porcine *ACTN4* gene was a transition from adenine (A) to cytosine (C) (accession: AY837723). These polymorphisms may affect the relevant proteins expression level (Geldermann 1996). Using the method "comparative sequencing of several individual animals of different breeds", Rothschild et al. (1996), Gerbens et al. (1997) and Wimmers et al. (2003) have already successfully identified the polymorphisms in the porcine estrogen receptor gene, the heart fatty acid binding protein gene and the C3 gene, respectively. Otherwise, polymorphisms can also be found in nucleotide sequence of animals having extreme phenotypic traits. For instance, polymorphisms in the porcine stress syndrome gene (Fujii et al. 1991), the bovine leukocyte adhesion deficiency gene (Shuster et al. 1992), the bovine double muscling gene (Grobet et al. 1997) as well as the human C3 gene (Botto et al. 1990a, 1990b, 1922a, 1992b) and the dog C3 gene (Ameratunga et al. 1998).

## 5.2 Hardy-Weinberg equilibrium

In this study, candidate gene loci *FSHB*, *ACTN1*, *ACTN4*, *GnRHR*, *RLN*, *LHB*, and *INHBA* were in Hardy-Weinberg equilibrium in both tested boar populations. Hardy-Weinberg equilibrium means a population with constant allele and genotype frequencies if the population satisfies the following conditions: panmixia and gametes encounter each other randomly; "infinite" population (very large: to minimize differences due to sampling); there must be no selection, mutation, migration (no allele loss/gain); successive generations are discrete (no crosses between different generations) (Falconer and Mackay 1996).

Selection and random genetic drift cause allele frequency to change. For loci *PRL*, *PRLR*, *INHA*, *RBP4*, and *OPNin6* not in Hardy-Weinberg equilibrium in this study, selection might have occurred for a particular trait and changed the allele frequency. In the absence of selection, the amount of drift depends on allele frequencies and effective population size. After drift begins, it is most probable that an allele frequency will continue changing in the same direction (Falconer and Mackay 1996).

In the past due to the breeding goals, Pietrain and Hampshire pigs were selected for pork quality and fattening traits with low emphasis on fertility (Schwark et al. 1975, Rothschild and Ruvinsky 1998). Even so, the selection might lead to changes of allele frequencies of these loci *PRL*, *PRLR*, *INHA*, *RBP4*, and *OPNin6* leading to significant deviation of the observed genotype frequencies from the expected.

For microsatellite markers *FST*, *INHBB*, *ACR*, *ACTG2*, and *OPNpro*, the significant deviation from Hardy-Weinberg equilibrium could result from many reasons, such as too much heterozygote. Negative *Fis*-value is caused by selection and systematic breeding, positive *Fis*-value can result from 'null alleles' at the locus or inbreeding or from subpopulations existing within a population (Weir 1996). The deviation from Hardy-Weinberg equilibrium should appear simultaneously at more loci in homologous magnitude due to inbreeding or hybridisation. For a locus, the selection affects normally the deviation from Hardy-Weinberg equilibrium in any population. In this study, deviations from Hardy-Weinberg equilibrium were not consistent at different loci *FST*, *INHBB*, *ACR*, *ACTG2* and *OPNpro* in both populations. Even though there is no clear evidence for effects of selection or existence of 'null alleles' for these microsatellite markers in the boar populations, but the boars do probably not give a good representation of the population/breed they belong to.

5.3 Allele and genotype frequencies

*FSHB* locus

The homozygote genotype AA of the *FSHB* gene was most frequent among the genotyped animals of both populations with allele A having a frequency of more than 0.80 in the PI and the PI×HA population. Kossakowska et al. (2003) reported allele A frequencies of more than 0.90 in a synthetic pig line, that was selected for reproductive traits. This might indicate the frequency of allele A of the reproductive gene *FSHB* is enhanced by selection of AI boars for reproduction traits.

*PRL* Locus

Two polymorphic sites were identified with restriction enzyme *BstUI* in fragments amplified from intron 2 of the *PRL* gene. A total of four possible alleles were observed by Vincent et al. (1998b). Three alleles A, B, C and five genotypes AA, AB, AC, BC, and CC were observed in the genotyped boar populations. The fourth allele, representing the undigested PCR product was not found in this study. This is in agreement with the findings in a polish synthetic pig line (Kossakowska et al. 2003). Vincent et al. (1998b) reported allele A frequency ranging from 0.41 to 1.0 in seven pig breeds of Chester White, Hampshire, Landrace, Large White, Yorkshire, and Meishan. In the present study, allele A appeared with frequencies of 0.49 and 0.59 in the PI and the PI×HA population, respectively.

*PRLR* Locus

The homozygote AA of locus *PRLR* varied with the frequency of 58.61 % in 244 PI boars and 86.04 % in 122 PI×HA boars. The allele A of the *PRLR* was prevailing in the PI and even more frequent in the PI×HA population. This result is in agreement with findings in the Duroc (0.79), Landrace (0.72) and Meishan (0.56) breed (Vincent et al. 1997). Linville et al. (2001) reported that allele B frequency of the *PRLR* gene increased within two lines selected for reproductive traits. The first line was selected eight generations for an index of ovulation rate and embryo survival, followed by eight generations of two-stage selection for ovulation rate and litter size; the second line was selected at random for eight generations, followed by eight generations of two-stage

selection; a control line was randomly selected for 16 generations. The frequencies in the control line were 0.42 of the allele A and 0.58 of the allele B. Compared with the control line, animals in the selection lines showed increased allele frequency for the allele B with 0.81 in the first line and 0.64 in the second line. The effect of selection, different allelic frequencies in the founder animals and random genetic drift could have caused allele frequencies to differ among lines (Linville et al. 2001).

### *RBP4* Locus

The frequency of allele A was higher in the PI (0.67) in the present experiment. This is consistent with the study of Steinheuer et al. (2002) (allele A 0.71 in PI boars). Similar results were also demonstrated by the study of Hamann et al. (2002), with allele A frequency close to those in genotyped animals of the different groups. In the present study, homozygote AA and heterozygote AB showed frequencies of 33.61% and 66.39% in the PI, and 2.69% and 97.34% in the PI×HA. Homozygote BB was not observed in both boar populations. Linville et al. (2001) reported that the frequent genotype heterozygote AB was 68.7% and 50.7% in two lines that were selected for reproductive traits; heterozygote AB and homozygote BB appeared at the same frequencies (38.9%) in animals that were mated randomly over 16 generations (control line). Therefore, in this study allele A of the *RBP4* gene could have been increased due to selection of these AI boars for reproduction traits and lead to lack of homozygote BB.

### *INHA* locus

The homozygote AA was very rare in the genotyped populations of PI and PI×HA boars. Allele A of the *INHA* gene was infrequent in the PI and the PI×HA with frequencies of 0.11 and 0.34, respectively. Hiendleder et al. (2002) found that allele A had a frequency of 0.24 in 29 unrelated individuals from the Duroc, German Landrace, Large White, Meishan, and Wild boar, but five Meishan pigs were homozygous for the allele B. These reported results are consistent with the findings of the present study. The Meishan pig breed is known for its better reproductive performance (Borg et al. 1993). This could indicate that the frequency of allele B of the reproductive gene *INHA* could have been increased in the selected AI boars from the AI station.

*ACTN1* and *ACTN4* locus

For the locus *ACTN1* and *ACTN4*, in the present study polymorphisms were for the first time determined within commercial pigs. Homozygote BB was rare in both populations, whereas allele A of both loci was more frequent. With regard to genetic variation of these polymorphisms in commercial breeds, it needs further genotyping in different breeds.

*GnRHR* Locus

It was observed that allele C of the *GnRHR* is the most abundant in the purebred Pietrain and the crossbred Pietrain × Hampshire animals. Jiang et al. (2001) reported that the frequency of the G allele of *GnRHR* was 0.42 in European Large White and 0.94 in Meishan, which is known for better reproductive performances (Borg et al. 1993). Heterozygotes GC were observed at similar frequencies in the PI and the PI×HA (38% and 33.99%, respectively). Homozygote GG appeared to be extremely rare in the PI population and be absent in the PI×HA population.

*LHB* Locus

A polymorphism (T>C) at position 177 of the porcine sequence U78106, within exon 2 of gene *LHB* was described by Jiang et al. (1999). The frequencies of alleles at this locus were unknown so far. Heterozygote TC was more than 40% in both populations. The allele frequencies were similar in both populations.

*RLN* locus

The frequencies of allele A of *RLN* locus were very low in the purebred Pietrain (0.09) and crossbred Pietrain × Hampshire (0.05). This result is in agreement with the finding reported by Wimmers et al. (2002b). Homozygote AA appeared to be extremely rare in the genotyped AI boars.

*OPNin6* locus

The polymorphism within intron 6 of the *OPN* gene is based on the presence or absence of a 305 bp block. Among 356 genotyped boars, the frequency of allele A at locus *OPNin6* reached 0.46 in the PI breed and 0.25 in the PI×HA crossbred, respectively.

Kossakowska et al. (2001) reported that the frequency of allele A at the *OPNin6* locus in 440 genotyped pigs of a synthetic line (line 990) reached 0.21. This line was selected for reproduction traits. These results might indicate that the frequency of allele B of the reproductive gene *OPN* could have been increased in these populations due to selection for reproduction traits. Knoll et al. (1999) found that the frequency of allele A at *OPNin6* locus was 0.57 in Landrace (n=40), 0.26 in Large White (n=31), 0.16 in Duroc (n=19) and 0.82 in Pietrain (n=14). These differences of frequencies might result from the small number of genotyped pigs or genetic drift.

*OPNpro* locus

Southwood et al. (1998) reported thirteen different alleles for the highly polymorphic microsatellite marker at the porcine *OPNpro* locus. Six of the thirteen alleles had frequencies less than 1% in their experiments. In this study seven alleles of the *OPNpro* locus were observed in PI and PI×HA boars. Steinheuer et al. (2002, 2003) found seven alleles of this locus in German Landrace. Hamann et al. (2000) reproted that seven alleles of the same marker could be observed in several pig breeds. In the studies of Southwood et al. (1998) and Hamann et al. (2000) the alleles were not indicated by their length, so it was impossible to compare with our results. However, their investigations are in agreement with the point that particular alleles segregated with very low frequency in those populations.

*AR* locus

Trakooljul et al. (2000) reported that out of the seven alleles for the microsatellite in the 5´-untranslated region of the androgen receptor gene, four alleles in Pietrain, four alleles in Large White and five alleles in German Landrace were observed, whereas the alleles *203 bp* and *228 bp* were not detected. The allele frequencies for the microsatellite in the 5´-UTR of *AR* locus in Pietrain and Pietrain × Hampshire were similar.

*FST* locus

An $(A)_n$ mononucleotide repeat in the porcine follistatin gene was reported by Moran (1993). Ellegren (1993b) also detected the $(A)_n$ mononucleotide repeat in the *FST* gene with six alleles. The heterozygosity was 0.69 among ten founder animals (two wild

boars and eight Large White sows) in the Swedish reference pedigree. In the present study, ten alleles were observed. Heterozygosity was 0.49 in the Pietrain breed, with nine alleles segregating, and 0.47 in Pietrain × Hampshire crossbred. In both populations, the heterozygosity was less than the expected. Allele frequencies were similar in both populations. The heterozygosity in this study was different from those in the experiment of Ellegren (1993b), because the magnitude of heterozygosity at a locus depends on the number of alleles and the allele frequency. The more alleles at a locus appear and the more similar the allelic frequencies are, the higher the heterozygote degree is (Wimmers et al. 1998).

*ACR* locus

Yasur et al. (1999) reported a microsatellite repeat sequence (CA)n within the porcine *ACR* gene. Ten alleles ranging from 186 bp to 232 bp were detected within the PiGMaP reference families. In our study, twelve alleles in the PI boars and eleven alleles in the PI×HA boars were found, allele length ranged from 186 bp to 228 bp. Allele 208 bp in both boar populations was the most frequent, frequencies of allele 202 bp and 228 bp differed between the Pietrain and the Pietrain × Hampshire population.

*ACTG2* locus

Sun et al. (1995) found that a dinucleotide repeat $(AC)_n$ exists within the porcine *ACTG2* gene. High polymorphism of this microsatellite marker was detected for the first time in the purebred PI and the crossbred PI×HA in this study. Heterozygosity was more than 0.75 in both groups of boars.

*INHBA* locus

A highly polymorphic microsatellite $(CA)_n$ exists in the porcine *INHBA* gene as described by Moran (1993). Campbell et al. (2001) reported six alleles and 78% heterozygous were in the parents of the USDA-MARC porcine linkage map experiment. In our study, five alleles were found in the PI breed and four alleles were found in the PI×HA crossbred. The lack of alleles in both populations might result from selection for reproduction traits of these AI boars or small population size. The heterozygosities

within both populations were different from that in the experiment of Campbell et al. (2001).

*INHBB* locus

Nonneman and Rohrer (2003) reported a polymorphism of repetitive sequences $(AG)_n$ within exon 2 of the *INHBB* gene with six alleles. In the present study, also six alleles appeared in the two groups of boars. Regarding the allele frequencies, allele 171 bp was the most frequent in both groups, but the distributions of the other alleles were different.

5.4 Candidate gene effects

Development of porcine genome maps offers the opportunity to identify individual genes controlling reproduction. Application of MAS will increase as more associations between markers and traits are identified (Rothschild, 1998).

Obviously female fertility has a greater impact on the reproductive performance realized in a commercial herd than boar fertility and therefore more efforts have been made to elucidate genetic components controlling female fertility. A number of QTL regions mainly for pig reproductive traits have been identified (Rothschild et al. 1996, Rathje et al. 1997, Wilkie et al. 1999, Rohrer et al. 1999, Rohrer 2000, Wada et al. 2000, Brauschweig et al. 2001, Cassady et al. 2001, Jiang et al. 2001, Hirooka et al. 2001, King et al. 2003, Campbell et al. 2003, Sato et al. 2003). Among them the QTL regions associated with the interesting pig reproductive traits such as litter size, fully formed pigs, testis weight, age at puberty and plasma FSH concentration, are summarized in Table 5.2. Furthmore, more and more QTL regions have been detected for male reproductive traits in pig (Rohrer et al. 2001; Bidanel et al. 2001; Ford et al. 2001; Sato et al. 2004). The QTL for male fertility detected in mice are on chromosomes 4, 10, 13, 18 and X (Zidek et al. 1998, Le Roy et al. 2001). In rat, QTL for sperm quality parameter and morphological testis and epididymides properties were found on chromosome 8q24, the region showed also effect on litter size (Zidek et al. 1999).

Table 5.2: Genomic scan detected significant and intimate QTLs for pig reproductive traits of interest

| Traits | Chromosome | Position (*c*M) | QTL span (*c*M) | Author |
|--------|-----------|-----------------|-----------------|--------|
| TNB | SSC1 | 19 | - | Rothschild et al. 1996 |
| LS | SSC8 | 127 | 92.4-127.7 | King et al. 2003 |
| NBL | SSC11 | 71 | - | Cassady et al. 2001 |
| NSB | SSC4 | 5 | 4.1-27.1 | Wilkie et al. 1999 |
| NSB | SSC5 | 131 | - | Cassady et al. 2001 |
| NSB | SSC13 | 101 | - | Cassady et al. 2001 |
| NFF | SSC11 | 52 | - | Cassady et al. 2001 |
| TEWE | SSC3 | 33 | 27.6-42.8 | Sato et al. 2003 |
| AEGP | SSC1 | 105 | 91-119 | Rohrer et al. 1999 |
| AEGP | SSC7 | 58 | 48-73 | Cassady et al. 2001 |
| AEGP | SSC8 | 101 | - | Cassady et al. 2001 |
| AEGP | SSC10 | 125 | 115-130 | Rohrer et al. 1999 |
| AEGP | SSC12 | 9 | - | Cassady et al. 2001 |
| FSH | SSC3 | 49 | 42.3-50.8 | Rohrer et al. 2001 |
| FSH | SSC8 | 12 | 11.1-12.4 | Rohrer et al. 2001 |
| FSH | SSC10 | 101 | 101-108 | Rohrer et al. 2001 |
| FSH | SSCX | 84 | 71.7-87.4 | Rohrer et al. 2001 |

LS: Litter size, NBL: Total number born alive; NSB: Number of still born; NFF: Fully formed pigs; TEWE: Testis weight; AGEP: Age at puberty; FSH: Plasma FSH concentration; -: No information.

*GnRHR* gene

The *GnRHR* locus was for the first time shown to be significantly associated with MOT, PDR and ASR. Heterozygote animals had higher sperm motility and were prone to lower abnormal sperm rate and immature sperm rate in boars. The tested polymorphism in the 3′-UTR of the *GnRHR* gene and might directly affect gene expression level and/or be a marker for potentially linked polymorphisms with impact on protein structure and/or expression level. In human, *GnRHR* mutations are significantly associated with male infertility (Layman et al. 1997). Furthermore, Rohrer et al. (2000)

suggested that QTL regions affecting the concentration of serum FSH in Meishan ×
Large white boars on different chromosomes, including SSC8 (Position 19 cM).
However, *GNRHR* was assigned to SSC8q1.1-1.2 (Rohrer 1999a) in far distance from
that QTL for FSH serum concentration. QTL for growth traits have been identified in
vicinity to *GNRHR* as well as QTL for other fertility traits like age at puberty, prenatal
survival and ovulation rate (Casas-Carrillo et al. 1997, Milan et al. 2002, Cassady et al.
2001, King et al. 2003, Rathje et al. 1997) supporting suggestions raised by our finding.

