Haplotype analysis of $\beta$-actin gene for its association with sperm quality and boar fertility

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Introduction

Fertility is economically one of the most important traits in swine production. Implementation of artificial insemination (AI) in swine production allows improved selection of boars for production traits. Meanwhile, AI sets high requirements for the individual boar reproduction performance and there needs to be a systematic evaluation of fertilization potential of semen samples for AI in boar stations.

The process from spermatogenesis to fertilization is complex, controlled by a large number of genes, including the $\beta$-actin ($ACTB$) gene. $\beta$-actin ($ACTB$) is expressed in sperm and is distributed in the acrosomal and postacrosomal regions of ejaculated spermatozoa where it is potentially involved in membrane changes during acrosome reaction with an important implication on sperm function (Casale et al. 1988). Castellani-Ceresa et al. (1993) showed that actin polymerization during capacitation and acrosome reaction is important for the fertilization process. The study by Liu et al. (2005) provided further evidence of the important role of actin in sperm function. It showed that approximately 10% of motile sperm exposes actin on the surface of the human sperm head, which is significantly correlated with sperm morphology in semen, capacitation and zona binding. Therefore, exposure of actin on the surface of motile sperm may provide a useful marker to sort sperm cells with good potential to fertilize (Liu et al. 2005).

The $ACTB$ gene was assigned to porcine chromosome 3 (Thomsen et al. 1998) close to several quantitative trait loci (QTL) for male and female reproductive traits (PigQTLDB: http://www.animalgenome.org/QTLdb/; Hu et al. 2005). Therefore, the $ACTB$ gene can be considered as a positional and functional candidate gene for reproductive traits in pig.

Summary

$\beta$-actin ($ACTB$) was examined as a direct functional candidate gene for the possible association with sperm concentration, motility (MOT), semen volume per ejaculate, plasma droplet rate, abnormal sperm rate (ASR) and the fertility traits, non-return rate and number of piglets born alive (NBA). Three polymorphisms in intron 3 (T>C) and one polymorphism in exon 4 (T>C) of porcine $ACTB$ gene were identified by comparative sequencing of animals of the breeds Pietrain and Hampshire. Association analysis revealed that haplotypes affected the variation of the traits MOT, ASR and NBA. The beneficial haplotypes may provide considerable improvement of sperm quality and fertility in the tested commercial boar population.
Compared with the observation of a particular individual, single-nucleotide polymorphism (SNP) and haplotype analysis, the observation of specific combinations of nucleotides on the same chromosome can provide more information on the complex relationship between DNA variation and phenotypes (Daly & Day 2001; Stephens et al. 2001; Grindflek et al. 2006). However, haplotypes are often ambiguous because of an unknown linkage phase of the measured sites along the chromosome when unrelated subjects are sampled. In this study, we are using haplotype analysis to elucidate the association of the ACTB gene with sperm quality and boar fertility.

Materials and methods

Phenotypic records
Sperm quality traits including sperm concentration (SCON, ×10⁹/ml), motility (MOT, %), semen volume per ejaculate (VOL, ml), plasma droplet rate (PDR, %) and abnormal spermatozoa rate (ASR, %) were obtained according to the guidelines of the World Health Organization from AI boars of the breed Pietrain (PI, n = 244) and its crossbreed with Hampshire (Pietrain × Hampshire, PI × HA, n = 112), born between 1990 and 1999 and used in commercial pig farms mainly in north-western Germany. For each boar, the repeated measures of sperm quality traits recorded between January 2000 and December 2001 were available (29–217 records per boar, mean 157). The examined boars produced between 12 and 4397 litters (mean 139) and the fertility data [non-return rate (NRR) and number of piglets born alive (NBA)] of each boar were collected and given as the deviation from the population mean values within breed, parity of sow, farm and season classes. The sperm quality traits and boar fertility traits are shown in Table 1. Using the procedure ‘PROC UNIVARIATE’ of the SAS software package (SAS system for Windows, release 8.02, SAS Inst., Cary, NC, USA) the traits were found to be normally distributed.

