CHAPTER 4

RESULTS AND DISCUSSION

4.1 PAC library screening

Probes of porcine *TAC1* and *BAX* were generated on porcine genomic DNA with *TAC1* F/R and *BAX* F/R primers, respectively. The *TAC1*-specific primers formed a 415-bp long amplicon (spanning exon 7 and 3'UTR) in pigs (GenBank Accession no: AM233488) and BAXF/R amplified a 501-bp long fragment spanning exons 3 to 4 (GenBank Accession no: AM233489). Sequencing and subsequent BLAST comparisons verified the porcine sequence identity with the human ortholog of 84% for *TAC1* and 94% for *BAX*.

```
    TAC1 F ⟶
  1 cagcttcatt tgtgtcaatg gctgatgaaa ggtaaaatga gacagacgct atgaagaata
 61 attatttatt taataataat tgttgttttg agttgaaaac tcaaaaagta tttatttttc
121 atattgtgcc aagatgtgtt gtaaaagtgt gttataattc taacatggca actccctcag
181 aaatagaaat cagtggtaat ttctcaacaa agcagtgttc aatgaagtgg taggaaccta
241 tcaatgatac agtctccaaa gaaagaaata atttctgttt ctcaagagca gtcatatcag
301 cgacgtgtga agaaaggaaa ctcacagata tgctgtgctt ctccatttgt tttcatggtg
361 aaaatgtact gagatttggt agcaaactgt ggtgtatctc tgaagcattt tcatg
                                                      ⟵ TAC1 R
```

Figure 4.1 *TAC1* probe sequence (GenBank Accession no: AM233488).

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    BAX F ⟶
  1 agctgagcga gtgtctcaag cgcattggag atgaactgga cagtaacatg gagctgcaga
 61 ggtgtggccc ctgggaccca ggagtggtct cttctccctc agaacccaat cgccacttcc
121 cctgggagcc tggagtccgg gcccacagcc ccttttccct cagacccaag gggtccaggt
181 cgctactcct cagctcagtg ctttgaactc ccaggcctcc cctcccctaa gatatggaaa
241 ccctcctcca gggagtcagt ttcctaaagg tccatcttgt ccctttcctg catggtgccc
301 tcttgatttc agcctggctc aggcctcagt gttcttgtct ttggtatgag ctgaacgcca
361 gagcttccac acgttgcccg atcctccttc ccagcacgac tctctcccct gcaggatgat
421 cgcagccgtg gacacggact cccccgaga agtctttttc cgagtggcgg ccgaaatgtt
481 tgctgacggc aacttcaact g
                ⟵ BAX R
```

Figure 4.2 *BAX* probe sequence (GenBank Accession no: AM233489).