## *PRL* gene

The polymorphism of *PRL* locus was shown for the first time to affect sperm
concentration in the PI×HA boars in this study. The tested polymorphism localizes in
intron 2 of the *PRL* gene, and might affect the gene expression level and/or be as a
marker for potentially linked polymorphisms with impact on protein structure and/or
expression level. Seminal plasma prolactin concentrations in man correlated directly to
sperm concentrations and motilities. These relationships were statistically significant
(Aiman et al. 1988). Moreover, positional evidence for association of the *PRL* gene with
sperm quality arises from a QTL region affecting the male development traits (testes
weight, epididymis weight and length of bulbo-urethral gland) in Meishan × Large
White boars that occur at position 66 *cM*, 86*cM*, 143 *cM* of SSC7 (Bidanel et al. 2001).
The detailed localization of the *PRL* gene is at position 68 *c*M of SSC7
(http://www.thearkdb.org/, PigMap for averaged male and female).

## *PRLR* gene

The tested SNP is within coding sequence region of *PRLR* gene (Vincent et al. 1997).
Huang et al. (2002) reported that the *PRLR* locus was significantly associated with
sperm motility ($P < 0.05$) and plasma droplets rate ($P < 0.01$) in 34 Duroc boars.
Steinheuer et al. (2003) reported that the polymorphism of *PRLR* locus had significant
effect on NBA in 51 boars of German Landrace breed. Functional evidence of
association with the *PRLR* gene arises from knock out experiments showing delayed
fertility in male mice (Goffin et al 1999). In this study, no significant effect of the *PRLR*
locus was observed on boar reproduction traits of the PI and the PI×HA population. The
allele effects differ between populations due to the genetic background. Different

linkage phases between the markers and a causal mutation due to recombination may explain the observed differences between the different populations. Also, still unknown quantitative trait loci with an effect on the traits could be linked to this gene (Linville et al. 2001).

*FSHB* gene

The tested mutation is located within intron 1 of the *FSHB* gene and might be a marker for potentially linked polymorphisms with impact on follicle stimulating hormone beta protein structure and/or expression level. In this study, the polymorphism of the *FSHB* locus was significantly associated with sperm concentration. The functional association evidence of the *FSHB* gene arises from knock out experiments showing that deficiency at the *FSHB* gene lead to decrease sperm concentration to 75% (Layman 2000). Ford et al. (1997) reported that a negative relationship in mature Meishan boars between FSH secretion and testicular size accompanied with decreased total daily sperm production; seminal FSH and testosterone concentrations were positively correlated with sperm output in man (Vasquez et al. 1986). FSH affected spermatogenesis in boar (Zanella et al. 1999), testis size and weight correlated with daily sperm production and total sperm reserves (Huang and Johnson 1996). The *FSHB* gene is located on SSC2, no positional evidence for linkage to these traits has been reported yet.

*LHB* gene

The tested polymorphism in exon 2 of the *LHB* gene is a silent mutation that might be a marker for potentially linked polymorphisms with impact on luteinizing hormone beta protein structure and/or expression level. A mutation causing inactivation of the LH beta subunit in human leads to absence of Leydig cells, lack of spontaneous puberty and infertility (Huhtaniemi et al. 1999). In this study, no significant effect of the *LHB* locus on sperm quality traits and boar fertility traits could be observed. The possible reason is that boar reproduction traits are complex traits affected by many genes and their interaction between them, and because of espistasis, the polymorphism of the *LHB* gene could have a small effect on the variation of phenotypes. Furthermore, linkage disequilibrium relationships could also cause the lack of effects on the tested traits in these boar populations.

*FST* gene

The microsatellite polymorphism of the *FST* gene significantly influences semen volume in this study. The tested polymorphism in intron 1 of the *FST* gene is a marker for potentially linked polymorphisms with impact on follistatin protein structure and/or expression level. Follistatin modulates secretion of follicle stimulating hormone in pig (Shimonaka et al. 1991, Marther et al. 1993, Hafez and Hafez 2000), follicle stimulation hormone regulates spermatogenesis (Zanella et al. 1999, Lunstra et al. 1997). This reflects the functional evidence for the marker *FST* locus association with the sperm quality traits. The *FST* locus is on SSC16, there is not any information for positional evidence yet.

*INHA*, *INHBA* and *INHBB* gene

The inbins are heterodimeric homeres composed of a alpha-subunit (α) and one of two beta-subunits (ßA or ßB), produced by sertoli cells and secreted via the lymph in male, which contribute to the endocrine regulation of the reproductive system (Hafez and Hafez 2000). Therefore *INHA*, *INHBA* and *INHBB* were analysed as candidate genes for boar reproduction.

The tested polymorphism in the *INHA* gene is a silent mutation that might be a marker for potentially linked polymorphisms with impact on inhibin alpha protein structure and/or expression level. There are a few functional evidences for significant association of the *INHA* gene with sperm quality traits. Activation of the *INHA* gene during testis development correlated with the histological maturation of the testis and the acquisition of fertility in male (Seok et al. 2004). Over expression of the *INHA* gene leads to a disruption of the normal inhibin-to-activin ratio and to reproductive deficiencies (Cho et al. 2001). The *INHA* gene is assigned on SSC15, but the positional evidence for association of the *INHA* locus has not been found yet.

The polymorphism of the *INHBA* was significantly associated with PDR and ASR in this study. The tested polymorphism is within 3´-UTR of the porcine *INHBA* gene and might be a marker for potentially linked polymorphisms with impact on inhibin beta A protein structure and/or expression level. Kaipia et al. (1992) reported that the

expression of inhibin beta A in different stages of seminiferous epithelial cycle regulated spermatogenesis. No other evidence could be found.

In this study, it was for the first time detected that the *INHBB* locus is significantly associated with SCON trait. The tested microsatellite marker polymorphism is in the 3′-UTR that might be a marker for potentially linked polymorphisms with impact on inhibin beta B protein structure and/or expression level. Serum inhibin B was suggested as marker for sperm production and infertility in man and monkey (Hu and Huang 2002, Plante and Marshall 2001).

*RBP4* gene

Steinheuer et al. (2003) reported that the *RBP4* locus is significantly associated with the breeding values for number of piglets born alive and number of piglets weaned in 51 German Landrace boars. Additive effect and dominance effect are shown to be significantly different from zero. In our tested boar populations, no effect of the *RBP4* locus on boar fertility traits could be found. The allele effects differ between populations due to the genetic background. Different linkage phases between the markers and a causal mutation due to recombination may explain the observed differences between the populations. Also, still unknown quantitative trait loci with an effect on the traits could be linked to this gene (Drogemuller et al. 2001).

The effect of the *RBP4* locus on sperm quality traits was firstly detected in our study, this locus is significantly associated with sperm motility. There is a functional evidence for it: the localization of cellular retinol-binding protein was shown within the testis and epididymis where retinol may play an important role in sperm formation and maturation (Kato et al. 1985). Otherwise, the *RBP4* is located on SSC14, there is no detected QTL region resided in this chromosome on boar reproduction traits yet.

*AR* gene

In this study, we observed that the *AR* gene marker is associated with the sperm quality trait. The tested polymorphism is in the 5′-UTR of the *AR* gene that might be a marker for potentially linked polymorphisms with impact on androgen receptor protein

structure and/or expression level. More than 300 different mutations (commonly missense mutations) in the human *AR* gene have been shown causing androgen insensitivity for example (Patterson et al., 1994). Men with short CAG repeats in the *AR* gene have the highest sperm output (Von Eckardstein et al. 2001).

A QTL region on SSCX near to the *AR* gene significantly affects boar FSH, testes size and weight, epididymis weight and seminal vesicles weight (Bidanel et al. 2001, Rohrer et al. 2001). Ford et al. (2001) reported that a gene (genes) regulating testicular development and pubertal gonadotropin FSH and LH concentrations might reside on chromosome X. They performed investigations on Chinese Meishan × White composite crossbred boars and showed that the boars that inherited X-linked alleles from the Meishan breed had smaller testes and a reduced daily sperm production. These could be evidence for the *AR* gene marker associated with abnormal sperm rate.

*RLN* gene

The polymorphism (C>A) at position 1 of codon 8 (nucleotide 22) in the first exon of the *RLN* gene led to amino acid exchange (L8I). In this study, a significant association of the *RLN* gene with semen volume has been detected for the first time. Other studies showed that the relaxin level in seminal plasma significantly affected sperm motility in boars (Bagnel et al. 1993, Sasaki et al. 2001). The *RLN* gene is located on SSC1 (http//www.thearkdb.org), a QTL region on SSC1 near to the *RLN* gene significantly affects age at puberty in pig (Rohrer et al. 1999). Further evidences for the *RLN* gene association with other porcine reproduction traits have not been found yet.

*ACR* gene

In this study, the *ACR* gene was found to be significantly associated with sperm concentration and motility as well as with the boar fertility trait of NRR. The polymorphism of the *ACR* gene potentially influenced expression level of acrosin affecting sperm quality and fertility capacity. Acrosin activity is an important index for the evaluation of male fertility (Cui et al. 2000). Acrosin has been used as molecular marker for male fertility in human (Braundmeier and Miller 2001). The lack of acrosin in sperm in combination with modifications to the zona pellucida (ZP) can affect

fertility in mouse (Nayernia et al. 2002). In vitro storage of bovine spermatozoa, a loss of acrosin may be responsible for the decreased fertility (Miller et al. 1998). The boar proacrosin and acrosin contents are correlated with sperm fertilizing capacity (Cechova et al. 1984). The *ACR* gene transcription products acrosin plays a very critical role as a binding protein for fertilization of sperm and egg in pig (Howes and Jones 2002) and the fluctuation of acrosin activity might probably relate to the effects of high summer temperatures during spermatogenesis and semen parameter (sperm concentration, ejaculate volume and number of spermatozoa) (Ciereszko et al. 2000) and in vitro fertilization (De Jonge et al. 1993). Acrosin activity can be used to evaluate the quality of boar semen (Glogowski et al. 1998). Otherwise, the *ACR* gene is located on SSC5, even though, Cassady et al. (2001) reported that a QTL region for the porcine number of still born trait resided in the position 131 *cM* of the SSC5, but no positional evidence for linked to these boar reproduction traits has been observed yet.

*OPN* gene

The microsatellite marker in the promoter region of the *OPN* gene is significantly associated with fertility traits in German Landrace boars (Steinheuer et al. 2003). The effects of the same marker on boar fertility trait could not be observed in our tested boar populations of Pietrain and Pietrain × Hampshire. However, the other polymorphism in the intron 6 of the *OPN* gene significantly affected the trait of number of piglets born alive. Therefore, the polymorphism of the *OPN*in6 locus may be a marker for potentially linked causative mutations with impact on osteopontin protein structure or expression level influencing boar fertility. Osteopontin, one of fertility-associated proteins has been proposed as molecular indictor for male fertility in human (Braundmeier and Miller 2001).

In addition, this experiment revealed for the first time that *ONPin6* locus is significantly associated with sperm motility and the *OPNpro* locus significantly affected abnormal sperm rate. The latter polymorphism may be also a marker for potentially linked polymorphisms with impact on osteopontin protein structure and/or expression level. There are some functional evidences for these loci to be associated with sperm quality traits. The *OPN* gene is expressed and regulated in testis, efferent ducts, and epididymis

(Luedtka et al. 2002). The presence of *OPN* mRNA and protein in testis and epididymis as well as on the surface of epididymal spermatozoa, could play a role in testicular cell adhesion during spermatogenesis and/or epididymal maturation, although other potential functions in the male reproductive tract are possible (Siiteri et al. 1995).

Furthermore, the *OPN* gene is located in the position 120.2 *cM* of SSC8 (http://www. ncbi. nlm. nih.gov/mapview/maps.cgi). King et al. (2003) reported a QTL region for litter size resided in the position 127 *cM* of SSC8 (QTL span 92.4-127.7 *cM*), it may be a positional evidence for the *OPN* gene associated with the fertility trait. Otherwise, Rohrer et al. (2001) showed that a QTL region affecting the concentration of serum follicle stimulating hormone in Meishan × Large white boars may also occur on different chromosomes including SSC8 (Position 19 *cM*), unfortunately, the *OPN* gene is not located in this QTL region.

*ACTN1* gene

The *ACTN1* gene association with boar fertility traits NRR (in MIXED-BOAR and PI) and NBA (in MIXED-BOAR and PI×HA) was detected for the first time in this study. The evaluation of the additive effect on NRR was carried out for allele A of the *ACTN1* gene. Estimates of the dominance effect demonstrated the superiority of the heterozygote in comparison with the means of the two homozygotes. Negative effects for allele A were observed in the PI, allele B was shown to exhibit favourable effects. The tested SNP is within intron 18 of the *ACTN1* gene that might be a marker for potentially linked polymorphisms with impact on alpha actinin 1 protein structure and/or expression level. The candidate gene *ACTN1* is expressed in sperm, and potentially involved in membrane changes during acrosome reaction with important implication on sperm function (Yagi and Paranko 1992). *ACTN1* has been assigned to SSC7 (Wimmers et al. 2005), but no QTL region for the traits have been found yet.

The *ACTN1* gene significantly affected the boar fertility traits only in one boar population, not in the other boar population. The possible reason is that the polymorphism in the studied gene may indirectly affect the trait, the polymorphism could be a maker linked with the causative mutation within the gene or a closely linked

gene. Different linkage relationships may be the reason that estimates of the effect of gene differ across boar populations (Linville et al. 2001).

No significant relationship between genotypes of the *ACTN1* locus and sperm quality traits could be observed.


*ACTN4* gene

The *ACTN4* gene had no significant effect on boar fertility traits and sperm quality traits in this study. The tested polymorphism in 3′-UTR of the *ACTN4* gene may have small effect or the polymorphism could be only a marker linked to causative mutation within this gene or a linked gene with unfavourable linkage disequilibrium relationship. The functional evidence arises from the *ACTN4* gene expressed in sperm and potentially involved in membrane changes during acrosome reaction with important implication on sperm function (Yagi and Paranko 1992). *ACTN4* was located on SSC6q12, but no positional evidence has been found yet.


*ACTG2* gene

In the present study, the *ACTG2* gene was firstly proven to affect VOL in PI and MOT in PI×HA population. Protein gamma actin is distributed in ejaculated boar spermatozoa (Casale et al. 1998) and the *ACTG2* mRNA is differentially expressed during spermatogenesis (Waters et al. 1985). The tested polymorphism in the *ACTG2* gene may not directly affect the sperm quality traits, it could be a marker linked to the causative mutation within this gene or a linked gene. The causative mutation has an impact on gamma actin protein structure and/or express level. The different linkage relationships between two boar populations could be a reason for significant effect of the polymorphism on sperm quality traits in one but not the other boar population. Otherwise, Rohre et al. (1999) and Sato et al. (2003) reported QTL for boar reproductive traits on SSC3 (49 and 33 cM), however, *ACTG2* has been assigned to 3q11-14 (Yerle et al. 1998) in far distance from that QTL for FSH serum concentration and testis weight.

5.5 The lack of effects

The lack of candidate gene effect could be due to several reasons. First of all, the gene does not affect boar fertility. It is also possible, that the genes do have effect on the traits of interest, but the study failed to elucidate them in both or one of the two groups of boars. This may be due to the fact that the genes' effects are too small to be detected with the given number and structure of animals.

Boar reproductive traits are complex traits and an interaction of many genes (epistasis) is responsible for them (Geldermann 1996), therefore the effect of the single gene locus can be hidden and not reveal significant association because of very small effect on the phenotype. Background effects of other genes and interactions of these genes with the markers can also cause estimates of gene effects to differ between boar populations. Little is known about the magnitude of epistatic variation in the traits studied here. But if epistasis exists, the effect of a particular allele depends on its frequency and the frequency of alleles at other loci. Because of epistasis, polymorphisms for a gene could have a small effect in one population, yet explain a significant portion of the variance across boar populations (Linville et al. 2001)

The other possible reason for the lack of effect in one population, by genes that had positive effects on boar reproductive traits in the other population, is that different linkage disequilibrium existed in these populations. The polymorphism in the studied genes may indirectly affect these traits. These polymorphisms could be markers linked with the causative mutation within the genes or a closely linked gene. Different linkage relationships may be the reason estimates of the effects of genes differ across boar populations (Drogemuller et al. 2001).

5.6 Analysis of relevant factors

5.6.1 Effect of relevant factors on fertility traits

In this study, factors `boar birth year´ and `breed´ were classified in the analysis model for association between candidate gene genotypes and fertility traits of NRR and NBA. Their effects were estimated with model I (association for fertility traits) and the statistical results given as least square mean (LSM) and standard deviation (SD) are shown in Table 9.40 in Appendix 9.8. There is no significant difference for NRR and NBA between `breeds´ and between `boar birth year´.

Differences of boar fertility traits between breeds Pietain and Pietrain × Hampshire have not been found yet. No significantly different effects were observed on fertility among the class `boar birth year´ which is in agreement with the result of Steinheuer et al. (2003).