Genotypic records
Genomic DNA was isolated from sperm samples of 356 boars. After defrosting from −20°C, sperm samples of 0.5 ml were mixed with 4 ml (0.9%) of sodium chloride solution and centrifuged at 5000 g for 10 min. The supernatant was discarded, the pellets were resuspended in 4 ml of lysis buffer containing 0.01% of sodium dodecyl sulphate, 0.01 M of Tris, 0.01 M of ethane diaminnetra acetic acid (EDTA), 0.045% of proteinase K and 2% of mercaptoethanol, and then 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 min. The aqueous supernatant was collected and the phenol–chloroform extraction was repeated, followed by precipitation with isopropanol. Finally, the DNA was dissolved in 1 ml of TE buffer and kept at 4°C.

For screening polymorphism within the ACTB gene, oligonucleotide primers were designed in accordance with porcine ACTB cDNA (GenBank accession no. AY550069) F: 5′-GAACCCCAAGCAACCGT-3′, R: 5′-CTGAGGTTCTGGCGATG-3′. Polymerase chain reaction (PCR) was performed in 15-µl reaction volume in 1x PCR buffer (Genecraft, Lüdinghausen, Germany), containing 50 ng genomic DNA templates, 0.3 µl of each dNTP (10 mM), 0.3 µl of each primer (10 µM), and 0.3 U of BioTherm DNA polymerase (Genecraft GmbH, Lüdinghausen, Germany) at 95°C for 5 min, 35× (94°C for 1 min, 62°C for 1 min, 72°C for 2 min), 72°C for 10 min. Comparative sequencing of animals of the breeds of PI and HA was performed on a Beckman Coulter CEQ8000 capillary sequencer (Beckman Coulter, Krefeld, Germany). The obtained genomic sequences (GenBank accession no. DQ452569) were investigated for SNPs.

In total, 301 boars were genotyped for the SNPs within the ACTB gene by single base extension (SBE) (Hirschhorn et al. 2000) using CEQ8000. Primers used for SNP genotyping were designed according to the partial genomic sequence of porcine ACTB (GenBank accession no. DQ452569) and included a poly T tail of different length for multiple analysis.

Table 1 Mean values, SD, sample size and ranges of boar traits

<table>
<thead>
<tr>
<th>Traits</th>
<th>Sample size</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRR (%)</td>
<td>356</td>
<td>0.554</td>
<td>6.217</td>
<td>−24.07</td>
<td>19.62</td>
</tr>
<tr>
<td>NBA (pig/litter)</td>
<td>356</td>
<td>0.012</td>
<td>0.550</td>
<td>−2.97</td>
<td>1.40</td>
</tr>
<tr>
<td>SCON (10⁹/ml)</td>
<td>49 416</td>
<td>2.987</td>
<td>0.979</td>
<td>1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>VOL (ml)</td>
<td>49 221</td>
<td>250.19</td>
<td>68.44</td>
<td>41</td>
<td>499</td>
</tr>
<tr>
<td>MOT (%)</td>
<td>49 671</td>
<td>85.43</td>
<td>3.49</td>
<td>75</td>
<td>92</td>
</tr>
<tr>
<td>PDR (%)</td>
<td>41 591</td>
<td>6.59</td>
<td>2.37</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>ASR (%)</td>
<td>43 011</td>
<td>6.64</td>
<td>2.55</td>
<td>3</td>
<td>18</td>
</tr>
</tbody>
</table>

NRR, non-return rate; NBA, number of piglets born alive; SCON, sperm concentration; MOT, motility; VOL, semen volume per ejaculate; PDR, plasma droplets rate; ASR, abnormal spermatozoa rate.

*Fertility (NRR, NBA) corrected with factors: parity, farm, season and breed.

bNumber of boars.

aNumber of ejaculates.
SNP1: 5'-TTTTTAGGCGGCTGGCAAGAG-3'
SNP2: 5'-TTTTTTTTTCTCTGATGTCGGGACATC-3'
SNP3: 5'-TTTTTTCCTCGGCTCCGCAGGTTGATCGACAG-3'
SNP4: 5'-TTTTTTTTTTTTTTTCGAGCAGGAGATGAGCCAC-3'

The target fragment was amplified and PCR products were purified (5 µl of PCR product added to 1 µl of ExoSAP-IT; GE Healthcare, Munich, Germany) at 37°C for 30 min followed by an inactivation at 80°C for 15 min. The multiplex primer extension reaction was performed in 10 µl of reaction volume containing 2 µl of purified PCR products, 2 µl of primer extension kit (Beckman Coulter), 4 µl of each SNP-specific primer (1 µM), and 2 µl of ddH₂O. 25× (96°C for 10 s, 50°C for 10 s, 72°C for 30 s). The multiplex PCR products were purified (2.5 µl of PCR product added to 1 µl of SAP; GE Healthcare) at 37°C for 30 min, 80°C for 10 min. The genotypes were determined using the CEQ8000 sequencer.