The porcine PAC library TAIGP714 (Al-Bayati *et al*., 1999) was screened uses *TAC1* and *BAX* specific probes. Two single positive clones 323H8 (*TAC1*) and 393C3 (*BAX*) were isolated. Pulsed field electrophoresis after *Not*I digestion of the isolated PAC clone containing the *BAX* gene revealed an insert size of about 36 kb and for the *TAC1* clone an insert size of about 71 kb (Figure 4.3).
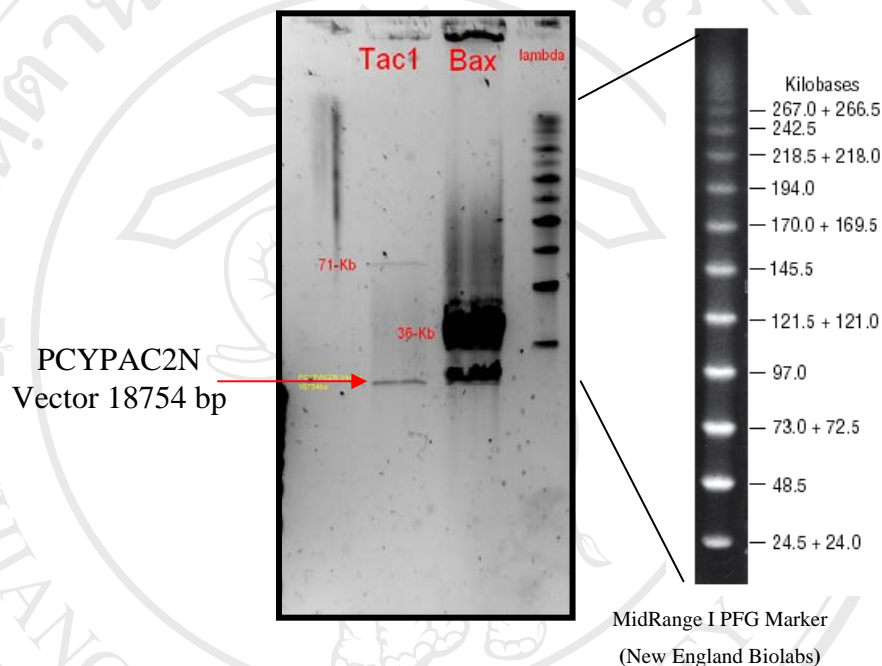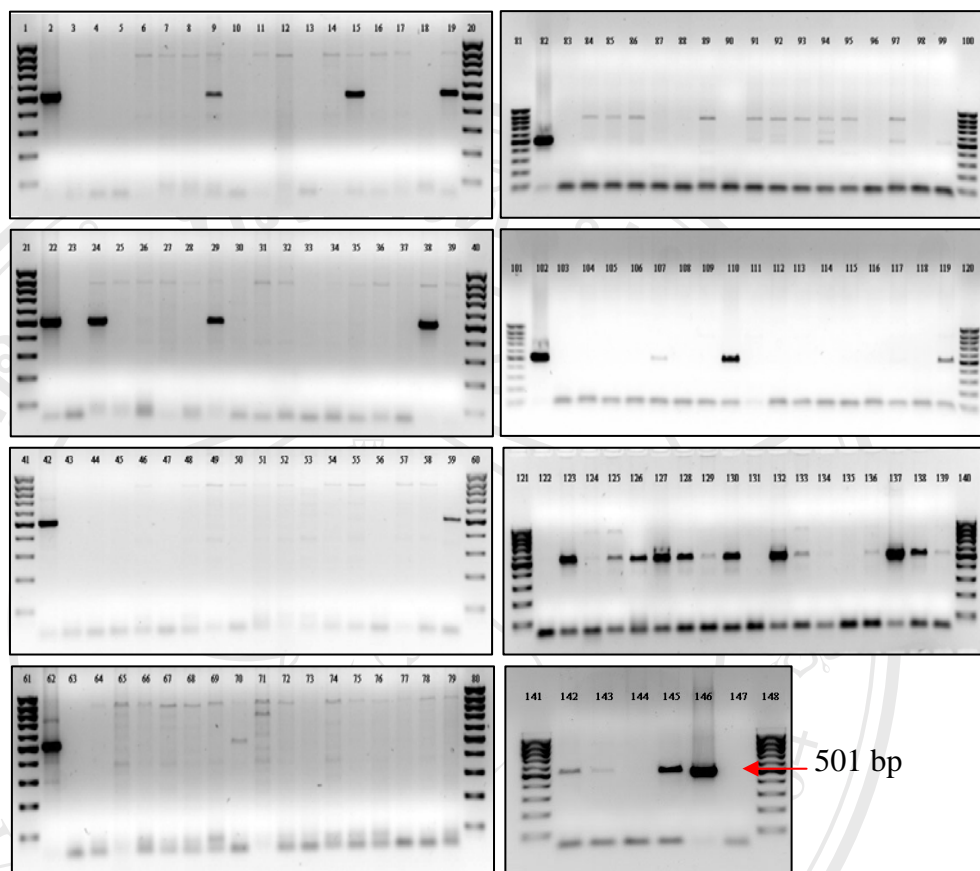

Figure 4.3 Pulsed field gel electrophoresis of PAC clone containing *TAC1* and *BAX*.

4.2 Chromosomal assignment

The RH and somatic panel results are shown in Figures 4.4 to 4.6. Detailed data analyses of the positive signals are as played in Tables 4.1 and 4.2, respectively. The RH map and somatic hybrid panel assignments are presented in Table 4.3 and 4.4, respectively. *BAX* was located on porcine chromosome 6q21 and linked to marker S0220 at a distance of 18 cR (LOD = 16.35, retention = 16%, percent error risk lower than 0.1 and maximal correlation of 0.86). *TAC1* was located on porcine chromosome 9q12-q14 (Knorr *et al*., 2006) and linked to marker SWR915 at a distance of 67 cR (LOD = 5. 79, retention = 49%, percent error risk lower than 0.5 and maximal correlation of 1.00).

To confirm the correctness of the localizations, fluorescence *in situ* hybridization of the PAC clones containing *TAC1* and *BAX* was done. Signals on *SSC9q12-14* and *SSC6q21,* respectively were detected (Figures 4.7 and 4.8).

415 bp

Figure 4.4 Analysis of the *TAC1* specific products using the 118 DNA from the radiation hybrid panels. Lane [2-147] = DNA of the porcine whole-genome radiation hybrid panels; Lane 1, 20, 21, 40, 41, 60, 61, 80, 81, 100, 101, 120, 121, 140, 141, 148 = marker 100 bp; Lane 2, 22, 42, 62, 82, 102, 122 = positive control; Lane 3, 23, 43, 63, 83, 103, 123 = negative control.