Effects of sperm traits, including SCON, VOL, MOT, PDR and ASR on fertility traits (NRR, NBA) were determined using the procedure 'PROC MIXED' of SAS. Estimates of sperm characters are represented as slope in Table 9.41 of Appendix 9.8. The results exhibited that all sperm characters significantly affect fertility traits except semen volume (VOL). This is because the same volume has been used for inseminating commercial sows in the AI process. Malmgren and Larsson (1984) proposed that semen evaluation could be used as an indicator of fertility of boars. The percentage of sperm with normal morphology also explained a large part of the variance in litter size born ($R^2$=0.59) (Xu et al. 1998). Sperm concentration, motility and normal sperm rate have usually been used as criteria for semen quality evaluation (Colenbrander et al. 1993, Flowers and Esbenshade 1993).

5.6.2 Effects of relevant factors on sperm quality traits

In this study, factors `boar age´, `breed´, and `season of semen collection´ were classified for association analysis between genotypes and sperm quality traits. Effects of

these factors were evaluated with model 3 (association for sperm quality traits) and are shown as least square means and their standard errors in Table 9.42 of Appendix 9.8. Their significant effect levels are presented in Table 9.43 of Appendix 9.8. `Boar age´ was used as covariable in the statistical analysis, effects of `boar age´ on sperm quality traits were shown as slope. The results showed that `boar age´ had highly significant effects on SCON ($P < 0.01$), VOL ($P < 0.01$), MOT ($P < 0.01$) and PDR ($P < 0.05$), but no significant effect on ASR ($P > 0.05$). `Semen collection season´ very significantly influenced any sperm quality traits ($P < 0.01$); `breed´ was significantly associated with VOL ($P < 0.01$) and MOT ($P < 0.05$), but not significantly associated with the other sperm quality traits ($P > 0.05$). Colenbrander et al. (1993) reviewed that sperm quality was influenced by many factors such as season, collection frequency, breed and age.

5.6.3 Estimation of repeatability for sperm quality traits

Repeatability is defined in VIM (International vocabulary of basic and general terms in metrology) vocabulary of metrology as the ability of a measuring instrument to provide closely similar indications for repeated applications of the same measurand under the same conditions of measurement (Shull 2001). The conditions included: (1). reduction to a minimum of the variation due to the observer; (2). the same measurement procedure; (3). the same observer; (4). the same measuring equipment used under the same conditions; (5). the same location; (6). repetition over a short period. The estimates of repeatability for sperm quality traits are shown in Table 9.44 of Appendix 9.8. Obviously, semen volume had the highest repeatability (0.43), the repeatability of MOT and SCON were middle (0.35 and 0.32), for PDR and ASR the repeatability were low (0.15 and 0.21) in this experiment.

# 6 Summary

The present study has been carried out to investigate the associations of 17 candidate genes with sperm quality traits. In addition, these genes were also tested for associations with boar fertility traits that reflect the success rate of artificial inseminations. The direct functional candidate genes gonadotropin releasing hormone receptor (*GnRHR*), follicle simulating hormone beta (*FSHB*), luteinizing hormone beta (*LHB*), relaxin (*RLN*), prolactin (*PRL*), prolactin receptor (*PRLR*), androgen receptor (*AR*), follistatin (*FST*), inhibin alpha (*INHA*), inhibin beta A (*INHBA*), inhibin beta B (*INHBB*), retinol-binding protein 4 (*RBP4*), acrosin (*ACR*), osteopontin (*OPN*), actin gamma (*ACTG2*), alpha-actinin 1 and 4 (*ACTN1*, *ACTN4*), were selected based on their biological correlations and/or physiological functions in the male reproductive gonadotropin axis.

The purebred Pietrain (PI) (n=244) and the crossbred Pietrain × Hampshire (PI×HA) (n=112) boars from an AI boar station, born between 1990 and 1999 and used in commercial pig herds mainly from North-Western Germany, were included in the study. The sperm quality traits included sperm concentration (SCON), semen volume per ejaculation (VOL), sperm motility (MOT), plasma droplets rate (PDR) and abnormal sperm rate (ASR), which were obtained from each ejaculate with light microscopy according to the guidelines of the World Healthy Organization. Fertility traits data (NRR and NBA) of each boar was given as the deviation from the population means within breed, parity of sow, farm and season classes.

Sperm samples of boars were collected from January 2000 to December 2001 and stored at -20 °C for DNA isolation. Genomic DNA was purified by a standard protocol using mercaptoethanol treatment and proteinase K digestion followed by phenol/chloroform extraction and precipitation with isopropanol.

For screening polymorphism within *ACTN1* and *ACTN4* gene, oligonucleotide primers were designed in accordance with porcine *ACTN1* (GenBank accession: BF442592) and porcine *ACTN4* (GenBank accession: BI338543). The PCR fragments were amplified from porcine genomic DNA. Comparative sequencing of animals of the breeds Pietrain

and Hampshire revealed polymorphisms in intron 18 (G>A) of porcine *ACTN1* gene and in 3′-UTR (A>C) of porcine *ACTN4* gene. These polymorphisms were confirmed by PCR-RFLP with restriction enzymes *Bst*EΠ and *Apa*I, respectively.

Genotypes of 356 boars were obtained for the eighteen candidate gene loci by means of PCR-RFLP or PCR-SSCP or microsatellite.

Association analysis revealed that loci *ACTN1*, *ACR* and *OPNin6* were significantly associated with boar fertility traits and loci *FSHB*, *PRL*, *ACR*, *INHA*, *INHBA*, *INHBB*, *FST*, *RLN*, *ACTG2*, *GnRHR*, *RBP4*, *OPNin6*, *AR* and *OPNpro* significantly affected sperm quality in different boar population. No significant effect of loci *LHB*, *PRLR* and *ACTN4* on any of boar reproductive traits were observed in this study.

In particular:

1. The candidate gene *ACTN1* locus significantly ($P < 0.05$) affected NRR in the MIXED population and in the PI population. Additive effect ($P < 0.01$) and dominance effect ($P < 0.05$) were significant on NRR in these boar populations. This locus also had significant effect ($P < 0.05$) on NBA in the MIXED population and in the PI×HA population, with significant additive ($P < 0.01$) and dominance effects ($P < 0.01$) on NBA in these populations.

2. The locus *OPNin6* was significantly ($P < 0.05$) associated with NBA in the MIXED population and in the PI population. However, only significant ($P < 0.05$) additive effect on NBA was observed in these boar populations, the dominance effect was not significant. This locus was also significantly ($P ≤ 0.05$) associated with MOT in the MIXED boar population, but no significant additive and dominance effects were observed on MOT in these boars.

3. The candidate gene *ACR* had significant effect ($P < 0.05$) on NRR in the MIXED population and in the PI population, but the significant allele substitution effect ($P < 0.05$) on NRR was only for allele `*208* bp´ observed in the PI population. The locus *ACR* also significantly ($P ≤ 0.01$) affected SCON in the PI×HA population. Allele `*208* bp´ and allele `*218* bp´ had significant allele substitution effects ($P < 0.05$) on SCON in this boar population. The *ACR* locus

had significant effect ($P < 0.01$) on MOT in the MIXED population and in the PI population. However, no significant allele substitution effect on MOT was observed in these boar populations.

4. The locus *ACTG2* was significantly associated ($P < 0.01$) with VOL in the PI boars, but only allele `*109* bp´ had significant allele substitution effect ($P < 0.05$) on VOL in this boar population. The *ACTG2* marker was also significantly ($P < 0.05$) associated with MOT in the PI×HA population. Whereas, no any significant allele substitution effect on MOT was observed.

5. The *FSHB* gene was significantly ($P \leq 0.01$) associated with SCON in the PI population, only additive effect was significant ($P < 0.05$) on SCON, dominance effect was not significant.

6. The *GnRHR* marker was significantly associated with MOT in the MIXED boar population ($P < 0.05$) and in the PI boar population ($P < 0.01$), and also significantly ($P < 0.01$) associated with PDR in the PI boars, as well as significantly ($P < 0.05$) associated with ASR in the MIXED boars and in the PI boars. The genetic effect was not estimated due to missing of the genotype GG of the *GnRHR* locus.

7. The locus *PRL* was significantly ($P < 0.01$) associated with SCON in the PI×HA boars, but only dominance effect between allele A and allele C was significant ($P < 0.01$) on SCON, no significant additive effect between allele A and C was observed.

8. The *RLN* gene significantly ($P < 0.05$) affected VOL in the PI×HA boar population. The genetics effect was not estimated due to missing of the genotype AA of the *RLN* gene.

9. The candidate gene *RBP4* had significantly effect ($P < 0.05$) MOT in the PI population.

10. The *INHA* locus was significantly ($P < 0.05$) associated with ASR in the PI boars and in the PI×HA boar population, but no significant additive and dominance effects were observed in these boar populations.

11. The locus *FST* significantly affected VOL ($P < 0.01$) in the PI population, only allele `*142* bp´ had significant allele substitution effect ($P < 0.05$) on VOL.

12. The candidate gene *INHBA* locus was significantly associated with PDR ($P <$ 0.05) in the MIXED boar population, but no significant allele substitution effect on PDR was observed. This locus was also significantly associated with ASR in the MIXED boar population ($P < 0.01$) and in the PI boars ($P < 0.05$) and in the PI×HA boars ($P < 0.05$). However, no significant allele substitution effect on ASR was observed in these boar populations.

13. The locus *INHBB* was significantly associated with SCON in the MIXED boar population ($P < 0.05$) and in the PI boar population ($P < 0.01$). Whereas, no significant allele substitution effect on SCON was observed in these boar populations.

14. The *OPNpro* locus significantly ($P < 0.05$) affected ASR in the PI population, however, no significant allele substitution effect on ASR was observed.

15. The candidate gene *AR* was significantly ($P < 0.05$) associated with ASR in the MIXED boar population.

The results of this study demonstrate the difficulties in confirming candidate gene effects in different genetic groups. Allele effects differ between lines or populations due to the genetic background. Different linkage phases between the intronic markers and a causal mutation due to recombination may explain the observed differences between the boar populations. Also, still unknown quantitative trait loci with an effect on boar reproductive traits could be linked to these genes. The candidate gene approach can be used to differentiate between paternal and maternal contribution to fertility components and to explain gene actions such as imprinting. The use of marker information in combination with traditional selection tools allows faster improvement of male fertility, and at the same time optimal progress on other economically important production traits can be made. The selection strategy should be designed separately for each line and should always regard possible.

7 Zusammenfassung

Die vorliegende Studie wurde durchgeführt, um Assoziationen von 17 Kandidatengenen mit Merkmalen der Spermaqualität zu untersuchen. Zusätzlich wurden diese Gene auf Assoziationen mit Fruchtbarkeitsmerkmalen der Ebern getestet, welche die Erfolgsrate der künstlichen Besamung widerspiegeln. Diese direkten funktionellen Kandidatengene Gonadotropin Releasing Hormonrezeptor (*GnRHR*), Follikel-stimulierendes Hormon beta (*FSHB*), Luteinizing-Hormon beta (*LHB*), Relaxin (*RLN*), Prolactin (*PRL*), Prolactin Rezeptor (*PRLR*), Androgen Rezeptor (*AR*), Follistatin (*FST*), Inhibin Alfa (*INHA*), Beta-Inhibin A and B (*INHBA, INHBB*), Retinol-binde Protein 4 (*RBP4*), Acrosin (*ACR*), Osteopontin (*OPN*), Actin Beta (*ACTB*), Actin Gamma (*ACTG2*), Alfa-Actinin 1 und 4 (*ACTN1, ACTN4*), wurden basierend auf ihren Biologischen Korrelationen und/oder physiologischen Funktionen in der männlichen Gonadotropin-Achse ausgewählt.

Die reinrassigen Pietraineber (PI) (n=244) und die Kreuzungseber Pietrain × Hampshire (PI×HA) (n=112) aus einer Besamungsstation, geboren zwischen 1990 und 1999, und in kommerziellen Herden hauptsächlich in Nordwest Deutschland verwendet, wurden als Tiermaterial in dieser Studie verwendet. Die Spermaqualitat-Merkmale, umfassen Spermienkonzentration (SCON), Spermavolumen je Ejakulat (VOL), Spermienmotilität (MOT), Plasmatröpfchenrate (PDR) und abnormale Spermienrate (ASR) und wurden von jedem Ejakulat mit einem Lichtmikroskop entsprechend der Richtlinien der Weltgesundheitsorganisation, erfasst. Die Fruchtbarkeitsmerkmale Non-Return-Rate (NRR) und Anzahl lebend geborener Ferkel (NBA) wurden aus den kommerziellen Tiergruppen zwischen 1991 und 1999 abgeleitet.

Spermaproben wurden von Jan 2000 bis Dez. 2001 gesammelt und bis zu der DNA-Isolierung bei -20°C aufbewahrt. Genomische DNA wurde aus den Spermaproben der einzelnen Eber mit dem Standardprotokoll mit Mecaptoethanol Behandlung und ProteinaseK Verdau gefolgt von Phenol/Phenolchloroform Extraktion und Ausfällung mit Isopropanol reinigt.

Um Polymorphismen innerhalb der *ACTN1*- und *ACTN4*-Gene zu screenen, wurden Oligonukleotid Primer in Übereinstimmung mit dem porcinen *ACTN1* (BF442592) und porcinen *ACTN4* (BI338543) designed. Die PCR-Fragmente stammten aus genomischer DNA. Durch komparative Sequenzierung von Tieren der Rassen Pietrain und Hampshire wurden Polymorphismen im Intron 18 (G>A) des porcinen *ACTN1* Gens und im 3´-UTR (A>C) des porcinen *ACTN4* Gens, identifiziert. Diese Polymorphismen wurden durch eine PCR-RFLP mit den Restriktionsenzymen B*st*EΠ und *Apa*I, bestätigt.

Genotypen von 356 Ebern wurden für die 18 Genorte mit Hilfe eines PCR-RFLP, PCR-SSCP oder Mikrosatellite analysiert.

Assoziationsanalysen ergaben, dass die Genorte *ACTN1*, *ACR* und *OPNin6* signifikant mit der Eberfruchtbarkeit assoziiert waren und die Genorte *FSHB*, *PRL*, *ACR*, *INHA*, *INHBA*, *INHBB*, *FST*, *RLN*, *ACTG2*, *GnRHR*, *RBP4*, *OPNin6*, *AR* und *OPNpro* signifikant mit der Spermaqualität in Abhängigkeit von der Rasse des Ebers assoziiert waren. Die Genorte *LHB*, *PRLR* und *ACTN4* hatten keinen signifikanten Effekt auf irgendeinen der reproduktiven Merkmale der Eber.

Im Einzelnen:

1. Kandidatengen *ACTN1* Locus beeinträchtigt signifikant ($P < 0.05$) die NRR in der MIXED-Population und in der PI-Population. Additive Effekte ($P < 0.01$) und dominante Effekte ($P < 0.05$) waren signifikant auf NRR.

2. Locus *OPNin6* war signifikant ($P < 0.05$) mit NBA in der MIXED-Population und in der PI-Population assoziiert. Jedoch war nur der additive Effekt in beiden Populationen signifikant ($P < 0.05$), der dominante Effekt war nicht signifikant. Dieser Locus war ebenfalls signifikant ($P \leq 0.05$) mit MOT in der MIXED-Population assoziiert, aber additive und dominante Effekte waren nicht signifikant.

3. Kandidatengen *ACR* hat einen signifikanten Effekt ($P < 0.05$) auf NRR in der MIXED-Population und in der PI-Population. Aber nur Allel `*208* bp´ hat einen signifikanten Allel-Substitutionseffekt ($P < 0.05$) auf die NRR in der PI-Population. Der Genort *ACR* beeinflusst ebenfalls SCON in der PI×HA-

Population signifikant ($P \leq 0.01$). Die Allele `*208* bp´ und `*218* bp´ hatten signifikante Allel-Substitutionseffekte ($P < 0.05$) auf SCON. Dieser Genort hatte einen signifikanten Effekt ($P < 0.01$) auf MOT in der MIXED-Population und in der PI-Population. Jedoch wurden keine Allel-Substitutionseffekte auf MOT gefunden.

*4.* Genort *ACTG2* war signifikant mit VOL in der PI-Population ($P < 0.01$) assoziiert. Aber nur Allel `*109* bp´ hatte einen signifikanten Allel-Substitutionseffekt auf das VOL. Dieser Marker war signifikant ($P < 0.05$) mit MOT in der PI×HA-Population assoziiert, während kein signifikanter Allel-Substitutionseffekt auf MOT beobachtet wurde.

5. Genort *FSHB* war signifikant ($P \leq 0.01$) mit SCON in der PI-Population assoziiert. Nur der additive Effekt war signifikant ($P < 0.05$) auf SCON, der dominante Effekt war nicht signifikant.

6. Der *GnRHR* Marker war signifikant mit MOT in der MIXED-Population assoziiert ($P < 0.05$) und in der PI-Population ($P < 0.01$), und signifikant ($P < 0.01$) mit PDR in der PI-Population assoziiert, ebenfalls signifikant ($P < 0.05$) mit ASR in der MIXED-Population und in der PI-Population assoziiert.

7. Genort *PRL* war signifikant ($P < 0.01$) mit SCON in der PI×HA-Population assoziiert. Aber nur der dominante Effekt zwischen Allel A und Allel C war signifikant ($P < 0.01$) auf SCON, es wurden keine signifikanten additiven Effekte zwischen Allel A und C gefunden.