Data analysis

Haplotype construction for the four polymorphisms of the ACTB gene was predicted based on inferences derived from the genotypes of homozygous individuals, because no pedigree information was available from the commercial pig farms (Ciobanu et al. 2004). Computational haplotype construction was obtained from an expectation maximization (EM) algorithm to calculate maximum likelihood (ML) estimates of haplotype frequencies using the snphap programme (version 1.2) described on the website: http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt.

Statistical analyses were performed with the procedure ‘PROC MIXED’ of the SAS software package (SAS system for windows, release 8.02) to address the effects on sperm quality including SCON, MOT, VOL, PDR and ASR. The substitution effects of haplotypes were estimated using the mixed model which included the fixed effects of breed, collected season (eight seasons within 2 years) and age (covariable), and the random permanent effect of the boar (repeated measurement). One variable was included for each haplotype, the animal having zero, one and two copies of the haplotype in question (i.e. multiple linear regression analysis). The effect of the haplotype variables was tested by calculating the likelihood ratio between the full model and the reduced model with the haplotype variables. Significant p-values were obtained from chi-squared distribution with 2 d.f. for the values of −2 times the log-likelihood ratio. Haplotype substitution effects were estimated for traits affected (p < 0.05 or p < 0.01) by the ACTB haplotypes. In addition, the fertility traits of NRR (%) and NBA (per litter) were only available as an average of the mated sows. The substitution effects of haplotypes were estimated using the multiple linear regression model including effects of breed and birth year. Rare haplotypes (frequencies < 0.01) were excluded from the analysis.

Results and discussion

Comparative sequencing of animals of the breeds of PI and HA was performed using CEQ8000 and revealed three polymorphisms in intron 3 (T>C) and one silent polymorphism in exon 4 (T>C) of the porcine ACTB gene. Initially, the partial porcine ACTB gene (GenBank accession no. AY550069) was divided into four segments, which were subsequently amplified on genomic DNA and completely sequenced (data not shown). However, only the amplified fragment described in this study, containing intron 3 and exon 4, resulted in the identification of SNPs.

Allele frequencies for the four SNPs in two boar populations are given in Table 2. In total, 11 haplotypes were predicted and eight haplotypes appeared with a frequency higher than 1% and their frequencies are shown in Table 3. The frequencies of other haplotypes ‘TTCT’, ‘CCCC’ and ‘TCTT’ were 0.008, 0.006 and 0.002 respectively. These rare haplotypes were excluded from the analysis.

Association analysis showed that the haplotype ‘CTCT’ had significant effects on NBA (p < 0.05), whereas the haplotypes ‘CTTT’ and ‘TTTC’ were significantly associated with lower ASR (p < 0.05) as shown in Table 3. The haplotype ‘TTTC’ was also significantly associated with higher MOT (p < 0.05). In addition, the haplotype ‘CTCC’ showed a slight effect on SCON (p < 0.08). These results were

Table 2 Identified single nucleotide polymorphisms (SNPs) in the β-actin gene and allele frequencies in the Pietrain (PI) and Pietrain × Hampshire (PI × HA) populations

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Substitutions</th>
<th>Access/position</th>
<th>PI</th>
<th>PI × HA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>SNP1</td>
<td>T/C</td>
<td>87</td>
<td>0.513</td>
<td>0.487</td>
</tr>
<tr>
<td>SNP2</td>
<td>T/C</td>
<td>427</td>
<td>0.556</td>
<td>0.444</td>
</tr>
<tr>
<td>SNP3</td>
<td>T/C</td>
<td>465</td>
<td>0.485</td>
<td>0.515</td>
</tr>
<tr>
<td>SNP4</td>
<td>T/C</td>
<td>807</td>
<td>0.944</td>
<td>0.056</td>
</tr>
</tbody>
</table>
confirmed in a multiple testing using the Tukey adjustment. An analysis of each SNP (data not shown) did not show any association with the traits under investigation. This confirms that the haplotype analysis is more powerful than the single-SNP analysis as previously shown both within genes (Daly & Day 2001; Stephens et al. 2001) as well as in the genomic regions (Grindflek et al. 2006).

