CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental animals

A total of 37 full and half sib piglets phenotypically affected with hernia inguinalis were collected from commercial pig breeders in the Northern part of Germany and from the Pig Research Farm of Animal Science Department, Faculty of Agriculture, Chiang Mai University, Thailand. Additionally, 101 unaffected pigs of 6 breeds housed at the Artificial Insemination station at the Institute of Veterinary Medicine, Göttingen, were collected. Animals of Chinese pig breeds as well as Thai Native pigs and Thai Wild pigs were also used as control.

Table 3.1 Experimental animals.

Breed	Abbreviations	Phenotype	Number of Sample
German Crossbred	HxP	Hernia pigs	33
Thai Crossbred	DxYxL	Hernia pigs	4
Thai Native pigs	TNP	Normal pigs	5
Thai Wild pigs	WP	Normal pigs	5
Angler Saddleback	AS	Normal pigs	7
Pietrain	PIT	Normal pigs	15
German Landrace	DLS	Normal pigs	8
German Edelschwein	DE	Normal pigs	7
Swabian-Haellian swine	SHS	Normal pigs	7
Bunte Bentheimer	BB	Normal pigs	5
Chinese Yushanhei	YS	Normal pigs	7
Chinese Luchuan	LC	Normal pigs	12
Chinese Rongechang	RC	Normal pigs	7
Chinese Jiangquhar	JQH	Normal pigs	6
Crossbred	CB	Normal pigs	10
Total			138

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3.2 Chemicals and equipments

3.2.1 Chemicals

1. Absolute ethanol (Roth, Germany)
2. Acetic acid (glacial) (Amersham Bioscience, Germany)
3. Agarose (ultra pure) (Merck, Germany)
4. Agar-Agar (Roth, Germany)
5. Ampicillin (Roth, Germany)
6. Boric acid (Roth, Germany)
7. Bromophenol blue (Sigma, U.S.A.)
8. Calf intestine phosphatase (CIP) (New England Biolabs Inc., USA)
9. Carbinicilin (Roth, Germany)
10. dNTPs (Fermentas, USA)
11. E. coli XL1-blue (Promega Germany)
12. EDTA (Roth, Germany)
13. Ethidium bromide (Roth, Germany)
14. Glycerol (Roth, Germany)
15. Glycerin (Merck, Germany)
16. HCl (Roth, Germany)
17. Isopropanol (Roth, Germany)
18. IPTG (Roth, Germany)
19. Kanamycin (Roth, Germany)
20. KCl (Roth, Germany)
21. KH_2PO_4 (Roth, Germany)
22. KOH (Roth, Germany)
23. MOPS (Roth, Germany)
24. NaCl (Roth, Germany)
25. Na_2HPO_4 (Roth, Germany)
26. NaOH (Roth, Germany)
27. Nylon hybridization transfer membrane (Hybond N^+) (Amersham Biosciences, Germany)
28. pGEM®-4Z vector (Promega Germany)

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29. Proteinase K (Qiagen, Germany)
30. RNaseA (Roth, Germany)
31. Restriction enzymes (New England Biolabs Inc., USA)
32. Sodium dodecyl sulphate (SDS) (Roth, Germany)
33. T4 DNA Ligase (New England Biolabs Inc., USA)
34. Taq DNA polymerase (New England Biolabs Inc., USA)
35. Tetracycline (Roth, Germany)
36. Tris (hydroxymethyl) aminomethane (Roth, Germany)
37. Tris (Roth, Germany)
38. Tween 20 (Roth, Germany)
39. X-Gal (Roth, Germany)
40. Yeast extract (Roth, Germany)

3.2.2 Kits

1. BigDye^{TM} -Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Germany)
2. DIG-Nick Translation Mix (Roche, Germany)
3. ECL Direct Nucleic Acid Labelling and Detection reagent (Amersham Biosciences, Germany)
4. QIAquick PCR purification Kit (Qiagen, Germany)
5. QIAEX II Gel Extraction Kit (Qiagen, Germany)
6. QIAGEN Plasmid Maxi Kit (Qiagen, Germany)
7. QIAprep Spin Miniprep Kit (Qiagen, Germany)