8. Genort *RLN* beeinflusst signifikant ($P < 0.05$) das VOL in der PI×HA-Population.

9. Das Kandidatengen *RBP4* hatte einen signifikanten Effekt ($P < 0.05$) hinsichtlich MOT in der PI-Population.

10. Der Locus *INHA* war signifikant ($P < 0.05$) mit ASR in der PI-Population und in der PI×HA-Population assoziiert. Aber additive und dominante Effekte waren in beiden Populationen nicht signifikant.

11. Der Genort *FST* beeinflusst VOL in der PI-Population signifikant ($P < 0.01$). Aber nur Allel `*142* bp´ hatte einen signifikanten Allel-Substitutionseffekt ($P < 0.05$) auf VOL.

12. Der Locus *INHBA* war signifikant ($P < 0.05$) mit PDR in der MIXED-Population assoziiert. Aber es wurde kein Allel-Substitutionseffekt auf PDR gefunden. Dieser Genort war signifikant mit ASR in der MIXED-Population ($P < 0.01$) und in der PI-Population ($P < 0.05$) und in der PI×HA-Population ($P < 0.05$) assoziiert. Jedoch wurde kein signifikanter Allel-Substitutionseffekt auf ASR gefunden.

13. Der Genort *INHBB* war signifikant mit SCON in der MIXED-Population ($P < 0.05$) und in der PI-Population ($P < 0.01$) assoziiert, wobei kein signifikantes Allel-Substitutionseffekt auf SCON gefunden wurde.

14. Locus *OPNpro* beeinflusste signifikant ($P < 0.05$) ASR in der PI-Population, jedoch hatte alle Allele keine signifikanten Allel-Substitutionseffekte auf ASR.

15. Das Kandidatengen *AR* war signifikant ($P < 0.05$) mit ASR in der MIXED-Population assoziiert.

Die Ergebnisse dieser Studie zeigen die Schwierigkeiten in der Bestätigung von Kandidatengeneffekten zwischen den unterschiedlichen genetischen Gruppen. Alleleffekte unterscheiden sich zwischen den Linien oder Populationen wegen des genetischen Hintergrundes. Unterschiedliche Verknüpfungsphasen zwischen den intronic Marker und verursachenden Mutationen der Rekombination können die beobachteten Unterschiede zwischen den Eberpopulationen erklären. Zudem konnten unbekannte QTL mit einem Effekt auf Reproduktionsmerkmale des Ebers mit diesen Genen verbunden werden. Der Kandidatengenansatz kann verwendet werden, um zwischen väterlichem und mütterlichem Beitrag zu den Fruchtbarkeiten zu unterscheiden und Gen-Aktivitäten zu erklären. Die Nutzung von Marker Informationen im Zusammenhang mit traditioneller Selektion erlaubt schnellere Verbesserung der männlichen Fruchtbarkeit, und gleichzeitig kann optimaler Fortschritt auf anderen ökonomischen wichtigen Produktionsmerkmalen erreicht werden. Die Selektions-strategie sollte für jede Linie separat bestimmt werden.

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# 9 Appendixes

## 9.1 Estimates of LSMs for genotypic effects on NRR

Table 9.1:      Estimates of NRR (%) for boars in dependence from the loci *FSHB*, *PRL*, *PRLR*, *INHA*, *RBP4*, *ACTN4*, *GnRHR*, *RLN*, *LHB*, *OPNin6*, and *AR*

| Locus | Genotype | MIXED | PI | PI×HA |
|-------|----------|-------|-----|-------|
| *FSHB* | AA | 0.599±0.466 | 0.486±0.585 | 0.390±0.591 |
|  | AB | 0.953±0.674 | 0.597±0.877 | 1.436±0.816 |
|  | BB | -1.749±1.705 | -2.763±2.609 | -0.784±1.719 |
| *PRL* | AA | 0.241±0.747 | 0.037±1.057 | 0.365±0.795 |
|  | AB | 0.513±0.889 | 1.008±1.396 | -0.118±0.882 |
|  | AC | 1.037±0.643 | 0.579±0.798 | 1.899±0.802 |
|  | BC | -5.593±2.827 | -5.567±3.126 | - |
|  | CC | 0.890±0.937 | 1.028±1.089 | -0.669±1.500 |
| *PRLR* | AA | 0.404±0.458 | 0.102±0.629 | 0.578±0.540 |
|  | AB | 1.644±0.753 | 1.699±0.892 | 0.877±1.115 |
|  | BB | -0.642±1.059 | -1.054±1.196 | 0.706±2.453 |
| *INHA* | AA | 0.323±3.076 | -1.860±4.886 | 2.473±2.999 |
|  | AB | 0.173±0.609 | -0.101±1.028 | 0.359±0.587 |
|  | BB | 0.852±0.520 | 0.539±0.551 | 1.028±0.748 |
| *RBP4* | AA | 0.636±0.778 | 0.497±0.801 | -1.208±2.436 |
|  | AB | 0.549±0.441 | 0.329±0.605 | 0.693±0.513 |
|  | BB | - | - | - |
| *ACTN4* | AA | 0.818±0.605 | 0.607±0.781 | 0.831±0.782 |
|  | AB | 1.344±0.556 | 1.045±0.696 | 1.649±0.723 |
|  | BB | 0.890±0.930 | 0.699±1.275 | 1.132±1.100 |
| *GnRHR* | CC | 0.615±0.450 | 0.351±0.528 | 0.790±0.569 |
|  | CG | 0.442±0.785 | 0.490±1.316 | 0.253±0.766 |
|  | GG | - | - | - |
| *RLN* | CC | 0.706±0.429 | 0.658±0.551 | 0.515±0.522 |
|  | CA | -0.238±0.900 | -0.762±1.095 | 1.621±1.348 |
|  | AA | - | - | - |
| *LHB* | CC | 0.665±0.557 | 0.558±0.794 | 0.669±0.648 |
|  | CT | 0.388±0.558 | -0.025±0.692 | 0.972±0.737 |
|  | TT | 0.788±0.821 | 1.130±1.068 | -0.420±1.060 |
| *OPNin6* | AA | 0.253±0.778 | 0.210±0.864 | -0.643±1.436 |
|  | AB | 1.066±0.621 | 0.945±0.825 | 0.997±0.744 |
|  | BB | 0.336±0.538 | 0.062±0.755 | 0.562±0.615 |
| *AR* | *196* bp | 1.315±0.633 | 0.701±0.606 | 1.534±1.101 |
|  | *186* bp | -0.162±0.600 | -1.110±1.126 | 0.496±0.533 |
|  | *173* bp | 1.112±1.286 | 0.786±1.576 | 0.452±1.933 |
|  | *172* bp | 1.241±1.303 | 0.580±1.375 | - |

-: The LSMs were not estimated since the genotype was absent or very rare in the boars

Table 9 2:      Estimates of NRR (%) for boars in dependence from the *FST* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 142/142 | -0.103±1.029 | 142/142 | -1.357±1.675 | 142/142 | 0.892±1.042 |
| 142/147 | -0.232±1.460 | 142/146 | -0.800±3.898 | 142/147 | -0.449±1.312 |
| 142/148 | -1.499±1.769 | 142/147 | 0.517±2.757 | 142/148 | -0.199±1.997 |
| 142/999 | 1.441±2.167 | 142/148 | -2.707±2.560 | 142/999 | 2.711±1.983 |
| 143/143 | 1.022±0.799 | 143/143 | 1.296±1.046 | 143/143 | 0.262±1.036 |
| 143/147 | 1.142±1.502 | 143/146 | 6.864±2.535 | 143/147 | 1.737±1.590 |
| 143/148 | 1.278±0.921 | 143/147 | 0.467±2.254 | 143/148 | 1.022±1.412 |
| 143/999 | 5.005±1.951 | 143/148 | 1.196±1.109 | 143/999 | -0.578±3.082 |
| 144/144 | 0.516±1.611 | 144/144 | 1.255±2.157 | 144/144 | -1.242±1.956 |
| 144/147 | -0.353±2.096 | 144/147 | -0.635±2.819 | 144/147 | 0.038±1.559 |
| 144/148 | 3.700±1.746 | 144/148 | 3.495±1.864 | 147/147 | 1.055±1.866 |
| 144/999 | 0.176±2.521 | 144/999 | -0.737±3.003 | 147/999 | 1.251±3.138 |
| 147/147 | -0.389±1.185 | 146/146 | 3.083±3.030 | 999/999 | 1.611±1.728 |
| 147/999 | -1.017±1.966 | 147/147 | -0.958±1.422 | | |
| 148/148 | -2.194±1.375 | 147/999 | -1.802±2.385 | | |
| 148/999 | -0.916±1.989 | 148/148 | -2.300±1.487 | | |
| 999/999 | -0.491±1.533 | 148/999 | -1.144±2.254 | | |
| | | 999/999 | 1.174±3.390 | | |

Table 9.3:      Estimates of NRR (%) for boars in dependence from the *INHBA* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 252/252 | 0.790±0.513 | 252/252 | 0.464±0.612 | 252/252 | 1.082±0.656 |
| 252/254 | -0.175±0.718 | 252/254 | -0.694±0.970 | 252/254 | 0.445±0.830 |
| 252/256 | 0.284±1.100 | 252/999 | 1.612±1.528 | 252/256 | -1.231±1.135 |
| 252/999 | 1.038±1.695 | 254/254 | 1.033±2.626 | 252/999 | 0.131±1.467 |
| 254/254 | 0.959±2.096 | 254/999 | 3.541±4.015 | 254/254 | 0.570±3.071 |

Table 9.4:       Estimates of NRR (%) for boars in dependence from the *ACTG2* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 93/93 | -0.329±1.965 | 93/93 | 1.893±2.643 | 93/93 | -5.711±2.346 |
| 93/103 | 0.942±1.823 | 93/103 | 1.117±2.022 | 93/97 | -1.796±1.772 |
| 93/109 | 2.664±1.051 | 93/109 | 3.209±1.548 | 93/103 | 2.356±1.082 |
| 93/111 | 0.961±1.899 | 93/111 | 1.744±2.660 | 93/111 | -0.322±2.054 |
| 93/117 | 4.935±2.771 | 93/117 | 3.659±3.473 | 97/103 | 1.890±1.412 |
| 93/999 | 0.003±2.049 | 93/999 | 2.873±4.022 | 97/109 | 0.877±2.311 |
| 103/103 | 1.107±2.075 | 103/103 | 1.308±2.295 | 97/111 | 0.381±1.324 |
| 103/109 | -0.237±0.976 | 103/109 | 0.314±1.374 | 97/117 | 1.914±2.888 |
| 103/111 | -1.050±1.407 | 103/111 | -0.845±1.537 | 97/999 | 1.852±2.331 |
| 103/999 | 2.246±1.443 | 103/999 | 0.818±2.309 | 103/109 | -1.225±1.048 |
| 109/109 | 0.216±1.143 | 109/109 | 0.443±1.488 | 109/109 | -0.288±1.412 |
| 109/111 | 0.740±0.989 | 109/111 | 0.780±1.298 | 109/111 | 0.835±1.173 |
| 109/117 | 3.279±1.734 | 109/117 | 4.392±2.472 | 109/117 | 2.263±1.866 |
| 109/999 | -1.750±1.424 | 109/999 | -2.937±1.843 | 109/999 | 2.881±2.841 |
| 111/111 | -3.280±1.561 | 111/111 | -3.365±1.773 | 111/999 | -0.597±2.346 |
| 111/117 | -1.093±2.058 | 111/117 | -1.352±2.436 | 117/999 | 1.448±2.815 |
| 111/999 | 0.675±1.273 | 111/999 | 1.109±2.081 | 999/999 | 2.640±2.804 |
| 117/999 | 0.176±2.332 | 117/999 | -0.624±3.995 | | |
| 999/999 | 2.430±1.695 | 999/999 | 3.606±2.609 | | |

Table 9.5:       Estimates of NRR (%) for boars in dependence from the *INHBB* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 161/163 | 1.686±2.051 | 171/171 | 0.510±0.915 | 159/159 | 0.181±1.826 |
| 161/171 | -0.397±1.481 | 171/173 | 0.714±0.960 | 159/171 | 0.323±1.146 |
| 161/173 | 2.301±2.595 | 171/175 | -0.070±1.004 | 159/173 | 1.675±2.517 |
| 163/171 | -1.356±1.837 | 171/999 | -4.291±2.348 | 159/999 | 0.846±2.173 |
| 163/173 | 2.423±2.610 | 173/173 | 0.947±2.010 | 161/163 | 1.792±1.393 |
| 163/175 | 2.808±2.610 | 173/175 | 0.327±1.478 | 161/171 | 0.114±1.026 |
| 163/999 | 0.107±2.627 | 173/999 | 1.054±2.626 | 161/173 | 3.794±1.970 |
| 171/171 | 0.807±0.756 | 175/175 | 1.287±1.971 | 163/171 | 0.110±1.970 |
| 171/173 | 0.712±0.815 | 175/999 | 2.267±2.846 | 171/171 | 0.967±0.955 |
| 171/175 | 0.299±0.987 | | | 171/173 | -0.271±1.137 |
| 171/999 | -0.705±1.638 | | | 171/999 | -0.544±2.550 |
| 173/173 | 1.098±1.721 | | | 173/173 | 0.017±3.064 |
| 173/175 | 0.739±1.422 | | | | |
| 175/175 | 1.752±1.840 | | | | |
| 175/999 | 0.807±3.175 | | | | |

Table 9.6:     Estimates of NRR (%) for boars in dependence from the *OPNpro* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 118/118 | 1.000±1.713 | 118/118 | 0.803±1.875 | 118/142 | 2.353±1.628 |
| 118/142 | 1.116±1.122 | 118/142 | 0.528±1.405 | 118/146 | 1.604±2.235 |
| 118/146 | 3.457±2.211 | 118/146 | 5.036±3.491 | 118/148 | 0.929±1.331 |
| 118/148 | 0.050±0.967 | 118/148 | -0.372±1.223 | 126/142 | 0.357±2.566 |
| 142/142 | 1.198±1.135 | 142/142 | 1.032±1.185 | 126/148 | 0.559±1.379 |
| 142/146 | -0.016±1.628 | 142/146 | -0.278±2.848 | 126/999 | 0.104±3.112 |
| 142/148 | -0.800±0.760 | 142/148 | -1.020±0.905 | 138/142 | 0.325±1.709 |
| 142/999 | 0.877±1.769 | 146/146 | 0.489±3.130 | 138/148 | 0.454±1.569 |
| 146/146 | -0.668±2.212 | 146/148 | 0.207±2.452 | 142/146 | -0.101±1.551 |
| 146/148 | 0.015±1.720 | 148/148 | 2.532±1.190 | 142/148 | 0.583±1.091 |
| 148/148 | 2.255±0.918 | 148/999 | -1.451±3.491 | 142/999 | 3.873±3.101 |
| 148/999 | 0.442±1.264 | | | 146/146 | -2.963±2.558 |
| | | | | 146/148 | 0.363±1.970 |
| | | | | 148/148 | 1.051±1.247 |
| | | | | 148/999 | 2.530±2.539 |

## 9.2 Estimates of LSMs for genotypic effects on NBA

Table 9.7:     Estimates of NBA (pig/litter) for boars in dependence from the loci
              *FSHB*, *PRL*, *PRLR*, *INHA*, *RBP4*, *ACTN4*, *GnRHR*, *RLN*, *LHB*, and *AR*

| Locus | Genotype | MIXED | PI | PI×HA |
|-------|----------|-------|----|----|
| *FSHB* | AA | 0.025±0.041 | 0.061±0.047 | -0.018±0.072 |
|  | AB | 0.033±0.060 | 0.007±0.071 | 0.119±0.099 |
|  | BB | 0.079±0.151 | 0.258±0.212 | -0.115±0.209 |
| *PRL* | AA | -0.013±0.062 | -0.058±0.084 | 0.058±0.084 |
|  | AB | 0.109±0.074 | 0.233±0.110 | -0.029±0.093 |
|  | AC | 0.052±0.054 | 0.053±0.063 | 0.049±0.085 |
|  | BC | 0.383±0.236 | 0.400±0.250 | - |
|  | CC | 0.056±0.078 | 0.059±0.086 | 0.067±0.159 |
| *PRLR* | AA | 0.021±0.041 | 0.051±0.051 | -0.005±0.065 |
|  | AB | 0.059±0.067 | 0.063±0.073 | 0.077±0.135 |
|  | BB | 0.054±0.094 | 0.064±0.097 | 0.284±0.296 |
| *INHA* | AA | 0.151±0.271 | 0.306±0.395 | -0.022±0.365 |
|  | AB | 0.027±0.014 | 0.085±0.083 | -0.009±0.071 |
|  | BB | 0.032±0.046 | 0.046±0.045 | 0.066±0.091 |
| *RBP4* | AA | 0.084±0.069 | 0.113±0.065 | -0.241±0.296 |
|  | AB | 0.019±0.039 | 0.026±0.049 | 0.026±0.062 |
|  | BB | - | - | - |
| *ACTN4* | AA | 0.036±0.056 | 0.079±0.066 | -0.023±0.099 |
|  | AB | 0.041±0.051 | 0.052±0.058 | 0.036±0.092 |
|  | BB | -0.100±0.085 | -0.170±0.107 | 0.017±0.140 |
| *GnRHR* | CC | 0.011±0.040 | 0.045±0.043 | -0.025±0.069 |
|  | CG | 0.089±0.069 | 0.070±0.107 | 0.108±0.092 |
|  | GG | - | - | - |
| *RLN* | CC | 0.020±0.038 | 0.034±0.045 | 0.029±0.063 |
|  | CA | 0.071±0.080 | 0.116±0.089 | -0.097±0.164 |
|  | AA | - | - | - |
| *LHB* | CC | 0.034±0.051 | 0.094±0.064 | -0.044±0.078 |
|  | CT | 0.022±0.049 | 0.041±0.056 | 0.036±0.089 |
|  | TT | 0.055±0.073 | 0.025±0.086 | 0.185±0.128 |
| *AR* | *196* bp | 0.040±0.056 | 0.042±0.049 | 0.103±0.131 |
|  | *186* bp | 0.010±0.053 | 0.091±0.091 | -0.020±0.064 |
|  | *173* bp | 0.138±0.113 | 0.061±0.127 | 0.469±0.230 |
|  | *172* bp | 0.047±0.114 | 0.058±0.111 | - |