Figure 4.5 Analysis of the *BAX* specific products using the 118 DNA from the radiation hybrid panels. Lane [2-147] = DNA of the porcine whole-genome radiation hybrid panels; Lane 1, 20, 21, 40, 41, 60, 61, 80, 81, 100, 101, 120, 121, 140, 141, 148 = marker 100 bp; Lane 2, 22, 42, 62, 82, 102, 146 = positive control; Lane 3, 23, 43, 63, 83, 103, 147 = negative control.

Figure 4.6 Analysis of the *TAC1* (above) and *BAX* (below) specific products using the 27
DNA of the somatic cell hybrid panel.

Table 4.1 RH results vectors for *TAC1* and *BAX* (1 = positive, 0 = negative).

Gene	Vectors
TAC1	110111101101000100110111011000010000000100000011000011011000 0110001010001100000001100100000001100011100100000101001111
BAX	000001000001000110000100000000100000000000000001000000100000 00000000000000100101000100100000000101011111101100111111001

Table 4.2 Somatic cell hybrid results vector (+ = positive, - = negative)

Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14
TAC1	+	+	+	+	+	+	+	+	+	+	+	+	-	-
BAX	-	-	-	-	-	-	-	-	+	-	-	-	-	-

Gene	15	16	17	18	19	20	21	22	23	24	25	26	27
TAC1	+	-	+	+	-	+	-	-	+	-	-	-	-
BAX	-	-	-	-	-	+	-	-	-	-	-	+	-

Table 4.3 Chromosomal localization by radiation hybrid panel.

Gene	Chromosome	Marker	p (Break)	Dist (Ray)	Lod-Score
TAC1	9	SWR915	0.49	0.67	5.79
		S0220	0.16	0.18	16.35
BAX	6	S0333	0.20	0.23	14.73
		SW782	0.25	0.29	12.07

Table 4.4 Chromosomal localization by somatic cell hybrid panel.

Gene	Chromosome	Region (p in %)	Error risk (%)	Correlation (%)
TAC1	9	1/2q21 (79)	< 0.5	100
BAX	6	q12-(1/3q21) (97.5)	< 0.1	86
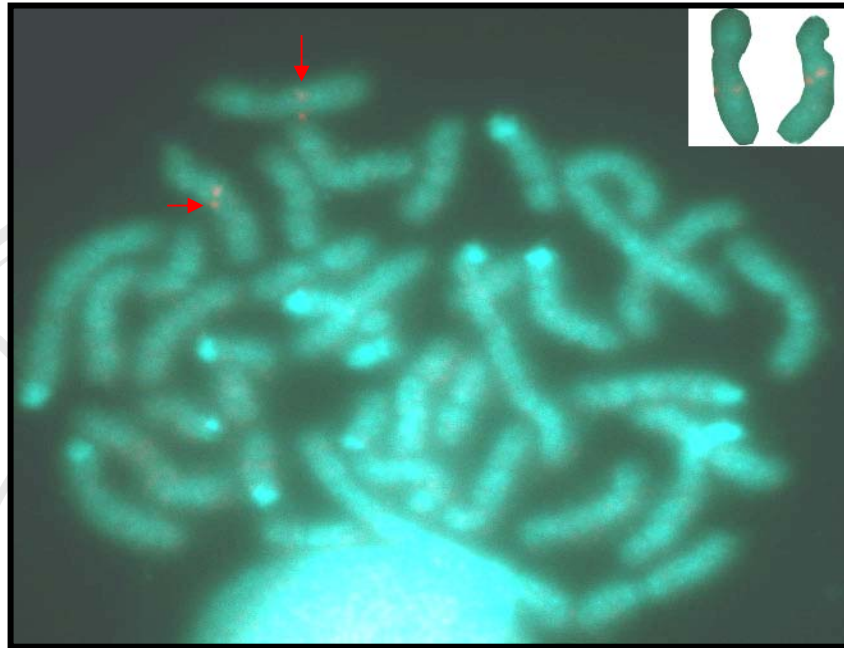
Figure 4.7 FISH mapping of PAC-clone (*TAC1*). Signals are marked by arrows. DNA was labeled by nick-translation, signals were detected using Anti-DIG-Cy3 (red signals).