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3.2.3 Equipment

1. Automated sequencer (ABI-3100 capillary analyzer) (Applied Biosystems, Germany)
2. Bioclave (Schütt Labortechnik, Germany)
3. Centrifuge 5424 (Eppendorf, Germany)
4. Deep freezer (Schütt Labortechnik, Germany)
5. Dest.-water (Biocell) (Millipore, Germany)
6. Developing machine Optimax TR (Schroeder + Henke, Germany)
7. Electrophoresis for agarose gel (Bio-Rad, Germany)

8. Electroporation Gene pulser II (Bio-Rad, Germany)
9. Heat block QBD 2 (Grant Instruments, England)
10. Hood (BDK Luft- und Reinraumtechnik, Germany)
11. Hybridization oven Hybrid (Bachofer, Germany)
12. Incubator Certomat BS 1 (Sartorius, Germany)
13. Kodak BioMax MR films (Amersham Biosciences, Switzerland)
14. Magnetic mixer KMO 2 (Janke und Klunkel, Germany)
15. Magnetic-mixer RCT basic (Schütt Labortechnik, Germany)
16. Megafuge 1.0 R (Thermo, Germany)
17. Set of Micropipetts (0.5 to 1000 µl) (Eppendorf, Germany)
18. Set of Micropipetts (0.5 to 1000 µl) (Gilson, USA)
19. Multifuge 1 sR (Thermo, Germany)
20. PCR gradient (T-gradient) (Biometra, Germany)
21. PCR thermocycler (T-3000) (Biometra, Germany)
22. pH meter PB 11 (Sartorius, Germany)
23. Power supply PowerPac (Bio-Rad, Germany)
24. Pulsed field gel electrophoresis CHEF-DR^{TM}II apparatus (Bio-Rad, Germany)
25. Refrigerator (Schütt Labortechnik, Germany)
26. Sorvall centrifuge RC-5B (Du Pont Instruments, Germany)
27. Speed Vac (Schütt Labortechnik, Germany)
28. UV-Transilluminator 312nm and 366 nm (Amersham Biosciences, Switzerland)
29. Vortex Genie 2 (Bender + Hobein, Germany)
30. Water bath (Gesellschaft für Labortechnik, Germany)
31. Zeiss Axioplan 2 microscope (Carl Zeiss, Germany)

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3.2.4 Softwares

1. Vision-Capt Software Version 12.8 (PEQLAB, Germany)
2. Blast Program (http://www.ncbi.nlm.nih.gov/BLAST)
3. SeqBuilder, Seqman, EditSeq; DNASTAR Lasergene 6 (DNASTAR Inc., Germany).
4. SAS Program (SAS Institute Inc., 1999-2001)
5. Somatic hybrid panel (http://www.toulouse.inra.fr/lgc/pig/pcr/pcr.htm)
6. Radiation hybrid panel (http://imprh.toulouse.inra.fr)