-: The LSMs were not estimated since the genotype was absent or very rare in the boars

Table 9.8: Estimates of NBA (pig/litter) for boars in dependence from the *FST* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 142/142 | 0.100±0.091 | 142/142 | 0.079±0.141 | 142/142 | 0.121±0.125 |
| 142/147 | -0.086±0.129 | 142/146 | 0.004±0.328 | 142/147 | -0.043±0.158 |
| 142/148 | 0.032±0.157 | 142/147 | -0.200±0.232 | 142/148 | 0.051±0.240 |
| 142/999 | -0.121±0.192 | 142/148 | 0.081±0.216 | 142/999 | -0.183±0.238 |
| 143/143 | 0.074±0.071 | 143/143 | 0.109±0.088 | 143/143 | 0.041±0.125 |
| 143/147 | 0.019±0.133 | 143/146 | 0.182±0.214 | 143/147 | 0.082±0.191 |
| 143/148 | -0.018±0.082 | 143/147 | 0.007±0.190 | 143/148 | -0.174±0.170 |
| 143/999 | 0.152±0.173 | 143/148 | 0.048±0.093 | 143/999 | 0.143±0.370 |
| 144/144 | -0.008±0.143 | 144/144 | -0.025±0.182 | 144/144 | 0.054±0.235 |
| 144/147 | -0.025±0.186 | 144/147 | 0.189±0.237 | 144/147 | -0.468±0.307 |
| 144/148 | -0.285±0.154 | 144/148 | -0.264±0.157 | 147/147 | 0.235±0.224 |
| 144/999 | -0.134±0.223 | 144/999 | -0.198±0.253 | 147/999 | 0.415±0.377 |
| 147/147 | 0.131±0.105 | 146/146 | 0.112±0.255 | 999/999 | 0.061±0.208 |
| 147/999 | 0.200±0.174 | 147/147 | 0.132±0.120 | | |
| 148/148 | 0.205±0.122 | 147/999 | 0.182±0.201 | | |
| 148/999 | -0.148±0.176 | 148/148 | 0.235±0.125 | | |
| 999/999 | 0.058±0.136 | 148/999 | -0.093±0.190 | | |
| | | 999/999 | 1.174±3.390 | | |

Table 9.9:    Estimates of NBA (pig/litter) for boars in dependence from the *INHBA* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 252/252 | 0.046±0.045 | 252/252 | 0.046±0.046 | 252/252 | 0.047±0.081 |
| 252/254 | 0.014±0.063 | 252/254 | 0.041±0.077 | 252/254 | -0.052±0.102 |
| 252/256 | 0.018±0.097 | 252/999 | 0.046±0.046 | 252/256 | 0.088±0.140 |
| 252/999 | 0.088±0.150 | 254/254 | 0.041±0.077 | 252/999 | 0.027±0.181 |
| 254/254 | -0.195±0.185 | 254/999 | -0.037±0.141 | 254/254 | 0.023±0.379 |

Table 9.10:    Estimates of NBA (pig/litter) for boars in dependence from the *ACTG2* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 93/93 | -0.032±0.175 | 93/93 | 0.078±0.217 | 93/93 | -0.160±0.312 |
| 93/103 | -0.110±0.162 | 93/103 | -0.056±0.166 | 93/97 | 0.263±0.236 |
| 93/109 | 0.029±0.094 | 93/109 | 0.091±0.127 | 93/103 | -0.060±0.144 |
| 93/111 | 0.205±0.169 | 93/111 | 0.163±0.218 | 93/111 | 0.301±0.274 |
| 93/117 | 0.274±0.247 | 93/117 | 0.279±0.285 | 97/103 | 0.156±0.188 |
| 93/999 | 0.331±0.183 | 93/999 | 0.590±0.330 | 97/109 | -0.174±0.308 |
| 103/103 | 0.142±0.185 | 103/103 | 0.173±0.188 | 97/111 | 0.058±0.176 |
| 103/109 | -0.036±0.087 | 103/109 | -0.117±0.113 | 97/117 | 0.106±0.384 |
| 103/111 | -0.051±0.125 | 103/111 | -0.003±0.126 | 97/999 | 0.035±0.310 |
| 103/999 | 0.118±0.129 | 103/999 | 0.054±0.189 | 103/109 | 0.148±0.140 |
| 109/109 | 0.157±0.102 | 109/109 | 0.216±0.122 | 109/109 | 0.095±0.188 |
| 109/111 | -0.175±0.088 | 109/111 | -0.068±0.106 | 109/111 | -0.383±0.156 |
| 109/117 | 0.041±0.154 | 109/117 | 0.065±0.203 | 109/117 | -0.032±0.248 |
| 109/999 | -0.044±0.127 | 109/999 | 0.096±0.151 | 109/999 | -0.642±0.378 |
| 111/111 | 0.131±0.139 | 111/111 | 0.156±0.145 | 111/999 | 0.150±0.312 |
| 111/117 | -0.052±0.183 | 111/117 | -0.057±0.200 | 117/999 | -0.033±0.375 |
| 111/999 | 0.061±0.113 | 111/999 | 0.119±0.171 | 999/999 | 0.129±0.373 |
| 117/999 | 0.022±0.208 | 117/999 | -0.113±0.328 | | |
| 999/999 | 0.082±0.151 | 999/999 | 0.111±0.214 | | |

Table 9.11:    Estimates of NBA (pig/litter) for boars in dependence from the *INHBB* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 161/163 | -0.157±0.178 | 171/171 | 0.038±0.074 | 159/159 | 0.046±0.217 |
| 161/171 | -0.096±0.129 | 171/173 | 0.106±0.077 | 159/171 | 0.262±0.136 |
| 161/173 | 0.109±0.225 | 171/175 | 0.051±0.081 | 159/173 | 0.357±0.299 |
| 163/171 | -0.042±0.160 | 171/999 | -0.009±0.190 | 159/999 | 0.173±0.258 |
| 163/173 | 0.076±0.227 | 173/173 | -0.114±0.162 | 161/163 | -0.149±0.165 |
| 163/175 | -0.349±0.227 | 173/175 | 0.154±0.119 | 161/171 | -0.126±0.122 |
| 163/999 | 0.041±0.228 | 173/999 | 0.080±0.212 | 161/173 | 0.135±0.234 |
| 171/171 | -0.009±0.066 | 175/175 | 0.207±0.159 | 163/171 | 0.211±0.234 |
| 171/173 | 0.079±0.071 | 175/999 | -0.310±0.230 | 171/171 | -0.104±0.113 |
| 171/175 | 0.027±0.086 | | | 171/173 | -0.271±1.137 |
| 171/999 | 0.312±0.142 | | | 171/999 | -0.544±2.550 |
| 173/173 | -0.057±0.149 | | | 173/173 | 0.017±3.064 |
| 173/175 | 0.126±0.123 | | | | |
| 175/175 | 0.173±0.160 | | | | |
| 175/999 | 0.155±0.276 | | | | |

Table 9.12:    Estimates of NBA (pig/litter) for boars in dependence from the *OPNpro* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotyp | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 118/118 | 0.321±0.151 | 118/118 | 0.341±0.150 | 118/142 | 0.252±0.196 |
| 118/142 | 0.048±0.099 | 118/142 | -0.004±0.113 | 118/146 | -0.049±0.270 |
| 118/146 | 0.003±0.195 | 118/146 | 0.062±0.280 | 118/148 | 0.288±0.161 |
| 118/148 | 0.178±0.085 | 118/148 | 0.148±0.098 | 126/142 | -0.150±0.310 |
| 142/142 | 0.057±0.100 | 142/142 | 0.065±0.095 | 126/148 | -0.097±0.166 |
| 142/146 | -0.101±0.143 | 142/146 | -0.227±0.228 | 126/999 | 0.135±0.375 |
| 142/148 | -0.016±0.067 | 142/148 | -0.024±0.073 | 138/142 | 0.140±0.206 |
| 142/999 | 0.029±0.156 | 146/146 | 0.447±0.251 | 138/148 | -0.109±0.189 |
| 146/146 | 0.280±0.195 | 146/148 | -0.211±0.197 | 142/146 | 0.003±0.187 |
| 146/148 | -0.136±0.152 | 148/148 | 0.110±0.095 | 142/148 | 0.020±0.132 |
| 148/148 | 0.006±0.081 | 148/999 | 0.167±0.280 | 142/999 | 0.004±0.374 |
| 148/999 | -0.044±0.111 | | | 146/146 | -2.963±2.558 |
| | | | | 146/148 | 0.363±1.970 |
| | | | | 148/148 | 1.051±1.247 |
| | | | | 148/999 | 2.530±2.539 |

Table 9.13: Estimates of NBA (pig/litter) for boars in dependence from the *ACR* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM + SE | Genotype | LSM + SE | Genotype | LSM + SE |
| 202/202 | 0.221±0.186 | 202/202 | 0.207 ±0.200 | 208/208 | 0.077±0.126 |
| 202/208 | 0.062±0.087 | 202/208 | 0.083±0.082 | 208/218 | 0.028±0.241 |
| 202/214 | -0.070±0.145 | 202/214 | -0.088±0.157 | 208/226 | 0.165±0.123 |
| 202/999 | -0.043±0.142 | 202/999 | 0.005 ±0.143 | 208/228 | -0.056±0.099 |
| 208/208 | 0.007±0.062 | 208/208 | 0.006±0.064 | 208/999 | 0.044±0.138 |
| 208/214 | 0.075±0.121 | 208/214 | 0.077±0.125 | 218/228 | -0.213±0.261 |
| 208/228 | -0.048±0.095 | 208/999 | 0.104 ±0.094 | 218/999 | -0.052±0.299 |
| 208/999 | 0.122±0.068 | 214/214 | 0.298±0.255 | 226/999 | 0.088±0.367 |
| 214/214 | 0.235±0.228 | 214/999 | 0.031±0.286 | 228/999 | -0.390±0.263 |
| 214/228 | -0.286±0.277 | | | 999/999 | 0.206±0.267 |
| 228/999 | -0.038±0.195 | | | | |

## 9.3 Estimates of LSMs for genotypic effects on SCON

Table 9.14: Estimates of SCON ($10^8$/ml) for boars in dependence from the loci *RLN*, *PRLR*, *RBP4*, *INHA*, *OPNin6*, *ACTN1*, *ACTN4*, *LHB*, *GnRHR*, and *AR*

| Locus | Genotype | MIXED | PI | PI×HA |
|---|---|---|---|---|
| *PRLR* | AA | 3.017±0.039 | 2.998±0.049 | 3.046±0.063 |
| | AB | 2.992±0.072 | 2.924±0.072 | 3.235±0.179 |
| | BB | 2.846±0.099 | 2.794±0.097 | 3.167±0.400 |
| *RBP4* | AA | 3.042±0.072 | 2.989±0.066 | 3.093±0.328 |
| | AB | 2.989±0.038 | 2.929±0.046 | 3.067±0.060 |
| | BB | - | - | - |
| *INHA* | AA | 2.964±0.287 | 3.258±0.410 | - |
| | AB | 3.024±0.055 | 2.972±0.082 | 3.090±0.075 |
| | BB | 2.980±0.047 | 2.939±0.043 | 3.051±0.097 |
| *OPNin6* | AA | 3.070±0.07 | 2.970±0.07 | 3.492±0.22 |
| | AB | 2.986±0.06 | 2.976±0.07 | 2.910±0.10 |
| | BB | 2.987±0.05 | 2.887±0.06 | 3.109±0.07 |
| *ACTN1* | AA | 2.990±0.043 | 2.929±0.053 | 3.065±0.070 |
| | AB | 3.025±0.060 | 2.966±0.064 | 3.098±0.127 |
| | BB | 2.969±0.139 | 2.871±0.154 | 3.165±0.330 |
| *ACTN4* | AA | 3.018±0.057 | 2.988±0.066 | 3.063±0.102 |
| | AB | 3.023±0.051 | 2.971±0.059 | 3.125±0.091 |
| | BB | 2.923±0.088 | 2.874±0.107 | 3.008±0.148 |
| *LHB* | CC | 3.022±0.053 | 2.959±0.063 | 3.116±0.093 |
| | CT | 3.001±0.050 | 2.949±0.056 | 3.083±0.091 |
| | TT | 2.941±0.076 | 2.926±0.088 | 2.922±0.142 |
| *GnRHR* | CC | 2.988±0.039 | 2.937±0.040 | 3.066±0.071 |
| | CG | 3.030±0.075 | 3.007±0.109 | 3.074±0.104 |
| | GG | - | - | - |
| *RLN* | CC | 3.006±0.079 | 2.998±0.085 | 3.054±0.105 |
| | CA | 3.117±0.168 | 3.007±0.109 | 3.174±0.221 |
| | AA | - | - | - |
| *AR* | *196* bp | 2.909±0.057 | 2.912±0.047 | 3.002±0.148 |
| | *186* bp | 3.087±0.055 | 3.123±0.092 | 3.084±0.066 |
| | *173* bp | 3.019±0.123 | 3.032±0.128 | 3.017±0.321 |
| | *172* bp | 2.801±0.120 | 2.840±0.112 | - |

-: The LSMs were not estimated since the genotype was absent or very rare in the boars

Table 9.15:     Estimates of SCON ($10^8$/ml) for the boars dependent on the *FST* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 142/142 | 3.015±0.091 | 142/142 | 2.843±0.130 | 142/142 | 3.190±0.124 |
| 142/143 | 2.768±0.566 | 142/143 | 2.693±0.556 | 142/147 | 3.253±0.197 |
| 142/147 | 3.029±0.151 | 142/146 | 2.856±0.318 | 142/148 | 3.453±0.257 |
| 142/148 | 2.973±0.164 | 142/147 | 2.789±0.228 | 142/999 | 2.928±0.263 |
| 142/999 | 2.820±0.200 | 142/148 | 2.667±0.210 | 143/143 | 3.025±0.130 |
| 143/143 | 2.963±0.074 | 143/143 | 2.830±0.086 | 143/147 | 3.111±0.225 |
| 143/147 | 3.123±0.145 | 143/146 | 3.018±0.195 | 143/148 | 2.840±0.197 |
| 143/148 | 2.976±0.086 | 143/147 | 3.120±0.184 | 143/999 | 3.765±0.398 |
| 143/999 | 3.114±0.172 | 143/148 | 2.953±0.090 | 144/144 | 2.715±0.246 |
| 144/144 | 2.682±0.146 | 143/999 | 2.637±0.557 | 144/147 | 3.051±0.393 |
| 144/147 | 3.338±0.201 | 144/144 | 2.644±0.175 | 144/999 | 2.678±0.553 |
| 144/148 | 2.837±0.160 | 144/147 | 3.395±0.227 | 147/147 | 2.661±0.251 |
| 144/999 | 2.831±0.230 | 144/148 | 2.777±0.153 | 147/999 | 2.982±0.391 |
| 147/147 | 2.910±0.111 | 144/999 | 2.814±0.247 | 148/148 | 1.933±0.550 |
| 147/999 | 3.423±0.179 | 146/146 | 3.331±0.246 | 999/999 | 2.813±0.225 |
| 148/148 | 3.025±0.124 | 146/148 | 2.110±0.550 | | |
| 148/999 | 3.173±0.181 | 146/999 | 3.455±0.551 | | |
| 999/999 | 3.046±0.141 | 147/147 | 2.914±0.118 | | |
| | | 147/999 | 3.485±0.195 | | |
| | | 148/148 | 3.023±0.120 | | |
| | | 148/999 | 3.225±0.184 | | |
| | | 999/999 | 2.896±0.228 | | |

Table 9.16:    Estimates of SCON ($10^8$/ml) for the boars dependent on the *INHBA* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 252/252 | 3.008±0.046 | 252/252 | 2.993±0.047 | 252/252 | 2.933±0.087 |
| 252/254 | 2.974±0.065 | 252/254 | 2.850±0.078 | 252/254 | 3.185±0.110 |
| 252/256 | 3.038±0.103 | 252/999 | 2.843±0.124 | 252/256 | 3.138±0.147 |
| 252/999 | 2.926±0.165 | 254/254 | 2.886±0.215 | 252/999 | 3.294±0.213 |
| 254/254 | 2.982±0.204 | 252/999 | 2.796±0.327 | 254/254 | 3.369±0.568 |
| 254/256 | 2.958±0.329 | 999/999 | 2.917±0.404 | 254/256 | 2.984±0.559 |
| 254/999 | 2.581±0.570 | | | 256/256 | 2.373±0.565 |
| 256/256 | 2.820±0.405 | | | 256/999 | 3.537±0.568 |
| 256/999 | 3.040±0.407 | | | | |

