CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

3.1.1 Chemicals

1) Absolute ethyl alcohol\ (Merck, U.S.A.)
2) Acetic acid (glacial)\ (Amersham Bioscience, Germany)
3) Agarose powder (ultra pure) \ (Biozyme, Germany)
4) Boric acid\ (Roth, Germany)
5) Bromophenol blue\ (Sigma, U.S.A.)
6) Dethyl pyrocabonate (DEPC)\ (Roth, Germany)
7) Dimethyl sulfoxide (DMSO)\ (Sigma, U.S.A.)
8) di-Sodium hydrogen phosphate\ (Roth, Germany)
9) Ethidium bromide\ (Roth, Germany)
10) Ethylenediaminetetraacetic acid (EDTA)\ (Roth, Germany)
11) Formamide\ (Roth, Germany)
12) Hydrochloric acid (HCl)\ (Roth, Germany)
13) Isopropanol\ (Sigma, U.S.A.)
14) N, N'- dimethyl-formamide\ (Roth, Germany)
15) Magnesium chloride\ (Qiagen, Germany)
16) Potasium dihydrogen phosphate\ (Roth, Germany)
17) Sodium acetate\ (Sigma, U.S.A.)
18) Sodium chloride\ (Roth, Germany)
19) Sodium dodecyl sulphate (SDS)\ (Roth, Germany)
20) Sodium hydroxide (NaOH)\ (Roth, Germany)
21) Tris\ (Roth, Germany)

3.1.2 Reagents

All solutions used in this investigation were prepared with deionized or demineralised water (ddH_2O or Millipore water). The pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl) (Reagent preparations see Appendix A)

1) 0.5M EDTA pH 8.0
2) 80% Ethanol
3) Digestion buffer
4) Phosphate buffer saline (PBS) pH 7.4
5) Proteinase K solution
6) 3M Sodium acetate pH 5.2
7) 2M Sodium chloride
8) 6M Sodium chloride
9) 9% Sodium chloride
10) 10% Sodium dodecyl sulfate (SDS)
11) 1x TBE buffer
12) 10x TBE buffer
13) TE buffer
14) 1M Tris pH 8.0

3.2 Enzymes, Nucleotides and Kits

3.2.1 Enzymes

1) Proteinase K\ (Qiagen, Germany)
2) *Taq*-DNA Polymerase\ (Qiagen, Germany)

3.2.2 Nucleotides

1) Deoxyribonucleotide triphosphate (dNTP)\ (Roth, Germany)
2) Oligonucleotides\ (MWG Biotech AG, Germany)

3.2.3 Kits

1) BigDye™ -Terminator Cycle Sequencing Ready Reaction Kit\ (Applied Biosystems, Germany)
2) DNA ladder and loading buffer\ (Amersham Biosciences, Germany)
3) PURE *Taq* Ready-To-Go PCR Beads®\ (Amersham Biosciences, Germany)
4) QIA-amp DNA mini Blood Kit\ (Qiagen, Germany)
5) QIA-quick PCR Purification Kit\ (Qiagen, Germany)

3.3 Instruments and Softwares

3.3.1 Instruments

1) Automated DNA sequencer\ (ABI-3100 capillary analyzer) (Applied Biosystems, Germany)
2) Automated spectrophotometer (ND-1000)\ (Nanodrop, Germany)
3) Bioclave\ (Schütt Labortechnik, Germany)
4) Centrifuge 5424\ (Eppendorf, Germany)
5) Deep freezer\ (Schütt Labortechnik, Germany)
6) Dest.-water (Biocell)\ (Millipore, Germany)
7) Electrophoresis equipment for agarose gel\ (Bio-Rad, Germany)
8) Functional micropipetter\ (Eppendorf, Germany)
9) Heat block QBD 2\ (Grant Instruments, England)
10) Incubator Certomat BS 1\ (Sartorius, Germany)
11) Magnetic mixer KMO 2\ (Janke und Klunkel, Germany)
12) Magnetic-mixer RCT basic\ (Schütt Labortechnik, Germany)
13) Megafuge 1.0 R\ (Thermo, Germany)
14) Set of Micropipette (0.5 µl to 1,000 µl)\ (Eppendorf, Germany)
15) Set of Micropipette (0.5 µl to 1,000 µl)\ (Gilson, USA)
16) Multifuge 1 sR\ (Thermo, Germany)
17) PCR gradient\ (T-gradient) (Biometra, Germany)
18) PCR thermocycler (T-3000)\ (Biometra, Germany)
19) pH meter PB 11\ (Sartorius, Germany)
20) Power supply PowerPac\ (Bio-Rad, Germany)