3.3 Methods

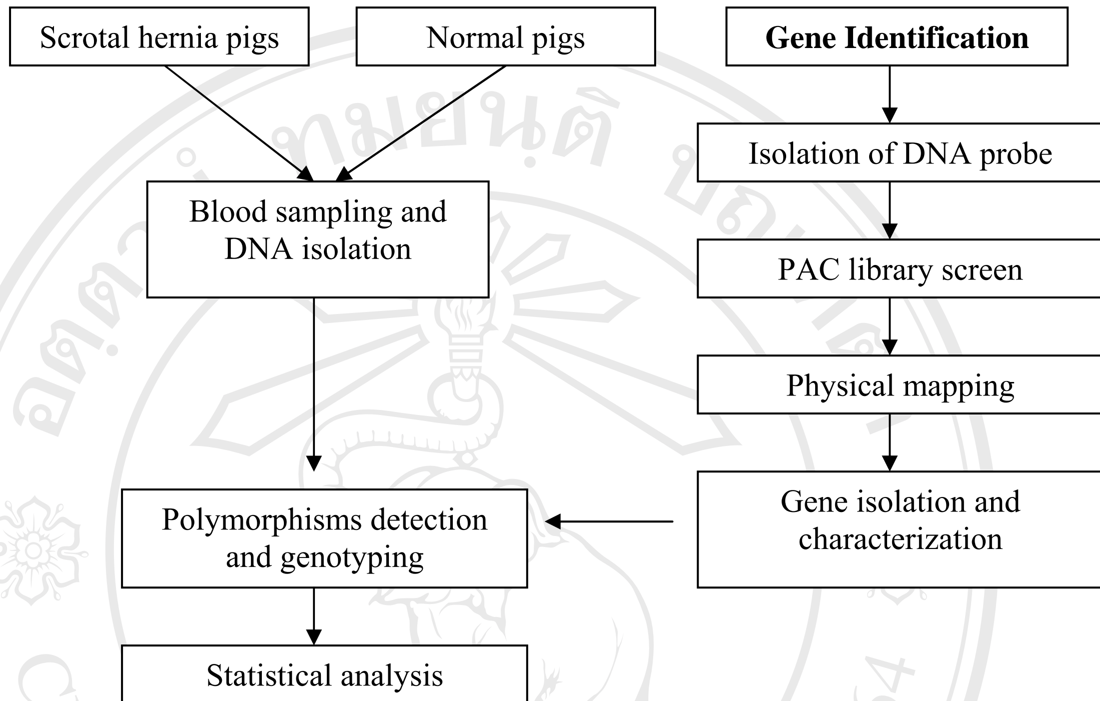

Figure 3.1 Schematic representation of the experimental design used for identification of the gene in this study.

3.3.1 Isolation of DNA and amplification probe

3.3.1.1 DNA isolation

Genomic DNA isolation was performed according to methods introduced by Sambrook *et al.* (1989) and Miller *et al.* (1988). Briefly, about 10 -15 ml blood was transtered into tubes containing 500 µl 0.5 M EDTA solution and centrifuged at 13,000 rpm for 15 min. The supernatant was subsequently discarded. Using a pasteur pipette, the buffy coat was carefully transfer to a fresh tube and centrifuged. The buffy coat is a broad band of white blood cells of heterogeneous density. The buffy coat was resuspend in 10 ml water to lyse red blood cells by vortexing 30 sec. The supernatant was discarded then 100 µl of 9% (w/v) NaCl was added. The samples were centrifuged at 13,000 rpm for 10 min and the supernatant discarded. The white pellet was resuspend with 1 ml PBS solution, centrifuged at 5,000 rpm, and the supernatant discarded. 800 µl digestion-

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buffer and proteinase K to a final concentration of 100 µg/ml were added as well as 10 % SDS (50 µl) followed by incubation at 55 °C overnight. Then 500 µl NaCl (6M) was transferred and centrifuged at 12,000 rpm for 10 min. The viscous supernatant was removed by a pasteur pipette and transfer to a 1.5 ml clean tube. 1:10 Na-acetate (3M) of pH 5.2 was added and after isopropanol precipitation, the DNA pellet was washed 2 times with 70% ethanol, dried and dissolved in 100 µl of TE buffer.

Before storing DNA at 4 °C the absorbance of the DNA-solution was measured to determine amount and quality of the DNA using a NanoDrop1000 spectrophotometer (V3.1). The ratio of sample absorbance at 260 and 280 nm is used to assess the purity of DNA. A ratio of about 1.8 is generally accepted as pure for DNA. Ratios of sample absorbance at 260 and 230 nm is a secondary measurement of nucleic acid purity. Ratios commonly in the range of 1.8-3.3 are general values for pure nucleic acid.