Table 9.17:    Estimates of SCON ($10^8$/ml) for boars dependent on the *OPNpro* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 118/118 | 3.060±0.159 | 118/118 | 3.011±0.153 | 118/138 | 3.167±0.596 |
| 118/142 | 2.914±0.105 | 118/142 | 2.776±0.116 | 118/142 | 3.294±0.224 |
| 118/146 | 2.879±0.203 | 118/146 | 3.068±0.285 | 118/146 | 2.696±0.298 |
| 118/148 | 3.019±0.091 | 118/148 | 3.010±0.096 | 118/148 | 2.914±0.211 |
| 118/999 | 3.558±0.405 | 118/999 | 3.949±0.570 | 126/142 | 3.224±0.344 |
| 142/142 | 2.899±0.105 | 142/142 | 2.851±0.096 | 126/148 | 3.132±0.198 |
| 142/146 | 2.808±0.153 | 142/146 | 2.818±0.231 | 126/999 | 3.036±0.418 |
| 142/148 | 2.994±0.069 | 142/148 | 2.941±0.071 | 138/142 | 3.242±0.267 |
| 142/999 | 3.082±0.176 | 142/999 | 3.495±0.566 | 138/146 | 3.288±0.592 |
| 146/146 | 2.814±0.202 | 146/14 | 2.662±2.534 | 138/148 | 3.328±0.270 |
| 146/148 | 3.028±0.165 | 146/148 | 2.961±0.214 | 142/146 | 2.830±0.211 |
| 146/999 | 3.226±0.571 | 148/148 | 3.070±0.094 | 142/148 | 3.086±0.153 |
| 148/148 | 3.087±0.084 | 148/999 | 2.659±0.283 | 142/999 | 2.576±0.422 |
| 148/999 | 3.042±0.127 | | | 146/146 | 3.051±0.342 |
| 999/999 | 2.969±0.405 | | | 146/148 | 3.129±0.269 |
| | | | | 148/148 | 3.070±0.165 |
| | | | | 148/999 | 3.118±0.348 |

Table 9.18:     Estimates of SCON ($10^8$/ml) for the boars in dependence from the
                *ACTG2* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 93/93 | 3.065±0.180 | 93/93 | 3.177±0.211 | 93/93 | 2.740±0.348 |
| 93/103 | 3.211±0.168 | 93/103 | 3.161±0.161 | 93/97 | 3.400±0.347 |
| 93/109 | 2.996±0.098 | 93/109 | 2.977±0.122 | 93/109 | 3.009±0.167 |
| 93/111 | 2.893±0.172 | 93/111 | 2.694±0.211 | 93/111 | 3.214±0.301 |
| 93/117 | 3.226±0.255 | 93/117 | 3.355±0.279 | 93/117 | 2.585±0.598 |
| 93/999 | 3.288±0.215 | 93/999 | 3.364±0.326 | 93/999 | 2.835±0.601 |
| 103/103 | 3.324±0.192 | 103/103 | 3.276±0.186 | 97/97 | 3.373±0.624 |
| 103/109 | 3.030±0.090 | 103/109 | 3.063±0.107 | 97/103 | 3.284±0.245 |
| 103/111 | 2.803±0.129 | 103/111 | 2.754±0.122 | 97/109 | 2.801±0.428 |
| 103/117 | 3.261±0.566 | 103/999 | 2.857±0.189 | 97/111 | 3.115±0.200 |
| 103/999 | 3.003±0.146 | 109/109 | 3.024±0.119 | 97/117 | 2.936±0.598 |
| 109/109 | 2.996±0.105 | 109/111 | 2.979±0.103 | 97/999 | 3.032±0.347 |
| 109/111 | 3.062±0.094 | 109/117 | 2.729±0.197 | 103/109 | 2.935±0.167 |
| 109/117 | 2.990±0.157 | 109/999 | 2.808±0.149 | 103/117 | 3.344±0.599 |
| 109/999 | 2.872±0.135 | 111/111 | 2.742±0.145 | 103/999 | 2.591±0.601 |
| 111/111 | 2.794±0.152 | 111/117 | 2.682±0.200 | 109/109 | 2.858±0.214 |
| 111/117 | 2.799±0.193 | 111/999 | 2.822±0.168 | 109/111 | 3.250±0.200 |
| 111/999 | 2.965±0.118 | 117/117 | 2.395±0.556 | 109/117 | 3.417±0.270 |
| 117/117 | 2.442±0.567 | 117/999 | 3.147±0.321 | 109/999 | 3.129±0.426 |
| 117/999 | 3.081±0.231 | 999/999 | 2.706±0.211 | 111/117 | 3.394±0.607 |
| 999/999 | 2.829±0.158 | | | 111/999 | 3.107±0.348 |
| | | | | 117/999 | 3.085±0.423 |
| | | | | 999/999 | 2.402±0.430 |

9.4 Estimates of LSMs for genotypic effects on VOL

Table 9.19:     Estimates of VOL (ml) for boars in dependence from loci the *PRLR*, *RBP4*, *INHA*, *OPNin6*, *ACTN1*, *ACTN4*, *LHB*, *GnRHR*, and *AR*

| Locus | Genotype | MIXED | PI | PI×HA |
|---|---|---|---|---|
| *PRLR* | AA | 259.33±3.13 | 246.80±3.56 | 271.51±5.86 |
| | AB | 258.83±5.68 | 248.33±5.28 | 259.35±16.74 |
| | BB | 260.50±7.86 | 246.06±7.09 | 310.40±37.50 |
| *RBP4* | AA | 254.31±5.66 | 241.27±4.72 | 313.17±30.36 |
| | AB | 260.54±2.96 | 250.16±3.32 | 269.65±5.51 |
| | BB | - | - | - |
| *INHA* | AA | 255.82±22.61 | 204.16±29.52 | - |
| | AB | 261.3 4±4.35 | 250.42±5.89 | 271.55±7.03 |
| | BB | 258.09±3.70 | 246.76±3.10 | 267.72±9.17 |
| *OPNin6* | AA | 253.49±5.70 | 243.33±5.00 | 253.49±5.70 |
| | AB | 262.55±4.49 | 249.24±4.75 | 262.55±4.49 |
| | BB | 259.36±3.77 | 248.33±4.11 | 259.36±3.77 |
| *ACTN1* | AA | 259.58±3.48 | 247.63±3.84 | 270.28±6.62 |
| | AB | 262.10±4.85 | 247.89±4.69 | 279.87±12.07 |
| | BB | 269.07±11.21 | 254.85±11.27 | 285.81±31.31 |
| *ACTN4* | AA | 261.63±4.39 | 249.43±4.63 | 270.84±9.18 |
| | AB | 256.98±3.93 | 242.15±4.08 | 272.84±8.22 |
| | BB | 262.10±6.71 | 248.54±7.47 | 273.80±13.42 |
| *LHB* | CC | 259.58±4.20 | 249.40±4.56 | 266.58±8.58 |
| | CT | 259.41±3.94 | 247.99±4.02 | 268.93±8.49 |
| | TT | 259.01±5.98 | 241.20±6.30 | 286.79±13.28 |
| *GnRHR* | CC | 259.43±3.13 | 248.05±2.92 | 268.21±6.63 |
| | CG | 258.80±5.94 | 239.56±7.89 | 277.06±9.69 |
| | GG | - | - | - |
| *AR* | *196* bp | 260.96±4.53 | 249.53±3.41 | 255.94±13.77 |
| | *186* bp | 257.67±4.37 | 237.76±6.67 | 273.86±6.11 |
| | *173* bp | 262.25±9.77 | 248.67±9.30 | 275.72±30.75 |
| | *172* bp | 260.49±9.53 | 247.32±8.16 | - |

-: The LSMs were not estimated since the genotype was absent or very rare in the boars

Table 9.20:     Estimates of VOL (ml) for boars in dependence from the *INHBA* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 252/252 | 258.76±3.61 | 252/252 | 244.50±3.40 | 252/252 | 276.84±8.43 |
| 252/254 | 263.76±5.12 | 252/254 | 256.24±5.65 | 252/254 | 265.24±10.72 |
| 252/256 | 243.93±8.08 | 252/999 | 234.60±9.05 | 252/256 | 264.30±14.27 |
| 252/999 | 266.94±13.01 | 254/254 | 263.01±15.66 | 252/999 | 268.42±20.64 |
| 254/254 | 272.22±16.03 | 252/999 | 252.31±23.89 | 254/254 | 258.91±54.88 |
| 254/256 | 261.67±25.92 | 999/999 | 274.38±29.40 | 254/256 | 293.13±54.50 |
| 254/999 | 290.37±44.93 | | | 256/256 | 262.09±54.86 |
| 256/256 | 239.25±31.86 | | | 256/999 | 297.38±54.90 |
| 256/999 | 316.80±31.93 | | | | |

Table 9.21:     Estimates of VOL (ml) for boars in dependence from the *INHBB* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 161/163 | 286.91±15.40 | 171/171 | 247.76±5.22 | 159/163 | 278.43±23.53 |
| 161/171 | 278.86±10.84 | 171/173 | 249.77±5.68 | 159/171 | 271.25±15.94 |
| 161/173 | 239.08±20.18 | 171/175 | 241.94±6.00 | 159/173 | 257.22±52.60 |
| 161/175 | 239.25±45.03 | 171/999 | 253.16±13.90 | 159/999 | 261.84±30.44 |
| 163/163 | 281.03±31.73 | 173/173 | 255.90±12.08 | 161/163 | 295.30±17.50 |
| 163/171 | 255.02±15.11 | 173/175 | 260.41±8.70 | 161/171 | 289.31±12.41 |
| 163/173 | 261.62±18.78 | 173/999 | 242.92±15.86 | 161/173 | 259.82±26.27 |
| 163/175 | 244.49±18.77 | 175/175 | 225.66±11.57 | 161/999 | 248.58±52.72 |
| 163/999 | 269.97±20.40 | 175/999 | 236.24±17.17 | 163/163 | 334.07±52.64 |
| 171/171 | 2584.64±5.44 | 999/999 | 228.82±41.78 | 163/171 | 229.68±37.27 |
| 171/173 | 253.76±5.68 | | | 171/171 | 274.82±12.82 |
| 163/175 | 251.53±7.06 | | | 171/173 | 247.38±12.78 |
| 163/999 | 264.49±13.33 | | | 171/999 | 276.87±30.62 |
| 173/173 | 263.97±12.30 | | | 173/173 | 269.14±37.20 |
| 173/175 | 269.04±10.08 | | | | |
| 173/999 | 250.71±45.01 | | | | |
| 175/175 | 234.36±12.99 | | | | |
| 175/999 | 209.14±26.19 | | | | |

Table 9.22:     Estimates of VOL (ml) for boars in dependence from the *ACR* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 202/202 | 238.08±15.28 | 202/202 | 229.74±14.78 | 208/208 | 267.49±13.20 |
| 202/208 | 252.73±7.04 | 202/208 | 242.88±5.95 | 208/218 | 217.86±30.51 |
| 202/214 | 267.11±11.95 | 202/214 | 241.62±11.65 | 208/226 | 269.12±14.65 |
| 202/214 | 293.53±32.12 | 202/999 | 267.43±10.46 | 208/228 | 275.42±9.38 |
| 202/999 | 274.68±11.60 | 208/208 | 248.78±4.66 | 208/999 | 274.16±14.12 |
| 208/208 | 259.30±5.15 | 208/214 | 244.24±9.36 | 218/228 | 234.23±27.21 |
| 208/214 | 253.63±10.35 | 208/999 | 245.11±6.72 | 218/999 | 281.72±30.47 |
| 208/228 | 267.29±8.02 | 214/214 | 263.16±18.72 | 226/999 | 292.05±37.31 |
| 208/999 | 255.38±5.78 | 214/999 | 243.63±20.96 | 228/999 | 245.91±30.43 |
| 214/214 | 276.14±18.61 | 999/999 | 261.51±29.55 | 999/999 | 319.85±26.60 |
| 214/228 | 231.90±26.16 | | | | |
| 214/999 | 259.89±32.11 | | | | |
| 228/999 | 245.72±16.13 | | | | |
| 999/999 | 283.76±26.31 | | | | |

Table 9.23:     Estimates of VOL (ml) for boars in dependence from the *OPNpro* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 118/118 | 250.83±12.59 | 118/118 | 240.36±11.31 | 118/138 | 266.22±52.56 |
| 118/142 | 260.82±8.32 | 118/142 | 260.09±8.56 | 118/142 | 235.74±19.80 |
| 118/146 | 275.33±16.05 | 118/146 | 244.67±21.04 | 118/146 | 306.44±26.30 |
| 118/148 | 257.65±7.20 | 118/148 | 245.99±7.10 | 118/148 | 270.80±18.60 |
| 118/999 | 236.06±32.06 | 118/999 | 270.21±42.07 | 126/142 | 280.19±30.30 |
| 142/142 | 252.78±8.32 | 142/142 | 242.00±7.12 | 126/148 | 244.77±17.50 |
| 142/146 | 282.33±12.12 | 142/146 | 255.78±17.11 | 126/999 | 249.56±36.98 |
| 142/148 | 257.79±5.45 | 142/148 | 247.54±5.21 | 138/142 | 271.59±23.52 |
| 142/999 | 266.92±13.92 | 142/999 | 202.78±41.85 | 138/146 | 208.54±52.33 |
| 146/146 | 282.51±16.02 | 146/14 | 263.84±18.76 | 138/148 | 260.70±23.73 |
| 146/148 | 259.66±13.07 | 146/148 | 262.79±15.83 | 142/146 | 304.98±18.63 |
| 146/999 | 197.21±45.31 | 148/148 | 241.64±6.99 | 142/148 | 264.80±13.53 |
| 148/148 | 257.34±6.64 | 148/999 | 263.65±20.94 | 142/999 | 314.69±37.19 |
| 148/999 | 253.81±10.08 | | | 146/146 | 306.21±30.23 |
| 999/999 | 239.55±32.13 | | | 146/148 | 249.23±23.70 |
| | | | | 148/148 | 280.89±14.56 |
| | | | | 148/999 | 298.21±30.56 |

9.5 Estimates of LSMs for genotypic effects on MOT

Table 9.24:     Estimates of MOT (%) for boars in dependence from the loci *FSHB*, *PRLR*, *ACTN1*, *ACTN4*, *LHB*, and *RLN*

| Locus | Genotype | MIXED | PI | PI×HA |
|-------|----------|-------|-----|-------|
| *FSHB* | AA | 84.99±0.15 | 85.32±0.17 | 84.61±0.25 |
|  | AB | 84.91±0.23 | 85.06±0.27 | 85.03±0.42 |
|  | BB | 85.31±0.57 | 85.42±0.78 | 85.30±0.82 |
| *PRLR* | AA | 84.98±0.15 | 82.26±0.19 | 84.74±0.22 |
|  | AB | 85.02±0.27 | 85.30±0.27 | 84.72±0.64 |
|  | BB | 84.96±0.37 | 85.18±0.37 | 85.44±1.43 |
| *ACTN1* | AA | 85.05±0.16 | 85.34±0.20 | 84.79±0.25 |
|  | AB | 84.88±0.23 | 85.17±0.24 | 84.74±0.45 |
|  | BB | 84.42±0.52 | 84.93±0.58 | 82.87±0.23 |
| *ACTN4* | AA | 84.90±0.21 | 85.27±0.25 | 84.53±0.35 |
|  | AB | 85.04±0.19 | 85.32±0.22 | 84.94±0.31 |
|  | BB | 85.13±0.32 | 85.52±0.40 | 84.77±0.51 |
| *LHB* | CC | 85.23±0.20 | 85.58±0.24 | 84.81±0.33 |
|  | CT | 84.84±0.18 | 85.16±0.21 | 84.48±0.32 |
|  | TT | 84.82±0.28 | 84.87±0.33 | 85.28±0.50 |
| *RLN* | CC | 84.99±0.14 | 85.28±0.16 | 84.69±0.22 |
|  | CA | 85.97±0.31 | 85.10±0.34 | 85.22±0.61 |
|  | AA | - | - | - |

-: The LSMs were not estimated since the genotype was absent or very rare in the boars

Table 9.25:     Estimates of MOT (%) for boars in dependence from the *INHBA* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 252/252 | 85.16±0.17 | 252/252 | 85.32±0.17 | 252/252 | 85.19±0.30 |
| 252/254 | 84.76±0.24 | 252/254 | 84.81±0.29 | 252/254 | 84.95±0.39 |
| 252/256 | 84.63±0.38 | 252/999 | 85.68±0.46 | 252/256 | 83.85±0.52 |
| 252/999 | 85.17±0.60 | 254/254 | 84.04±0.81 | 252/999 | 83.96±0.75 |
| 254/254 | 83.64±0.75 | 252/999 | 86.98±1.23 | 254/254 | 82.05±2.00 |
| 254/256 | 86.95±1.20 | 999/999 | 85.27±1.51 | 254/256 | 86.41±1.96 |
| 254/999 | 86.10±2.08 | | | 256/256 | 83.06±1.98 |
| 256/256 | 85.27±1.48 | | | 256/999 | 83.02±1.99 |
| 256/999 | 82.98±1.49 | | | | |