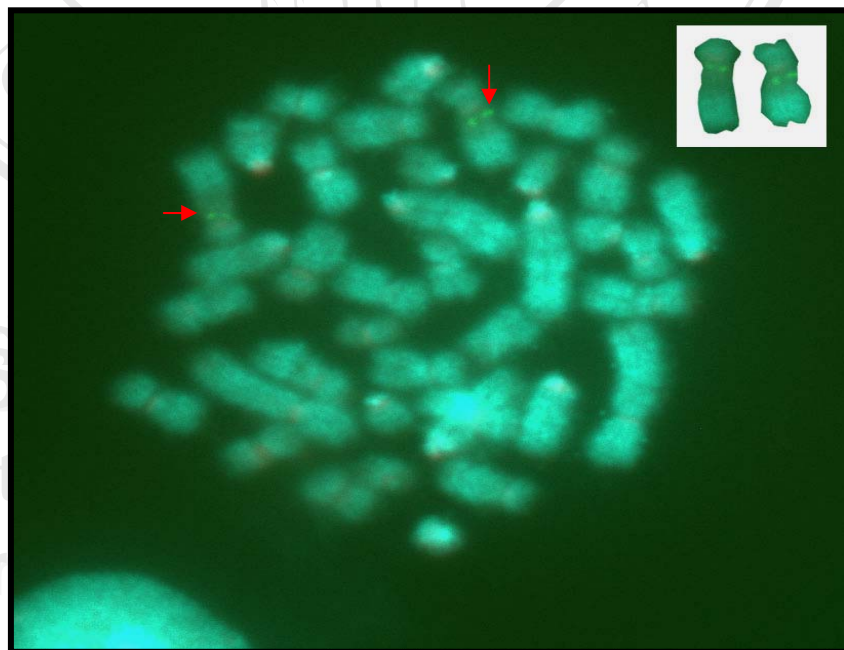
Figure 4.8 FISH mapping of PAC-clones (*BAX*). Signals are marked by arrows. DNA was labeled by nick-translation, signals were detected using avidin FITC (green signals).

A recent genome scan with DNA-markers and affected siblings revealed five chromosomal regions that are associated with the hernia phenotype on porcine chromosomes (Ssc) 3, 6, 7, 12 and 15 in German pig breeds (Bornemann-Kolatzki, 2004; Knorr *et al*., 2001). In order to selection potential candidate genes, or to identify regions of potential association, physical mapping gives the possibility of locating and identification of genes responsible for the trait. One popular approach is to identify positional candidate genes by comparative mapping. Correspondences between porcine and human chromosomes were already determined by chromosome painting (Goureau *et al*., 1996; Yerle *et al*., 1992).

The physical mapping of porcine *BAX* to porcine chromosome 6q21 (Figure 4.9) confirms the comparative correspondence between human chromosome 19 and porcine chromosome 6. In contrast, the human *TAC1* maps to chromosome 7q21-22 (Bonner *et al*., 1987) which shows homology to either porcine chromosomes 3 or 9. The porcine *TAC1* was assigned chromosome 9q12-14 and confirms synteny with human chromosome 7q21-22.

Because of it chromosomal assignment, *TAC1* can no longer be regarded as candidate gene for the scrotal hernia defect in the investigated population. Confirmed to tract, *BAX* formed out to be a positional candidate gene by its chromosomal position. Thus, only the porcine *BAX* was further isolated from the PAC library.
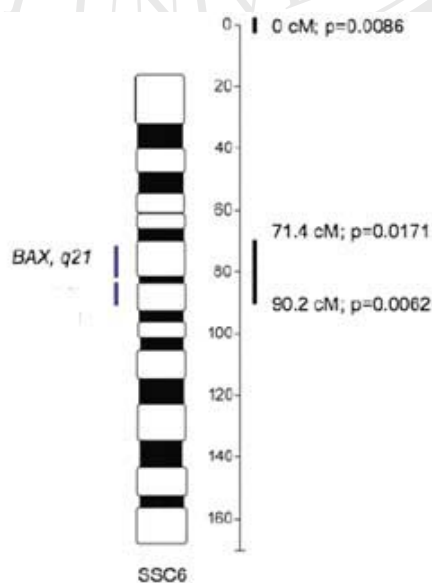

Figure 4.9 Mapping of *BAX* gene on *Ssc 6*.

4.3 Molecular characterization of *BAX*

4.3.1 Gene characterization

Figure 4.10A displays the fragment patterns of the BAX containing PAC clone after digestion with several endonucleases. On the right side (B) the audioradiogram is shown after hybridization with the 501-bp long probe. The white bar indicates fragments containing path of the gene. A 10 kb band after *Xba*I and *EcoR*I digestion was cut off the gel and analyzed by sub-cloning and sequencing. 2298 bp were generated and the total 10741 bp was done by the company Medigenomix at Munich, Germany. The structure of BAX gene containing contig is shown in Figure 4.11. The full composition of sequence is shown in Appendix B. The isolated sequence consists of the 5'-UTR, exons 1-4, introns 1-3 and part of intron 4 of the porcine BAX gene. Exons are ranging in length from 52 bp to 136 bp. An exon-intron boundaries are conserved (Table 4.5). The lengths of the exons are highly conserved between humans and pig. A comparison of the porcine *BAX* coding region with mammalian orthologs revealed nucleotide sequence identities of 94% with *Bos taurus* (NM_173894.1) and 93% with *Homo sapi*ens (NM_004324.3). The screening for CpG islands that span the promoter region and parts of exon 1 reveals a CG positions 7418-7619. A TATA box (TATAA) is located at positions 6635-6649, a CAAT box (CAAT) is located at positions 6962-6965 and a GC box (GGGCGGG) is located at positions 7659-7665. Microsatellite marker S0220 (GenBank Accession no. L31355) is located in the 5'-flanking region.