3.3.1.2 Polymerase chain reaction (PCR)

Primers were designed from the human *BAX* (GenBank Accession no: NC000019.8) and *TAC1* (GenBank Accession no: NC000007.12). A 25 µl PCR reaction contained at the final concentration the following reagents: 100 ng of purified genomic DNA, 200 ng of each of the primers (forward and revere primer), 200 µM dNTPs, 50 mM $MgCl_2$, 2.5 units Taq DNA polymerase and 1x of a PCR buffer. The PCR reactions were perform used TGradient/T-3000 PCR machine with the following PCR cycling conditions.

Initiate denature	94 °C 2 min	
Denature	94 °C 30 sec	30 cycles
Annealing	the primer-specific temperature 30 sec	30 cycles
Extension	72 °C 30 sec	30 cycles
Final extension	72 °C 10 min	

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Table 3.2 Primer sequences used for amplification of BAX and TAC1 probe.

Primer Name	Primer Sequence (5'→ 3')	Fragment (bp)	Tm (ºC)
BAX for	AGC TGA GCG AGT GTC TCA A	501	60.8
BAX rev	CAG TTG AAG TTG CCG TCA G		
TAC1 for	CAG CTT CAT TTG TGT CAA TGG	415	50.9
TAC1 rev	CAT GAA AAT GCT TCA GAG ATA C		

3.3.1.3 PCR product purification for sequencing

Before the specific PCR product can be used for sequencing it is necessary to purify it. To purify the PCR products from residual reaction components such as primers, unincorporated nucleotides, enzymes, salts, mineral oil and nonspecific amplification product the QIAquick PCR purification kits was used as recommended by the manufacture.

3.3.1.4 DNA sequencing

Sequencing of the plasmid and PCR products were done with the respective specific primers and 200 ng DNA-template directly after purification using the QiAQuick PCR purification Kit. All sequencing reactions were done on an ABI 3100 capillary analyzer.

3.3.2 Screening of the Porcine P1-derived artificial chromosome (PAC) library

A porcine P1-derived artificial chromosome (PAC) library TAIGP714 (Appendix B), originally generated from a male German Landrace pig (Al-Bayati *et al.*, 1999) was screened by a three-dimensional PCR strategy with specific primers. The average insert sizes of the positive PAC-clones was determined by digested with *Not*I, and pulsed field gel electrophoresis using the CHEF-DR^{TM}II apparatus under conditions: 1% agarose gel, 0.5xTBE as running buffer, 6 V/cm, and 6 sec switching interval for 20 hr at 14 °C.

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3.3.3 Physical mapping

3.3.3.1 Radiation hybrid panel and somatic hybrid panel

A porcine rodent somatic cell hybrid panel (Yerel *et al*., 1996) and a porcine whole genome radiation hybrid panel (Yerel *et al*., 1998) were screened for *TAC1* and *BAX by* PCR. For *TAC1* 415 bp fragment was amplified using primers 5'-CAG CTT CAT TTG TGT CAA TGG-3' (forward) and 5'-CAT GAA AAT GCT TCA GAG ATA C-3' (reverse). A 501 bp long fragment for the BAX gene was amplified using primers 5'-AGC TGA GCG AGT GTC TCA A-3' (forward) and 5'-CAG TTG AAG TTG CCG TCA G-3' (revere). The PCR conditions were optimized by gradient PCR on a TGradient machine so that only porcine DNA amplified. PCR results were evaluated using the interpreting web-pages http://imprh.toulouse.inra.fr (radiation hybrid panel) and http://www.toulouse.inra.fr/lgc/pig/hybrid.htm (somatic cell hybrid).

3.3.3.2 Fluorescence *in situ* hybridization (FISH)

Fluorescence in situ hybridization was performed according to the method introduced by Toldo *et al*. (1993) and Solinas-Toldo *et al*. (1995). Swine metaphase spreads were prepared from peripheral lymphocytes obtained from a normal, healthy boar. Probes (1 µg TAIGP714 DNA) were labeled with digoxigenin-11-dUTP by nick-translation using the DIG-Nick Translation Mix. Labelled probes were hybridized with 30-fold excess of porcine Cot-DNA and 6 µg of salmon sperm DNA. Immunodetection was performed using digoxigenin-antibodies conjugated to Cy3. Chromosomes were counterstained with DAPI and examined with a Zeiss Axioplan 2 microscope. The G-like banding pattern generated by DAPI staining was used for chromosome identification and for regional assignment of the hybridization signals. FISH experiments were carried out twice, using duplicate slides.