Table 9.26: Estimates of MOT (%) for boars in dependence from the *FST* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 142/142 | 85.13±0.34 | 142/142 | 85.35±0.50 | 142/142 | 84.92±0.47 |
| 142/143 | 82.69±2.10 | 142/143 | 82.93±2.14 | 142/147 | 84.89±0.74 |
| 142/147 | 85.59±0.56 | 142/146 | 85.09±1.22 | 142/148 | 85.30±0.97 |
| 142/148 | 84.38±0.61 | 142/147 | 86.59±0.88 | 142/999 | 84.57±0.99 |
| 142/999 | 84.67±0.74 | 142/148 | 83.95±0.81 | 143/143 | 84.54±0.49 |
| 143/143 | 85.28±0.27 | 143/143 | 85.72±0.33 | 143/147 | 84.25±0.85 |
| 143/147 | 84.33±0.54 | 143/146 | 85.62±0.75 | 143/148 | 84.87±0.74 |
| 143/148 | 84.79±0.32 | 143/147 | 84.46±0.71 | 143/999 | 87.13±1.50 |
| 143/999 | 85.71±0.64 | 143/148 | 84.93±0.35 | 144/144 | 84.80±0.93 |
| 144/144 | 84.92±0.54 | 143/999 | 85.52±2.14 | 144/147 | 82.48±1.49 |
| 144/147 | 83.49±0.75 | 144/144 | 85.12±0.67 | 144/999 | 87.07±2.09 |
| 144/148 | 86.39±0.59 | 144/147 | 83.96±0.87 | 147/147 | 84.68±0.95 |
| 144/999 | 85.14±0.85 | 144/148 | 86.60±0.59 | 147/999 | 83.64±1.48 |
| 147/147 | 85.27±0.41 | 144/999 | 84.91±0.95 | 148/148 | 87.11±2.08 |
| 147/999 | 83.98±0.66 | 146/146 | 85.12±0.95 | 999/999 | 84.35±0.85 |
| 148/148 | 85.06±0.46 | 146/148 | 87.80±2.12 | | |
| 148/999 | 84.99±0.67 | 146/999 | 83.15±2.12 | | |
| 999/999 | 84.27±0.52 | 147/147 | 85.54±0.45 | | |
| | | 147/999 | 84.21±0.75 | | |
| | | 148/148 | 85.17±0.46 | | |
| | | 148/999 | 84.91±0.71 | | |
| | | 999/999 | 83.61±1.07 | | |

Table 9.27:     Estimates of MOT (%) for boars in dependence from the *INHBB* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 161/163 | 84.81±0.72 | 171/171 | 85.32±0.27 | 159/163 | 84.16±0.92 |
| 161/171 | 84.98±0.51 | 171/173 | 85.55±0.29 | 159/171 | 85.17±0.62 |
| 161/173 | 85.52±0.94 | 171/175 | 84.73±0.31 | 159/173 | 87.72±2.05 |
| 161/175 | 84.70±2.10 | 171/999 | 86.03±0.71 | 159/999 | 85.95±1.19 |
| 163/163 | 82.24±1.48 | 173/173 | 84.29±0.62 | 161/163 | 84.54±0.68 |
| 163/171 | 86.22±0.71 | 173/175 | 85.76±0.44 | 161/171 | 84.83±0.48 |
| 163/173 | 85.33±0.88 | 173/999 | 85.57±0.81 | 161/173 | 85.34±1.02 |
| 163/175 | 85.06±0.88 | 175/175 | 84.43±0.59 | 161/999 | 84.32±2.06 |
| 163/999 | 84.47±0.95 | 175/999 | 85.30±0.87 | 163/163 | 81.27±2.05 |
| 171/171 | 84.95±0.25 | 999/999 | 83.18±2.13 | 163/171 | 84.61±1.46 |
| 171/173 | 85.13±0.27 | | | 171/171 | 84.44±0.50 |
| 163/175 | 84.58±0.33 | | | 171/173 | 84.45±0.50 |
| 163/999 | 85.15±0.62 | | | 171/999 | 86.43±1.20 |
| 173/173 | 84.15±0.57 | | | 173/173 | 84.77±1.45 |
| 173/175 | 85.46±0.47 | | | | |
| 173/999 | 87.96±2.10 | | | | |
| 175/175 | 84.12±0.61 | | | | |
| 175/999 | 86.12±1.22 | | | | |

Table 9.28:     Estimates of MOT (%) for boars in dependence from the *OPNpro* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 118/118 | 84.80±0.59 | 118/118 | 85.13±0.58 | 118/138 | 82.72±2.11 |
| 118/142 | 84.91±0.39 | 118/142 | 85.26±0.44 | 118/142 | 84.57±0.79 |
| 118/146 | 85.05±0.75 | 118/146 | 85.51±1.08 | 118/146 | 84.59±1.06 |
| 118/148 | 84.92±0.34 | 118/148 | 85.11±0.37 | 118/148 | 85.30±0.75 |
| 118/999 | 82.97±1.49 | 118/999 | 83.11±2.17 | 126/142 | 84.62±1.22 |
| 142/142 | 84.86±0.39 | 142/142 | 85.19±0.37 | 126/148 | 84.87±0.70 |
| 142/146 | 84.49±0.56 | 142/146 | 85.04±0.88 | 126/999 | 86.82±1.48 |
| 142/148 | 84.62±0.25 | 142/148 | 84.84±0.27 | 138/142 | 85.20±0.94 |
| 142/999 | 84.82±0.65 | 142/999 | 84.02±2.15 | 138/146 | 87.19±2.10 |
| 146/146 | 84.87±0.75 | 146/14 | 85.49±0.96 | 138/148 | 85.32±0.95 |
| 146/148 | 86.09±0.61 | 146/148 | 86.62±0.81 | 142/146 | 83.90±0.75 |
| 146/999 | 87.57±2.11 | 148/148 | 85.73±0.36 | 142/148 | 84.83±0.54 |
| 148/148 | 85.18±0.31 | 148/999 | 86.20±1.08 | 142/999 | 82.99±1.49 |
| 148/999 | 85.43±0.47 | | | 146/146 | 84.10±1.21 |
| 999/999 | 87.14±1.49 | | | 146/148 | 85.68±0.95 |
| | | | | 148/148 | 84.20±0.58 |
| | | | | 148/999 | 85.21±1.23 |

9.6 Estimates of LSMs for genotypic effects on PDR

Table 9.29: Estimates of PDR (%) for boars in dependence from the loci *FSHB*, *PRLR*, *ACTN1*, *ACTN4*, *LHB* and *RLN*

| Locus | Genotype | MIXED | PI | PI×HA |
|---|---|---|---|---|
| *FSHB* | AA | 6.71±0.07 | 6.60±0.08 | 6.80±0.12 |
| | AB | 6.73±0.11 | 6.62±0.12 | 6.85±0.20 |
| | BB | 6.50±0.27 | 6.51±0.36 | 6.41±0.40 |
| *PRLR* | AA | 6.73±0.07 | 6.64±0.08 | 6.80±0.11 |
| | AB | 6.57±0.12 | 6.48±0.13 | 6.64±0.30 |
| | BB | 6.71±0.17 | 6.61±0.17 | 6.90±0.69 |
| *ACTN1* | AA | 6.61±0.07 | 6.52±0.09 | 6.67±0.12 |
| | AB | 6.79±0.11 | 6.65±0.11 | 6.93±0.22 |
| | BB | 7.11±0.25 | 6.90±0.27 | 7.94±0.56 |
| *ACTN4* | AA | 6.73±0.10 | 6.65±0.11 | 6.75±0.17 |
| | AB | 6.68±0.10 | 6.56±0.10 | 6.80±0.15 |
| | BB | 6.62±0.15 | 6.46±0.18 | 6.79±0.25 |
| *LHB* | CC | 6.67±0.09 | 6.51±0.11 | 6.93±0.15 |
| | CT | 6.75±0.09 | 6.64±0.10 | 6.82±0.15 |
| | TT | 6.63±0.13 | 6.64±0.15 | 6.36±0.24 |
| *RLN* | CC | 6.71±0.06 | 6.60±0.07 | 6.81±0.11 |
| | CA | 6.68±0.14 | 6.63±0.16 | 6.59±0.29 |
| | AA | - | - | - |

-: The LSMs were not estimated since the genotype was absent or very rare in the boars

Table 9.230:   Estimates of PDR (%) for boars in dependence from the *FST* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 142/142 | 6.47±0.16 | 142/142 | 6.41±0.24 | 142/142 | 6.51±0.21 |
| 142/143 | 7.52±1.00 | 142/143 | 7.36±1.02 | 142/147 | 6.84±0.33 |
| 142/147 | 6.57±0.27 | 142/146 | 6.98±0.57 | 142/148 | 7.13±0.43 |
| 142/148 | 7.14±0.29 | 142/147 | 6.24±0.42 | 142/999 | 6.79±0.45 |
| 142/999 | 6.70±0.35 | 142/148 | 7.21±0.38 | 143/143 | 7.03±0.22 |
| 143/143 | 6.66±0.13 | 143/143 | 6.43±0.15 | 143/147 | 7.49±0.38 |
| 143/147 | 7.27±0.25 | 143/146 | 6.79±0.35 | 143/148 | 6.33±0.34 |
| 143/148 | 6.66±0.15 | 143/147 | 7.09±0.33 | 143/999 | 6.35±0.67 |
| 143/999 | 6.71±0.30 | 143/148 | 6.63±0.16 | 144/144 | 6.78±0.42 |
| 144/144 | 6.78±0.26 | 143/999 | 6.40±1.01 | 144/147 | 7.88±0.67 |
| 144/147 | 7.10±0.35 | 144/144 | 6.73±0.32 | 144/999 | 5.66±0.94 |
| 144/148 | 6.17±0.28 | 144/147 | 6.79±0.41 | 147/147 | 6.08±0.44 |
| 144/999 | 6.58±0.40 | 144/148 | 6.08±0.28 | 147/999 | 6.45±0.67 |
| 147/147 | 6.53±0.20 | 144/999 | 6.67±0.45 | 148/148 | 6.00±0.93 |
| 147/999 | 6.91±0.31 | 146/146 | 6.38±0.44 | 999/999 | 7.30±0.38 |
| 148/148 | 6.67±0.22 | 146/148 | 5.28±0.99 | | |
| 148/999 | 6.78±0.32 | 146/999 | 6.65±0.99 | | |
| 999/999 | 6.92±0.25 | 147/147 | 6.53±0.21 | | |
| | | 147/999 | 6.95±0.35 | | |
| | | 148/148 | 6.63±0.22 | | |
| | | 148/999 | 6.86±0.33 | | |
| | | 999/999 | 7.05±0.50 | | |

Table 9.31: Estimates of PDR (%) for boars in dependence from the *INHBB* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 161/163 | 7.07±0.34 | 171/171 | 6.60±0.12 | 159/163 | 6.99±0.43 |
| 161/171 | 6.63±0.24 | 171/173 | 6.48±0.13 | 159/171 | 6.34±0.29 |
| 161/173 | 6.33±0.44 | 171/175 | 6.81±0.14 | 159/173 | 6.31±0.96 |
| 161/175 | 8.44±0.98 | 171/999 | 6.10±0.33 | 159/999 | 6.96±0.55 |
| 163/163 | 8.19±0.69 | 173/173 | 7.04±0.29 | 161/163 | 7.14±0.32 |
| 163/171 | 6.24±0.33 | 173/175 | 6.38±0.21 | 161/171 | 6.73±0.23 |
| 163/173 | 6.40±0.41 | 173/999 | 6.23±0.37 | 161/173 | 6.49±0.48 |
| 163/175 | 6.79±0.41 | 175/175 | 6.68±0.27 | 161/999 | 8.45±0.96 |
| 163/999 | 6.94±0.44 | 175/999 | 6.67±0.40 | 163/163 | 8.45±0.96 |
| 171/171 | 6.71±0.12 | 999/999 | 7.86±0.98 | 163/171 | 7.13±0.70 |
| 171/173 | 6.64±0.12 | | | 171/171 | 6.82±0.24 |
| 163/175 | 6.86±0.15 | | | 171/173 | 6.87±0.23 |
| 163/999 | 6.27±0.29 | | | 171/999 | 5.88±0.59 |
| 173/173 | 7.05±0.27 | | | 173/173 | 6.52±0.68 |
| 173/175 | 6.49±0.22 | | | | |
| 173/999 | 6.16±0.98 | | | | |
| 175/175 | 6.78±0.28 | | | | |
| 175/999 | 6.86±0.57 | | | | |

Table 9.32: Estimates of PDR (%) for boars in dependence from the *ACR* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 202/202 | 6.95±0.33 | 202/202 | 7.02±0.34 | 208/208 | 6.68±0.24 |
| 202/208 | 6.54±0.15 | 202/208 | 6.45±0.14 | 208/218 | 7.35±0.57 |
| 202/214 | 6.76±0.26 | 202/214 | 6.76±0.27 | 208/226 | 6.90±0.27 |
| 202/214 | 6.45±0.70 | 202/999 | 6.27±0.24 | 208/228 | 6.93±0.17 |
| 202/999 | 6.38±0.26 | 208/208 | 6.65±0.11 | 208/999 | 6.72±0.26 |
| 208/208 | 6.69±0.11 | 208/214 | 6.36±0.22 | 218/228 | 6.41±0.50 |
| 208/214 | 6.47±0.23 | 208/999 | 6.84±0.16 | 218/999 | 6.54±0.58 |
| 208/228 | 6.79±0.18 | 214/214 | 5.67±0.44 | 226/999 | 7.71±0.69 |
| 208/999 | 6.88±0.13 | 214/999 | 7.18±0.49 | 228/999 | 6.14±0.55 |
| 214/214 | 5.74±0.41 | 999/999 | 7.23±0.68 | 999/999 | 6.17±0.49 |
| 214/228 | 6.44±0.57 | | | | |
| 214/999 | 7.67±0.69 | | | | |
| 228/999 | 6.53±0.35 | | | | |
| 999/999 | 6.83±0.57 | | | | |

Table 9.33: Estimates of PDR (%) for boars in dependence from the *OPNpro* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 118/118 | 6.85±0.27 | 118/118 | 6.73±0.27 | 118/138 | 7.82±0.95 |
| 118/142 | 6.76±0.18 | 118/142 | 6.76±0.20 | 118/142 | 6.52±0.36 |
| 118/146 | 6.68±0.35 | 118/146 | 6.70±0.50 | 118/146 | 6.63±0.48 |
| 118/148 | 6.60±0.16 | 118/148 | 6.54±0.17 | 118/148 | 6.32±0.34 |
| 118/999 | 8.05±0.70 | 118/999 | 8.21±0.99 | 126/142 | 6.94±0.56 |
| 142/142 | 6.85±0.18 | 142/142 | 6.75±0.17 | 126/148 | 7.23±0.32 |
| 142/146 | 7.04±0.26 | 142/146 | 6.80±0.40 | 126/999 | 6.62±0.67 |
| 142/148 | 6.76±0.12 | 142/148 | 6.69±0.12 | 138/142 | 6.64±0.43 |
| 142/999 | 6.93±0.30 | 142/999 | 7.14±0.98 | 138/146 | 5.30±0.94 |
| 146/146 | 6.40±0.35 | 146/14 | 6.23±0.44 | 138/148 | 6.48±0.43 |
| 146/148 | 6.07±0.28 | 146/148 | 5.95±0.37 | 142/146 | 7.27±0.34 |
| 146/999 | 5.25±0.98 | 148/148 | 6.27±0.16 | 142/148 | 6.73±0.25 |
| 148/148 | 6.57±0.14 | 148/999 | 6.81±0.49 | 142/999 | 8.01±0.67 |
| 148/999 | 6.74±0.22 | | | 146/146 | 6.62±0.55 |
| 999/999 | 6.53±0.69 | | | 146/148 | 6.21±0.43 |
| | | | | 148/148 | 7.21±0.26 |
| | | | | 148/999 | 5.99±0.56 |