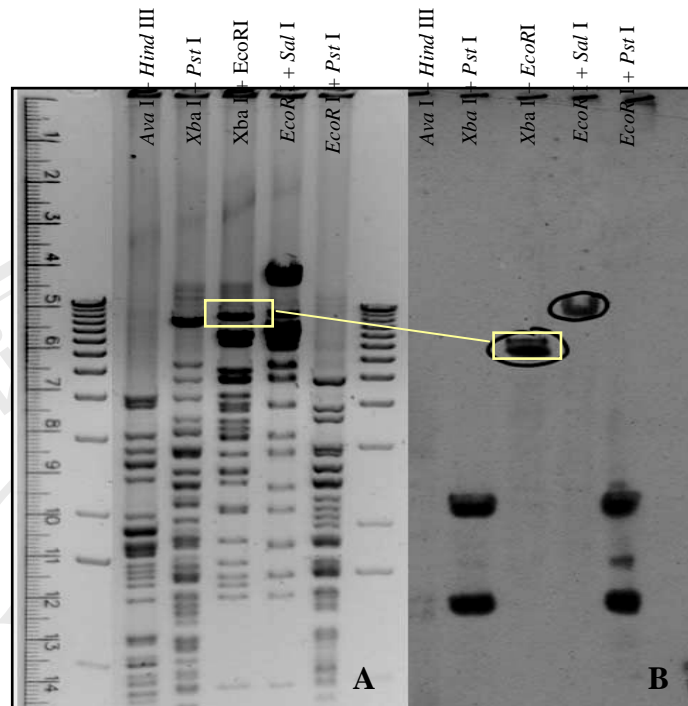
Figure 4.10 Fragment patterns before and after hybridization as southern blot of the BAX-containing PAC clone. Boxes indicate the positions of bands that were cut off the gel.
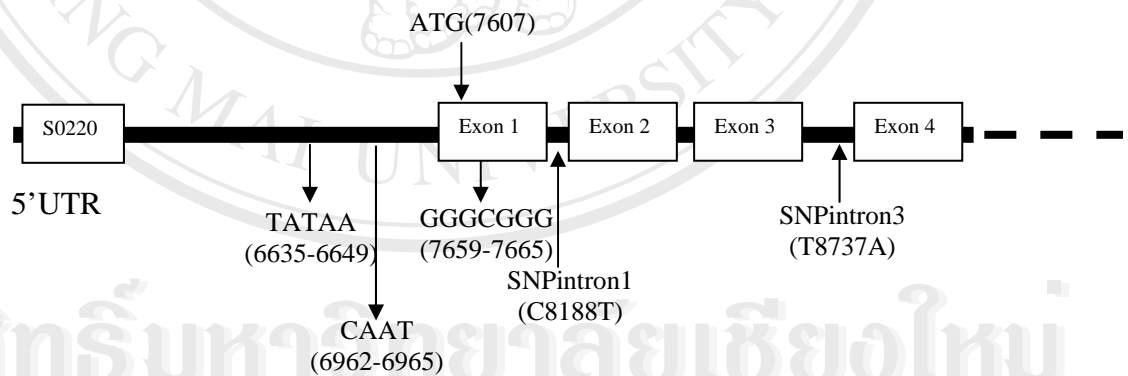
Figure 4.11 Genomic structures of porcine BAX. Positions correspond to the isolated sequence shown in index B. The start codon [ATG] locates at position 7607-7609. Two SNPs (SNPintron1:C8188T and SNPintron3:T8737A) are present.
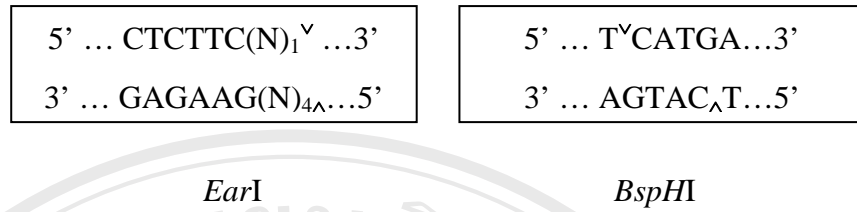
Table 4.5 Intron-exon boundaries and exon lengths of porcine *BAX* gene.