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3.3.4 Gene isolation and characterization

3.3.4.1 Large-scale plasmid purification (Maxi preparation)

Plasmids were prepared from a single colony from a freshly streaked plate containing Kanamycin (50 µg/ml).

1. Inoculate a starter culture of 5 ml LB medium containing Kanamycin (50 µg/ml) and incubate for 8 hr at 37 °C.
2. Dilute the starter culture to 1/1000 with LB medium and incubate for 10 hr at 37 °C under vigorous shaking.
3. Measure cell density (OD600 nm) of the plasmid DNA by spectrophotometer. Readings should be between 0.5-0.8.
4. Use 4 vessels to harvest the bacterial cells by centrifugation at 6000 rpm for 15 min at 4°C.
5. Romove the supernatant and resuspend each bacterial pellet in 25 ml resuspension buffer (P1) and add 25 ml lysis buffer (P2) incubate for 2-5 min. The bacterial cells are lysed in NaOH-SDS in the presence of RNaseA (100 µg/ml) and SDS solubilizes the phospholipids and protein components of the cell membrane. The lysate is neutralized by the addition of 50 ml chilled neutralization buffer (P3).
6. Incubate the mixture for 20 min on ice. The lysate becomes white precipitate, which contains genomic DNA, proteins, cell debris and SDS
7. Centrifuge the mixture at 6000 rpm for 40 min at 4°C.
8. The cleared lysate supernatant is applied to a QIAGEN-tip 500, which was equilibrated before with buffer QBT.
9. Wash the QIAGEN-tip 2 times with 30 ml buffer QC to remove all contaminants.
10. Elute DNA with 15 ml buffer QF
11. Add 10.5 ml isopropanol to precipitate DNA, mix and centrifuge at 6000 rpm for 60 min at 4°C.
12. Wash DNA pellet with 5 ml 70% ethanol dry and dissolved the pellet in 150 µl TE buffer.

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The PAC clone was then restricted with several restriction endonucleases and the resulting fragments were subsequently sub cloned into the polylinker site of pGEM®-4Z after southern blotting and hybridization with a *BAX* specific probe. Recombinant plasmids were used to transform *Escherichia coli* XL1-Blue. Purified plasmids DNA were done using QIAprep Spin Miniprep Kit. Isolated plasmids DNA were sequence with M13 primers shown in Table 3.3.

Table 3.3 Primer sequences used for amplification clone fragments.

Primer Name	**Primer Sequence (5'→ 3')**
M13 F	AAATGTAAAACGACGGCCAGT
M13 R	AAACAGGAAACAGCTATGACC

3.3.4.2 Agarose gel electrophoresis

Agarose powder was mixed with 1xTBE buffer to the desired concentration (0.8, 1 or 2%), and then heated in a microwave oven until it was completely melted. Ethidium bromide (final concentration 0.5 μg/ml) was added to the gel at this point to facilitate visualization of DNA after electrophoresis. After cooling, the solution was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the gel has solidified, the comb was removed; the gel was inserted into the electrophoresis chamber and covered with 1xTBE buffer. Samples containing DNA were mixed with the loading dye and pipette into the sample wells. The lid was placed on the chamber and current was applied. Electrophoresis was performed at 80-120 V, for a period of time that an allowed optimal separation. The fragments were visualized directly by UV light and photographed. DNA ladders (100 bp or 1 kbp ladder, Invitrogen, Germany) were used for estimation of fragment lengths.