Table 9.34:    Estimates of PDR (%) for boars in dependence from the *ACTG2* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 93/93 | 6.88±0.31 | 93/93 | 6.89±0.37 | 93/93 | 6.65±0.54 |
| 93/103 | 6.66±0.29 | 93/103 | 6.53±0.29 | 93/97 | 6.70±0.53 |
| 93/109 | 6.63±0.17 | 93/109 | 6.47±0.22 | 93/109 | 6.83±0.26 |
| 93/111 | 6.98±0.30 | 93/111 | 7.22±0.37 | 93/111 | 6.48±0.47 |
| 93/117 | 6.33±0.45 | 93/117 | 6.40±0.50 | 93/117 | 5.76±0.92 |
| 93/999 | 6.96±0.38 | 93/999 | 7.14±0.59 | 93/999 | 7.21±0.93 |
| 103/103 | 7.52±0.33 | 103/103 | 7.39±0.33 | 97/97 | 5.23±0.97 |
| 103/109 | 6.70±0.16 | 103/109 | 6.71±0.19 | 97/103 | 7.13±0.38 |
| 103/111 | 6.87±0.23 | 103/111 | 6.75±0.22 | 97/109 | 7.10±0.68 |
| 103/117 | 6.60±0.98 | 103/999 | 6.35±0.33 | 97/111 | 7.06±0.31 |
| 103/999 | 6.65±0.25 | 109/109 | 6.49±0.21 | 97/117 | 5.28±0.92 |
| 109/109 | 6.57±0.18 | 109/111 | 6.46±0.18 | 97/999 | 6.87±0.53 |
| 109/111 | 6.73±0.16 | 109/117 | 6.42±0.35 | 103/109 | 6.59±0.26 |
| 109/117 | 6.36±0.28 | 109/999 | 6.32±0.26 | 103/117 | 6.72±0.92 |
| 109/999 | 6.68±0.24 | 111/111 | 6.63±0.26 | 103/999 | 5.48±0.93 |
| 111/111 | 6.77±0.26 | 111/117 | 6.34±0.36 | 109/109 | 6.57±0.33 |
| 111/117 | 6.71±0.34 | 111/999 | 6.69±0.30 | 109/111 | 7.35±0.31 |
| 111/999 | 6.93±0.21 | 117/117 | 4.97±1.00 | 109/117 | 6.21±0.43 |
| 117/117 | 5.07±1.00 | 117/999 | 7.18±0.57 | 109/999 | 8.15±0.67 |
| 117/999 | 6.59±0.40 | 999/999 | 6.25±0.38 | 111/117 | 8.66±0.98 |
| 999/999 | 6.23±0.28 | | | 111/999 | 7.39±0.55 |
| | | | | 117/999 | 6.39±0.66 |
| | | | | 999/999 | 5.65±0.68 |

9.7 Estimates of LSMs for genotypic effects on ASR

Table 9.35:     Estimates of ASR (%) for boars in dependence from the loci *FSHB*, *PRL*, *PRLR*, *ACTN1*, *ACTN4*, *LHB*, and *RLN*

| Locus | Genotype | MIXED | PI | PI×HA |
|---|---|---|---|---|
| *FSHB* | AA | 6.82±0.09 | 6.69±0.10 | 7.02±0.16 |
| | AB | 6.91±0.14 | 6.90±0.16 | 6.74±0.27 |
| | BB | 7.02±0.35 | 7.43±0.46 | 6.44±0.53 |
| *PRL* | AA | 6.89±0.17 | 6.74±0.20 | 7.04±0.29 |
| | AB | 6.42±0.18 | 6.11±0.24 | 6.76±0.27 |
| | AC | 7.00±0.13 | 6.89±0.14 | 7.04±0.26 |
| | BC | 7.36±0.58 | 7.18±0.57 | 6.73±0.54 |
| | CC | 6.93±0.19 | 6.82±0.19 | 7.04±0.29 |
| *PRLR* | AA | 6.90±0.09 | 6.84±0.11 | 6.94±0.14 |
| | AB | 6.68±0.16 | 6.65±0.16 | 6.58±0.40 |
| | BB | 6.80±0.22 | 6.69±0.22 | 7.76±0.90 |
| *ACTN1* | AA | 6.85±0.10 | 6.75±0.12 | 6.94±0.16 |
| | AB | 6.87±0.14 | 6.79±0.14 | 6.80±0.29 |
| | BB | 6.93±0.31 | 6.63±0.34 | 8.01±0.75 |
| *ACTN4* | AA | 6.95±0.13 | 6.85±0.15 | 7.03±0.22 |
| | AB | 6.79±0.11 | 6.69±0.13 | 6.87±0.22 |
| | BB | 6.84±0.19 | 6.73±0.23 | 6.94±0.33 |
| *LHB* | CC | 6.82±0.12 | 6.71±0.14 | 6.96±0.21 |
| | CT | 6.93±0.11 | 6.81±0.12 | 7.10±0.20 |
| | TT | 6.72±0.17 | 6.78±0.19 | 6.63±0.32 |
| *RLN* | CC | 6.82±0.08 | 6.73±0.09 | 6.73±0.09 |
| | CA | 7.06±0.18 | 7.03±0.20 | 7.03±0.20 |
| | AA | - | - | - |

-: The LSMs were not estimated since the genotype was absent or very rare in the boars

Table 9.36:     Estimates of ASR (%) for boars in dependence from the *FST* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 142/142 | 6.68±0.21 | 142/142 | 6.58±0.30 | 142/142 | 6.77±0.29 |
| 142/143 | 7.03±1.29 | 142/143 | 7.01±1.27 | 142/147 | 7.33±0.45 |
| 142/147 | 6.86±0.34 | 142/146 | 7.24±0.72 | 142/148 | 6.78±0.59 |
| 142/148 | 7.53±0.37 | 142/147 | 6.22±0.52 | 142/999 | 6.93±0.60 |
| 142/999 | 7.06±0.45 | 142/148 | 8.05±0.48 | 143/143 | 7.17±0.30 |
| 143/143 | 6.66±0.17 | 143/143 | 6.42±0.20 | 143/147 | 7.56±0.52 |
| 143/147 | 7.88±0.33 | 143/146 | 6.87±0.45 | 143/148 | 6.57±0.46 |
| 143/148 | 6.74±0.19 | 143/147 | 8.07±0.42 | 143/999 | 6.78±0.91 |
| 143/999 | 6.95±0.39 | 143/148 | 6.75±0.21 | 144/144 | 7.61±0.57 |
| 144/144 | 6.96±0.33 | 143/999 | 7.66±1.27 | 144/147 | 7.86±0.91 |
| 144/147 | 7.23±0.46 | 144/144 | 6.61±0.40 | 144/999 | 5.52±1.30 |
| 144/148 | 6.38±0.36 | 144/147 | 6.98±0.52 | 147/147 | 5.68±0.58 |
| 144/999 | 6.66±0.52 | 144/148 | 6.35±0.35 | 147/999 | 6.42±0.90 |
| 147/147 | 6.65±0.25 | 144/999 | 6.86±0.56 | 148/148 | 5.03±1.28 |
| 147/999 | 6.94±0.40 | 146/146 | 7.10±0.56 | 999/999 | 6.85±0.52 |
| 148/148 | 6.74±0.28 | 146/148 | 5.07±1.25 | | |
| 148/999 | 6.65±0.41 | 146/999 | 6.15±1.25 | | |
| 999/999 | 7.03±0.32 | 147/147 | 6.84±0.27 | | |
| | | 147/999 | 7.04±0.44 | | |
| | | 148/148 | 6.79±0.27 | | |
| | | 148/999 | 6.79±0.42 | | |
| | | 999/999 | 7.39±0.63 | | |

Table 9.37:     Estimates of ASR (%) for boars in dependence from the *INHBB* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 161/163 | 7.10±0.44 | 171/171 | 6.81±0.16 | 159/163 | 6.68±0.60 |
| 161/171 | 6.77±0.31 | 171/173 | 6.63±0.17 | 159/171 | 6.83±0.41 |
| 161/173 | 7.27±0.58 | 171/175 | 6.95±0.18 | 159/173 | 6.59±1.36 |
| 161/175 | 7.18±1.29 | 171/999 | 6.21±0.42 | 159/999 | 6.51±0.78 |
| 163/163 | 8.37±0.91 | 173/173 | 7.46±0.37 | 161/163 | 7.18±0.45 |
| 163/171 | 6.47±0.43 | 173/175 | 6.43±0.26 | 161/171 | 6.84±0.32 |
| 163/173 | 6.82±0.54 | 173/999 | 7.04±0.48 | 161/173 | 6.99±0.67 |
| 163/175 | 6.28±0.54 | 175/175 | 6.99±0.35 | 161/999 | 7.33±1.35 |
| 163/999 | 6.58±0.59 | 175/999 | 6.24±0.52 | 163/163 | 7.72±1.35 |
| 171/171 | 6.87±0.16 | 999/999 | 9.09±1.27 | 163/171 | 7.37±0.98 |
| 171/173 | 6.80±0.16 | | | 171/171 | 6.84±0.33 |
| 163/175 | 7.00±0.20 | | | 171/173 | 7.17±0.33 |
| 163/999 | 6.77±0.35 | | | 171/999 | 6.94±0.80 |
| 173/173 | 7.23±0.35 | | | 173/173 | 5.56±0.96 |
| 173/175 | 6.50±0.29 | | | | |
| 173/999 | 6.58±1.30 | | | | |
| 175/175 | 7.07±0.37 | | | | |
| 175/999 | 6.49±0.75 | | | | |

Table 9.38:     Estimates of ASR (%) for boars in dependence from the *ACR* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 202/202 | 7.31±0.43 | 202/202 | 7.53±0.44 | 208/208 | 6.61±0.31 |
| 202/208 | 6.54±0.20 | 202/208 | 6.52±0.18 | 208/218 | 7.80±0.74 |
| 202/214 | 6.55±0.33 | 202/214 | 6.72±0.35 | 208/226 | 6.60±0.35 |
| 202/214 | 6.38±0.89 | 202/999 | 6.58±0.32 | 208/228 | 7.31±0.23 |
| 202/999 | 6.63±0.33 | 208/208 | 6.90±0.14 | 208/999 | 7.04±0.34 |
| 208/208 | 6.83±0.14 | 208/214 | 6.65±0.28 | 218/228 | 6.36±0.65 |
| 208/214 | 6.70±0.29 | 208/999 | 6.93±0.20 | 218/999 | 6.74±0.73 |
| 208/228 | 7.25±0.23 | 214/214 | 5.57±0.56 | 226/999 | 7.74±0.89 |
| 208/999 | 6.90±0.16 | 214/999 | 7.82±0.63 | 228/999 | 6.37±0.72 |
| 214/214 | 5.47±0.52 | 999/999 | 6.71±0.89 | 999/999 | 5.60±0.64 |
| 214/228 | 6.88±0.73 | | | | |
| 214/999 | 8.11±0.90 | | | | |
| 228/999 | 6.61±0.45 | | | | |
| 999/999 | 7.34±0.73 | | | | |

Table 9.39: Estimates of ASR (%) for the boars in dependence from the *ACTG2* locus

| MIXED | | PI | | PI×HA | |
|---|---|---|---|---|---|
| Genotype | LSM ± SE | Genotype | LSM ± SE | Genotype | LSM ± SE |
| 93/93 | 7.97±0.40 | 93/93 | 8.06±0.48 | 93/93 | 7.65±0.73 |
| 93/103 | 6.91±0.38 | 93/103 | 6.77±0.37 | 93/97 | 6.09±0.72 |
| 93/109 | 6.64±0.22 | 93/109 | 6.25±0.28 | 93/109 | 7.20±0.35 |
| 93/111 | 6.80±0.38 | 93/111 | 6.67±0.48 | 93/111 | 6.93±0.62 |
| 93/117 | 6.70±0.57 | 93/117 | 6.93±0.64 | 93/117 | 5.28±1.24 |
| 93/999 | 6.82±0.48 | 93/999 | 7.87±0.75 | 93/999 | 6.05±1.25 |
| 103/103 | 7.38±0.43 | 103/103 | 7.24±0.43 | 97/97 | 4.74±1.30 |
| 103/109 | 6.71±0.20 | 103/109 | 6.64±0.25 | 97/103 | 6.73±0.51 |
| 103/111 | 7.11±0.29 | 103/111 | 6.95±0.28 | 97/109 | 8.27±0.89 |
| 103/117 | 4.65±1.27 | 103/999 | 6.65±0.43 | 97/111 | 6.81±0.42 |
| 103/999 | 6.58±0.32 | 109/109 | 6.89±0.27 | 97/117 | 4.82±1.25 |
| 109/109 | 6.99±0.24 | 109/111 | 6.75±0.24 | 97/999 | 7.52±0.71 |
| 109/111 | 7.06±0.21 | 109/117 | 6.41±0.45 | 103/109 | 6.63±0.35 |
| 109/117 | 6.64±0.35 | 109/999 | 6.86±0.34 | 103/117 | 4.74±1.24 |
| 109/999 | 7.15±0.30 | 111/111 | 7.18±0.33 | 103/999 | 5.17±1.25 |
| 111/111 | 7.31±0.34 | 111/117 | 6.65±0.46 | 109/109 | 6.95±0.44 |
| 111/117 | 7.01±0.44 | 111/999 | 6.74±0.39 | 109/111 | 7.68±0.42 |
| 111/999 | 6.83±0.27 | 117/117 | 5.68±1.27 | 109/117 | 6.84±0.56 |
| 117/117 | 5.83±1.27 | 117/999 | 6.19±0.74 | 109/999 | 7.59±0.89 |
| 117/999 | 5.89±0.52 | 999/999 | 6.34±0.48 | 111/117 | 9.18±1.28 |
| 999/999 | 6.58±0.35 | | | 111/999 | 7.42±0.73 |
| | | | | 117/999 | 5.92±0.87 |
| | | | | 999/999 | 6.40±0.89 |

## 9.8 Analysis for the relevant factors

Table 9.40:    Estimates of the effects of the other relevant factors on fertility traits

| Factor | Classes | n | NRR | NBA |
|--------|---------|---|-----|-----|
| Breed | PI | 244 | -0.284±0.687 | 0.066±0.061 |
| | PI×HA | 112 | 0.153±0.793 | 0.025±0.070 |
| Birth year | < 96 | 49 | -0.348±1.026 | 0.099±0.091 |
| | 96 and 97 | 137 | 0.089±0.753 | 0.055±0.067 |
| | 98 and 99 | 170 | 0.063±0.725 | -0.019±0.064 |

Table 9.41:    Estimates of effects of sperm quality characters on boar fertility traits

| Traits | NRR | | NBA | |
|--------|-----|--|-----|--|
| | Estimate | Significance | Estimate | Significance |
| SCON | -0.00156±0.000190 | $P < 0.001$ | 0.000385±0.000047 | $P < 0.001$ |
| VOL | 5.12E-6±2.897E-6 | n.s. | -1.27E-6±0.000000 | n.s. |
| MOT | -0.00048±0.000046 | $P < 0.001$ | 0.000119±0.000011 | $P < 0.001$ |
| PDR | 0.000599±0.000075 | $P < 0.001$ | -0.00015±0.000019 | $P < 0.001$ |
| ASR | 0.000312±0.000069 | $P < 0.001$ | -0.00028±0.000017 | $P < 0.001$ |

n.s. (no significant): $P > 0.05$, $P < 0.001$: highly significant

Table 9.42:    Estimates for effects of age, breed and season on sperm quality traits

| Factor | | SCON ($10^8$/ml) | VOL (ml) | MOT (%) | PDR (%) | ASR (%) |
|---|---|---|---|---|---|---|
| Age (slope) | | 0.01425 | 0.5088 | 0.0365 | 0.0093 | 0.0068 |
| covariable | | ±0.0020 | ±0.1522 | ±0.0074 | ±0.0036 | ±0.0047 |
| Breed | PI | 2.934±0.035 | 247.18±2.81 | 85.22±0.13 | 6.59±0.06 | 6.72±0.08 |
| | PI×HA | 3.040±0.052 | 273.23±4.59 | 84.69±0.21 | 6.81±0.10 | 6.92±0.13 |
| Season | 2000/1 | 2.934±0.035 | 247.18±2.81 | 85.57±0.19 | 6.42±0.10 | 6.48±0.12 |
| | 2000/2 | 3.040±0.052 | 273.23±4.59 | 85.84±0.19 | 6.26±0.09 | 6.15±0.12 |
| | 2000/3 | 3.249±0.052 | 242.43±4.10 | 85.26±0.18 | 6.35±0.09 | 6.93±0.11 |
| | 2000/4 | 3.175±0.050 | 237.64±3.97 | 85.33±0.18 | 6.75±0.09 | 7.05±0.11 |
| | 2001/1 | 2.974±0.049 | 252.52±3.89 | 84.46±0.18 | 6.92±0.09 | 7.01±0.11 |
| | 2001/2 | 3.104±0.049 | 268.97±3.88 | 84.40±0.19 | 6.71±0.09 | 6.99±0.12 |
| | 2001/3 | 3.240±0.050 | 254.59±3.92 | 84.41±0.20 | 7.38±0.09 | 7.12±0.12 |
| | 2001/4 | 3.059±0.051 | 245.36±4.01 | 84.09±0.21 | 7.37±0.10 | 7.21±0.13 |

Table 9.43:    The level of significant effect of factors on sperm quality traits

| Factor | SCON | VOL | MOT | PDR | ASR |
|---|---|---|---|---|---|
| age | *** | *** | *** | ** | n.s |
| breed | n.s | *** | * | n.s | n.s. |
| season | *** | *** | *** | *** | *** |

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s (no significant): $P > 0.05$.

Table 9.44:    Estimates of repeatability for sperm quality traits

| Factors | SCON | VOL | MOT | PDR | ASR |
|---|---|---|---|---|---|
| Permanent environmental effect ($\delta_p^2$) | 0.30 | 1938.15 | 4.18 | 0.83 | 1.46 |
| Residual ($\delta_e^2$) | 0.65 | 2570.09 | 7.60 | 4.74 | 5.40 |
| Repeatability $\delta_p^2/(\delta_p^2+\delta_e^2)$ | 0.32 | 0.43 | 0.35 | 0.15 | 0.21 |

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