Exon	Length (bp)	Splice acceptor site	Splice donor site	Intron	Length (bp)
1	86		AGGCGGGG*gt*gaggcg	1	563
2	52	tcctct*ag*GGCCCACC	CTTCAGGG*gt*gagtgt	2	91
3	149	cactct*ag*TTTCATCC	GCAGAGGT*gt*ggcccc	3	351
4	136	ccctgc*ag*GATGATCG	TGCTCAAG*gt*gggcga		

4.3.2 SNPs detection

Comparative sequencing of the experimental pigs DNA was employed using gene-specific porcine primer (Table 3.4). PCR products covered exon 2 to 4 and intron 2 to 3, and part of intron 4. No polymorphism could be detected in the exonic regions. Two single nucleotide polymorphisms were detected in intron 1 (SNPintron1) and intron 3 (SNPintron3). The SNPintron1 is a transition from cytosine (C) to thymine (T) and SNPintron 3 is a tranversion from thymine (T) to adenine (A).

Simple PCR-RFLPs were established to facilitate large scale genotyping with the specific primers (Figure 4.12). An SNPintron 1, allele C comprises a restriction site for the enzyme *Ear*I and An SNPintron 3, allele T comprises a restriction site for the enzyme *BspH*I (Figure 4.13).
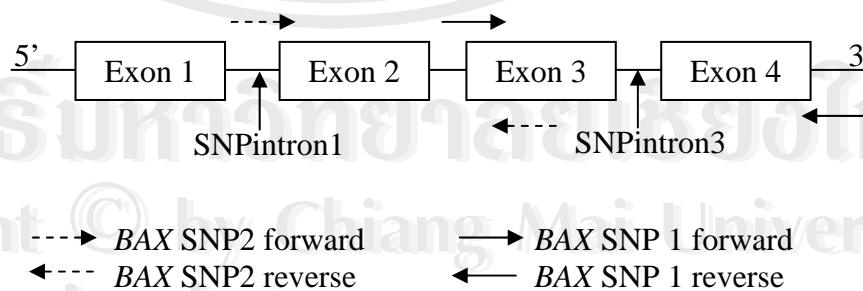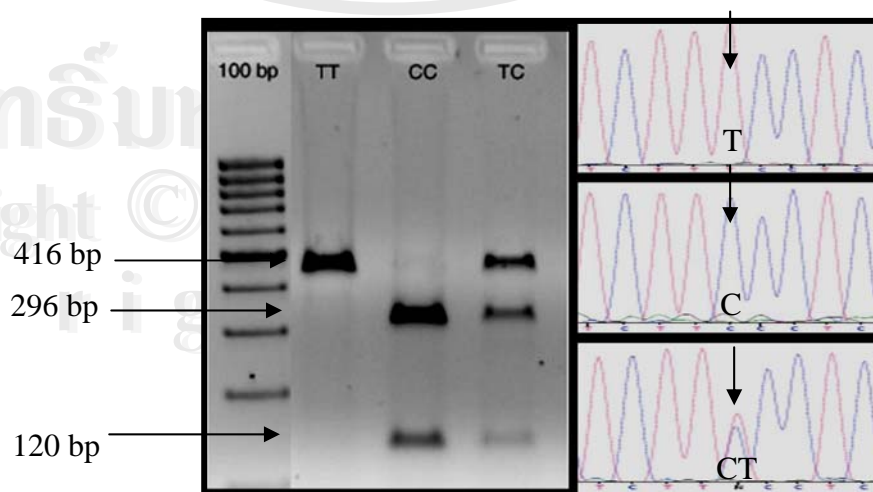

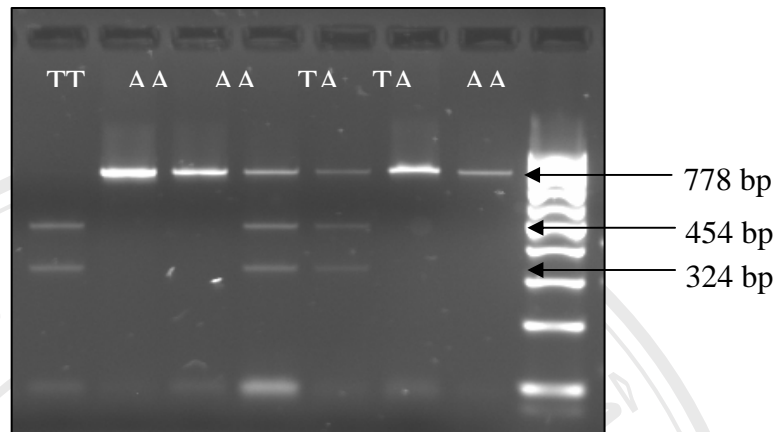**Figure 4.12** Positions of the primers used for SNP detection and genotype.