21) Refrigerator\ (Schütt Labortechnik, Germany)
22) Sorvall centrifuge RC-5B\ (Du Pont Instruments, Germany)
23) Speed Vac\ (Schütt Labortechnik, Germany)
24) UV-Transilluminator (312nm and 366 nm) and Gel documentation\ (Amersham Biosciences, Switzerland)
25) Vortex Genie 2\ (Bender + Hobein, Germany)
26) Water bath\ (Gesellschaft für Labortechnik, Germany)

3.3.2 Softwares

1) Vision-Capt Software (version 12.8)\ (PEQLAB, Germany)
2) BLAST Program\ (http://www.ncbi.nlm.nih.gov/BLAST)
3) ClustalW multiple sequence alignment\ (http://searchlauncher.bcm.tmc.edu/multialign/Options/clustalw.html)
4) PHYLIP software package (version 3.6a)\ (http://evolution.gs.washington.edu/phylip/software.html)
5) Primer express software (version 2.0)\ (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi)
6) SAS Program (version 8.0)\ (SAS Institute Inc, Cary, NC)
7) SeqBuilder, SeqMan, EditSeq; DNASTAR Lasergene 6\ (DNASTAR, Inc., Germany)
8) TREEVIEW\ (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html)

3.4 Experimental animals

3.4.1 Sample collections

For this study, a total of 55 Thai native pigs and wild boars DNA samples were used. Blood, ear clip or hair samples collections were collected from seven locations (Muang Mae Hongson, Tung Hua-chang, Jhom Thong, Chiang Dao, Fang, Viang Chai and Chiang San) in four Northern Thailand provinces i.e. Chiang Mai, Chiang Rai, Lamphun and Mae Hongson (Figure 3.1 and Table 3.1).