Various effects of the ACTB gene on sperm quality and fertility traits have been indicated, which might be in close relationship with previously identified QTL. The ACTB gene is mapped on SSC3p15–p17 (Thomsen et al. 1998) close to several QTL for male and female reproductive traits (PigQTLDB; http://www.animalgenome.org/QTLdb/; Hu et al. 2005).

For the male reproductive traits, two QTL were mapped, one at 33 cM for testis weight (Sato et al. 2003) and the other at 49 cM for plasma follicle-stimulating hormone (FSH) concentration (Rohrer et al. 2001). The QTL for the female reproductive trait ovulation rate is mapped at 36 cM with a confidence interval ranging from 3 to 70 cM (Rohrer et al. 1999). A significant association of the plasma FSH concentration was found with testis weight as well as its closely related traits, testis size and volume (Borg et al. 1993; Zanella et al. 1999). In addition, a positive correlation was found between ovulation rate and FSH concentration in boars (Mariscal et al. 1996; Cassady et al. 2000). These studies indicated that plasma FSH, testis characteristics and ovulation rate can be controlled by a similar set of genes. The ACTB gene might be one of them, especially because we found a significant association with NBA, which had correlated responses to selection for ovulation rate (Ruiz-Flores & Johnson 2001).

Nevertheless, it cannot be concluded that the ACTB gene is the one causing the QTL on SSC3, but there is some support for locating the QTL.

Therefore, the polymorphisms of the ACTB gene should be introduced in a QTL analysis. Furthermore, it cannot be excluded that the polymorphisms/haplotypes examined are in linkage disequilibrium with an unidentified susceptibility gene/polymorphism(s) that is/are responsible for the observed significant association and the additional complexity of heterogeneous interaction observed within these haplotypes (Zee et al. 2004).

**Acknowledgements**

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**References**


Zee et al. 2004.

### Table 3

Haplotype substitution effects on the number of piglets born alive (NBA), sperm cell motility (MOT) and abnormal spermatozoa rate (ASR) the commercial Pietrain (PI) and Pietrain × Hampshire (PI × HA) boars

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Frequencies</th>
<th>NBA</th>
<th>MOT</th>
<th>ASR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per litter</td>
<td>p-value*</td>
<td>%</td>
</tr>
<tr>
<td>TTTT</td>
<td>0.414</td>
<td>0.441 ± 0.342</td>
<td>0.1987</td>
<td>1.217 ± 0.758</td>
</tr>
<tr>
<td>CCCT</td>
<td>0.384</td>
<td>0.403 ± 0.350</td>
<td>0.2515</td>
<td>1.203 ± 0.776</td>
</tr>
<tr>
<td>TTCCT</td>
<td>0.076</td>
<td>0.234 ± 0.370</td>
<td>0.5274</td>
<td>1.122 ± 0.820</td>
</tr>
<tr>
<td>CCTCT</td>
<td>0.038</td>
<td>1.031 ± 0.415</td>
<td>0.0155</td>
<td>1.338 ± 0.936</td>
</tr>
<tr>
<td>CTCCT</td>
<td>0.022</td>
<td>0.315 ± 0.461</td>
<td>0.4955</td>
<td>0.395 ± 0.996</td>
</tr>
<tr>
<td>CTTCT</td>
<td>0.022</td>
<td>0.361 ± 0.431</td>
<td>0.4027</td>
<td>1.261 ± 0.948</td>
</tr>
<tr>
<td>TCTCT</td>
<td>0.015</td>
<td>0.325 ± 0.483</td>
<td>0.5011</td>
<td>1.278 ± 1.098</td>
</tr>
<tr>
<td>CCTTC</td>
<td>0.014</td>
<td>0.177 ± 0.545</td>
<td>0.7460</td>
<td>2.950 ± 1.255</td>
</tr>
</tbody>
</table>

*Significant p-values are in bold.
Haplotype analysis of β-actin gene

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