| 5' … CTCTTC(N)$_1$$^\vee$ …3' | 5' … T$^\vee$CATGA…3' |
| 3' … GAGAAG(N)$_{4\wedge}$…5' | 3' … AGTAC$_\wedge$T…5' |

*Ear*I   *BspH*I

Figure 4.13 *Ear*I and *BspH*I recognition sites.

A 416 bp long PCR fragment was amplified by primer combination *BAX* SNP2. Two alleles could be distinguished after *Ear*I digestion. Individual with allele T has posses no recognition site for *Ear*I and show an undigested PCR product (416bp), where as individual with allele C have a recognition site for *Ear*I and show after digestion the fragments of 120 and 296 bp. Heterozygous individuals possess all three possible fragments. Figure 4.14 shows the sample picture of the PCR product after digestion with *Ear*I and the corresponding sequence chromatogram.

A 778 bp long PCR fragment was amplified by primer combination *BAX* SNP1. Two alleles could be distinguished after *BspH*I digestion. Individual with allele T posses a recognition site for *BspH*I and show after digestion the fragments of 324 and 454 bp length, where as individual with allele A have no recognition site for *BspH*I and show the undigested PCR product. Heterozygous individuals possess all three possible fragments. Figure 4.15 shows the sample picture of the PCR product after digestion with *BspH*I.

Figure 4.14 Restriction patterns and sequencing chromatograms for SNPsintron1.

Figure 4.15 Restriction patterns for SNPsintron3.

A total of 138 animals (see table 3.1) was genotyped (data show in the appendix). The genotype frequencies are shown in Table 4.6. SNPintron 1 reveals genotypes CC, CT and TT. SNPintron3 is characterized by genotype TT, TA and AA.

Table 4.6 Genotype data of SNPs in the porcine *BAX* gene.

Breed	abbreviations	N	SNPintron1			SNPintron3		
			CC	CT	TT	TT	TA	AA
Hernia inguinalis piglets	HIP	37	20	10	7	37	0	0
Thai Native Pig	TNP	5	5	0	0	5	0	0
Thai Wild Pig	WP	5	5	0	0	5	0	0
Angler Saddleback	AS	7	6	1	0	6	1	0
Pietrain	PIT	15	4	7	4	15	0	0
German Landrasse	DLS	8	6	2	0	8	0	0
German Edelschwein	DE	7	5	2	0	7	0	0
Swabian- Haellian swine	SHS	7	3	4	0	7	0	0
Bunte Bentheimer	BB	5	5	0	0	5	0	0
Chiness Yushannhei	YS	7	7	0	0	7	0	0
Chiness Luuchuan	LC	12	12	0	0	7	4	1
Chiness Rongchang	RC	7	7	0	0	7	0	0
Chiness Jiangquhai	JQH	6	3	2	1	6	0	0
Crossbred	CB	10	8	2	0	10	0	0
Total		138	96	30	12	132	5	1

Allele frequencies were estimated by the simple gene count method directly from the genotype number and the following equation:

$$p(A_1) = p = \frac{2 \times D + H}{2N}$$

$$p(A_2) = q = \frac{2 \times R + H}{2N}$$

$p + q = 1$ where: $p(A_1) = p$ = allele frequency allele 1

$p(A_2) = q$ = allele frequency allele 2

D = number of A_1A_1 animals

H = number of A_1A_2 animals

R = number of A_2A_2 animals

N = number of animals in the sample

A test for Hardy-Weinberg equilibrium is to ensure that there is no population stratification and that each marker reveals the expected genotype distribution for the observed allele frequencies. Expected genotype frequencies are calculated from the allele frequencies under the assumption of $p^2 + q^2 + 2pq = 1$, where p and q are the allele frequencies and 2pq corresponds to the frequency of the heterozygote.

The total allele frequencies of SNPintron1 (C8188T) are p(C) = 0.8043 and q(T) = 0.1957 and for SNPintron3 (T8737A) p(T) = 0.9746 and q(A) = 0.0254. The distribution of allele frequencies is shown in Table 4.7 and the distribution of allele frequencies between hernia inguinal piglet and normal pigs is displayed in Table 4.8. Some breed differences in allele frequencies at both SNPs specific differences between PIT animal and TNP, WP, BB, YS, RC (p≤ 0.05) and LC (p≤ 0.01) and between LC and JQH (p≤ 0.05) exist for SNPintron1 are displayed in Table 4.9. A SNPintron1 significant difference in allele frequencies exist between German hernia inguinalis piglets and normal pigs (p≤ 0.05) (Table 4.10). A SNPintron3 only LC and HIP (p≤ 0.01) and PIT (p≤ 0.05) showed differences significant. With respective SNpintron3 no significant differences allele frequencies exist between normal pigs, German and Thai herniated pigs.

Table 4.7 Total number of animals and distribution of allele frequencies.