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3.3.4.3 Southern blot and hybridization

For southern blot analysis, 10-15 μg of PAC DNA was digested with selected restriction endonucleases. DNA was separated on a 0.8 % agarose gel. Gel was placed in denaturation solution, shaken slowly for 30 min. The gel was then equilibrated twice for 15 min in neutralization solution on the shaker. The DNA was transferred

from agarose to an nylon membrane (Hybond N+) via capillary action. The DNA was transferred overnight. The membrane was washed with 2xSSC buffer for 1 min with gentle agitation and allow to air dry. DNA fixation was done with baking at 80 °C for 2 hr. The membrane was prehybridized in hybridization buffer (ECL gold buffer, Amersham Biosciences, Germnay) at a ratio of 25 ml solution per 100 cm^2 membrane for 1 hr at 42 °C. probe was generated from *BAX* specific primer and denatured by heating in heat block for 5 min and quickly cooling on ice. 10 µl of DNA labeling reagent was added follow with 10 µl of glutaraldehyde solution. The labeled probe was incubated at 37 °C for 10 min.

The membrane was hybridized with labeled probe at 42 °C for overnight. Following hybridization, the membrane was washed twice with washing buffer I at a ratio of 2 ml solution per 1 cm^2 for 10 min at 55 °C. Then, the membrane was washed twice with washing buffer II at a ratio of 2 ml solution per 1 cm^2 for 5 min at room temperature. After the second washed, the membrane was allowed to air dry. The membrane was incubated in 40 ml of detection reagent (ECL detection reagents, Amersham Biosciences, Germnay) for 1 min. The membrane was placed in the film cassette. The film was placed on the top of the blot and exposed for 10 min. Then exposed film was processed in developing machine.

3.3.4.4 Preparation of pGEM®-4Z vector

The pGEM®-4Z Vector was provided with a glycerol stock of bacterial strain JM109. Prior to usage, appropriate pGEM®-4Z vectors were prepared by digestion with the respective enzymes.

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Normal digestion

pGEM®-4Z	10 µl (250 ng)
Enzyme	3 µl (10 U)
Enzyme specific buffer (10X)	3 µl
(BSA) (100X)	3 µl
ddH_2O	14/11 µl
Total	**30 µl**

Double Digestion

pGEM®-4Z	15 μl (375 ng)
Each Enzyme	3 μl (10 U)
Enzyme specific buffer (10X)	3 μl
(BSA) (100X)	3 μl
ddH_2O	9/6 μl
Total	**30 μl**

After digestion dephospholation of the vector were done by calf intestine phosphatase (CIP). The reaction was separated on a 0.8 % agarose gel. Then the vector prepare of about 2746 bp length was extracted from agarose gel using a clean scalpel and purified with QIAEX II Gel Extraction Kit. The purified vector was adjusted to a concentration of 25 ng/μl.

3.3.4.5 Ligation

DNA ligations were performed by incubating DNA fragments and appropriately linearized cloning vector with T4 DNA ligase in a ratio of 1:1 DNA, vector. The ligation reaction contains the following components:

Enzyme specific vector	1 μl (25 ng/μl)
10x ligation buffer	2 μl
T4 DNA ligase	1 μl (400,000 U/ml)
DNA fragments	X ng/μl (Y μl)
ddH_2O	Z μl
Total	**20 μl**

The concentration of the DNA fragment is calculated as follows:

$$X = \frac{\text{Length of Insert (kb)}}{\text{Length of Vector (2.746 kb)}} \times \text{Vector Concentration (25 ng/μl)}$$

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The amount of DNA fragment is calculated as follows:

$$Y= X / \text{DNA concentration (ng/}\mu\text{l)}$$

The amount of ddH_2O is calculated as follows:

$$Z= 20 \text{ (total volume; }\mu\text{l)} – (4+Y)$$

Ligation was performed overnight at 16°C. Then, the ligation mixture was transformed into XL1-blue competent cells.