Breed	SNPintron 1				SNPintron 3		
		Frequency		± S.E.	Frequency		± S.E.
	N	C	T		T	A	
Hernia inguinalis piglets (HIP)	37	0.6757	0.3243	0.0255	1.0000	0.0000	0.0000
Thai Native Pig (TNP)	5	1.0000	0.0000	0.0000	1.0000	0.0000	0.0000
Thai Wild Pig (WP)	5	1.0000	0.0000	0.0000	1.0000	0.0000	0.0000
Angler Saddleback (AS)	7	0.9286	0.0714	0.0177	0.9286	0.0714	0.0177
Pietrain (PIT)	15	0.5000	0.5000	0.0456	1.0000	0.0000	0.0000
German Landrasse (DLS)	8	0.8750	0.1250	0.0273	1.0000	0.0000	0.0000
German Edelschwein (DE)	7	0.8571	0.1429	0.0327	1.0000	0.0000	0.0000
Swabian-Haellian Swine (SHS)	7	0.7143	0.2857	0.0545	1.0000	0.0000	0.0000
Bunte Bentheimer (BB)	5	1.0000	0.0000	0.0000	1.0000	0.0000	0.0000
Yushanhei (YS)	7	1.0000	0.0000	0.0000	1.0000	0.0000	0.0000
Luchuan (LC)	12	1.0000	0.0000	0.0000	0.7500	0.2500	0.0383
Rongehang (RC)	7	1.0000	0.0000	0.0000	1.0000	0.0000	0.0000
Jiangquhar (JQH)	6	0.6667	0.3333	0.0642	1.0000	0.0000	0.0000
Cross Bred (CB)	10	0.9000	0.1000	0.0201	1.0000	0.0000	0.0000
Total	138	0.8043	0.1957	0.0095	0.9746	0.0254	0.0015

Table 4.8 Allele frequency of hernia inguinal piglets (German and Thai) and normal pig.

Animals	SNPintron 1				SNPintron 3		
		Frequency		± S.E.	Frequency		± S.E.
	N	C	T		T	A	
HIP Ger	33	0.6515	0.3485	0.0279	1.0000	0.0000	0.0000
HIP Thai	4	0.8750	0.1250	0.0387	1.0000	0.0000	0.0000
Normal	101	0.8515	0.1485	0.0089	0.9653	0.0347	0.0024
Total	138	0.8043	0.1957	0.0095	0.9746	0.0254	0.0015

Table 4.9 Significant differences of allele frequencies between origins.

SNPintron 1

	HIP	TNP	WP	AS	PIT	DLS	DE	SHS	BB	YS	LC	RC	JQH	CB
HIP		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
TNP	n.s.		n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
WP	n.s.	n.s.		n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AS	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
PIT	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	*	*	**	*	n.s.	n.s.
DLS	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DE	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SHS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BB	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.
YS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.
LC	**	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	*	n.s.
RC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.
JQH	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.
CB	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

SNPintron 3

Note: n.s.=nonsignificant, * p ≤ 0.05, ** p ≤ 0.01

Table 4.10 Significant differences of allele frequencies between HIP and normal pig.

SNPintron 1

Breed	HIP Ger	HIP Thai	Normal
HIP Ger		n.s.	*
HIP Thai	n.s.		n.s.
Normal	n.s.	n.s.	

SNPintron 3

Note: n.s.=nonsignificant, * p ≤ 0.05

No SNPs were detected in regulatory units of the gene. The variation A at SNPintron3 could only be detected at a low frequency. With one exclusion this allele ii specific for LC and must so far be regarded as a private or breed-specific allele. The significant differences between LC and the HIP Thai pig is possibly attribute to the small number of observation (HIP Thai, n =4).

There is some possibility that SNPintron1 may influence the predisportion for scrotal hernia in pigs because the position of the gene and significant differences in allele frequencies exist between German hernia inguinalis piglets and normal pigs. Although, this SNP is located in the intronic region, it is possible that it might affect the splice process and that are alterations of alternative splicing lead to disease. However, it is necessary to examine the biological role of the BAX protein to conduct a potential function of the characterized SNP. However, as the BAX gene maps to the hernia associated region on *Ssc6* the SNP is useful as a marker to fine map the region.

Most phenotypes of medical importance can be measured quantitatively (Mott *et al*., 2000). Scrotal hernia is a complex disease and several effects might contribute to the phenotype. Most traits of economic importance are affected by many different loci and the effects of these genes are influenced by environmental effects. If several genes contribute to the etiology of the disease, then there will be a positive relationship between the chance of an individual being affected and the extent to which that individual has genes in common with other affected individuals (Nicholas, 1999). However, only large-scale studies in large populations can help to isolate genes that are associated with a disease, and to select against the disorder.