3.3.4.6 Preparation of competent *E. coli* (XL-1 Blue)

Electrocompetent cells (*E. coli* XL-1 Blue) were used for transformation. A single colony of the strain was grown overnight at 37°C in 5 ml NZCYM containing 1 μg/ml tetracycline. 5 ml of the overnight culture was transferred into 20 ml Tym containing 1 μg/ml tetracycline and incubated at 37°C for further 2-3 hr until a OD600 of 0.5-0.8 was reached. The culture was transfer to 80 ml Tym containing 1 μg/ml tetracycline and incubated at 37°C for further 2-3 hr until a OD600 of 0.5-0.8 was reached. Transferred the culture to 400 ml Tym medium containing 1 μg/ml tetracycline and incubated at 37°C for further 2-3 hr until a OD600 of 0.6. The bacterial cells were harvest by centrifuge for 10 min 2 °C at 4000 rpm. The bacterial pellet was washed with 100 ml cold TFB-I. Then the bacteria were centrifuged once again and resuspended with 20 ml cold TFB-II. After that, bacteria were divided into 200 μl aliquotes and frozen at –80°C.

3.3.4.7 Transformation

The transformation step is necessary to transfer the ligated DNA into competent cells, which can then reproduce large numbers of cells with the inserted DNA. electroporation cuvets (Bio-Rad, Germany) were cooled on ice before usage and NZCYM was warmed to 37°C. 50 μl aliquots of electrocompetent cells were thawed on ice. 2.5 μl ligation product was added to the cells, mixed thoroughly and immediately transferred into a cooled electroporation cuvet. The Gene Pulser

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apparatus electroporation parameters were set at 2.5 kV, 25 µF and 200 Ohm. Optimal impulse time proved to be 4.8 msec. After the electric pulse bacteria were immediately mixed with 500 µl pre-warm NZCYM and incubated at 37°C for 2 hr.

For comparisons, also chemical competent cell were used. Aliquots of 50 µl competent cells were slowly defrosted and 5 µl of the ligation reaction were transferred to the tube containing the bacteria. The solution was subsequently incubated on ice for 20 min, to allow the recombinant plasmid DNA to attach to the bacterial membrane. After warming the samples to 42°C for 45 sec, the tubes were immediately transferred on ice for 5 min to produce a thermo-shock, allowing the recombinant plasmid to be included into the cells. Subsequently, 500 µl of NZCYM was added and incubation at 37 °C for 2 hr, with gentle shaking. To select positive white colonies, 200 µl and 300 µl of the cell suspension were then plated out onto TCXI plates and incubated overnight at 37°C.

3.3.5 Polymorphisms detection and genotyping

Comparative sequencing of affected and normal pigs was performed to identify polymorphisms in *BAX*. Porcine specific primers were designed from the obtained sequences as shown in Table 3.4.

Table 3.4 Primer sequences used for amplification of DNA fragments for SNPs screen

Primer Name	Primer Sequence (5'→ 3')	Fragment (bp)	Tm (ºC)
BAX SNP-1 F	AAATGTAAAACGACGGCCAG TTGCGGATTTGAGGCGTAATG	778	62.9
BAX SNP-1 R	AAACAGGAAACAGCTATGAC CGGTCCTCACAGGTCTGAG		
BAX SNP-2 F	TCAGTTCATCTAGCAGGGAC	416	54
BAX SNP-2 R	CCATGTTACTGTCCAGTTCATC		

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For large-scale SNP genotyping, PCR-RFLP tests were developed. RFLP reactions were performed in a total reaction volume of 30 µl using the following components:

1. 10 µl of PCR product.
2. 3 µl of appropriate 10_x restriction buffers,
3. 1.5 Unit of restriction enzyme (New England Biolabs Inc., USA)
4. ddH_2O to give a total volume of 30 µl per sample.

Incubate in an incubator oven at 37 °C, overnight. The digested products were subsequently separated on 2% agarose gels containing ethidium bromide.

3.4 Statistical analysis

Allele frequencies were calculated by the simple gene counting method. χ^2- Analysis was used to test for Hardy-Weinberg equilibrium. Statistical analyses of alleles between groups were performed using Fisher's Exact Test in the SAS procedure (SAS Institute Inc, 1999-2001).

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