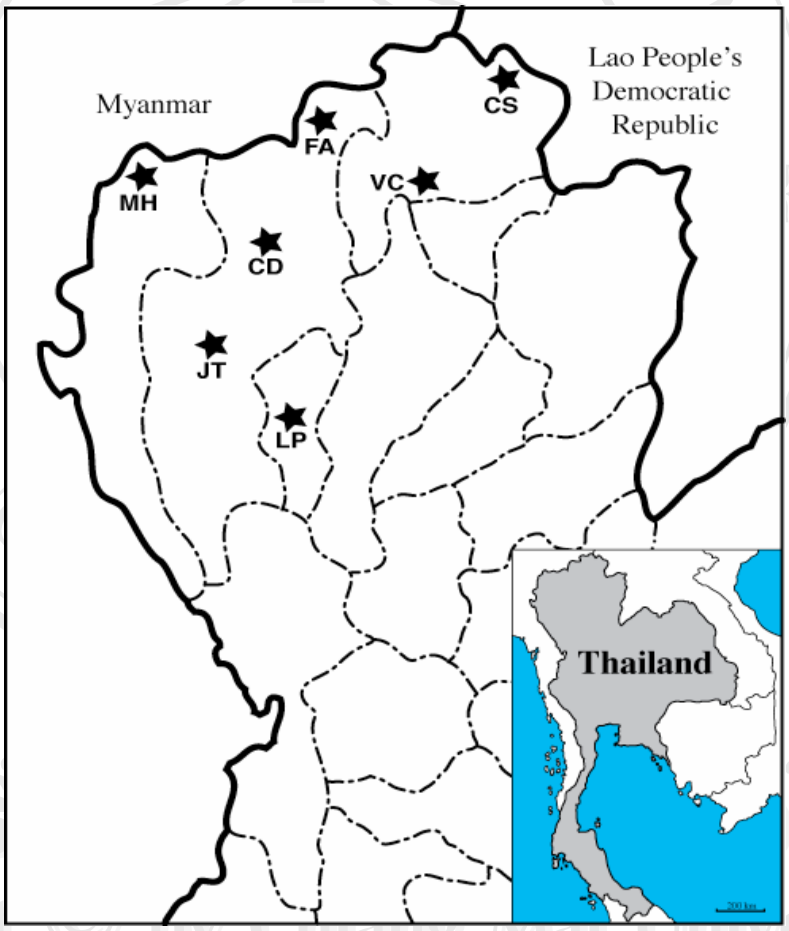

Figure 3.1 Map of sampling locations in Northern Thailand; CS = Chiang San (Chiang Rai Province), VC = Viang Chai (Chiang Rai Province), FA = Fang (Chiang Mai Province), JT = Jhom Thong (Chiang Mai Province), CD = Chiang Dao (Chiang Mai Province), MH = Muang Mae Hongson (Mae Hongson Province), LP = Tung Hua-chang (Lamphun Province).

Table 3.1 Samples of Thai pigs used in the mtDNA analysis.

Code	Types	Sampling location (City/Province)	n
CS	Thai native pig	Chiang San/Chiang Rai	8
VC	Thai native pig	Viang Chai/Chiang Rai	3
FA	Thai native pig	Fang/Chiang Mai	4
JT	Thai native pig	Jhom Thong/Chiang Mai	12
LP	Thai native pig	Tung Hua-chang/Lamphun	1
MH	Thai native pig	Muang/Mae Hong Son	6
CD	Thai native pig	Chiang Dao/Chiang Mai	16
TWB	Thai wild boar	San Sai/Chiang Mai	5
Total			55

3.4.2 Porcine mitochondrial genome from GenBank

To generate a phylogenetic framework suitable for comparison of the Thai native pig sequences, mtDNA D-loop sequences of 7 European (*Sus Scrofa*, Hampshire, Large White, Pietrain, Duroc, Landrace and Berkshire), 3 Chinese (Jin Hua, Tong Cheng, and Meishan), 1 Korean (Che Ju), 1 Japanese (Satsuma), and 1 Vietnamese (Mon Cai) indigenous pig breeds as well as 3 Asian and 2 European wild boars taken from GenBank were analysed. The Warthog (*Phacochoerus aethiopicus*) mtDNA D-loop sequence was used as an outgroup. The code assigned to each mtDNA D-loop sequence and GenBank accession numbers are shown in Table 3.2.

Table 3.2 The code assigned to each porcine mtDNA D-loop haplotype and GenBank accession numbers

Haplotype	Breed	Accession number	Type
SS	*Sus Scrofa*	AJ002189	European indigenous pig
HS	Hampshire	AB041488	European indigenous pig
LW	Large White	AB041492	European indigenous pig
PT	Pietrain	AB041489	European indigenous pig
DR	Duroc	AB041486	European indigenous pig
LR	Landrace	AB041496	European indigenous pig
BS	Berkshire	AB041484	European indigenous pig
EWB1	European wild boar 1	AB015094	European wild boar
EWB2	European wild boar 2	AB059651	European wild boar
RWB	Ryukyu wild boar	AB015087	Asian wild boar
JWB	Japanese wild boar	AB015085	Asian wild boar
KWB	Korean wild boar	AY574047	Asian wild boar
CJ	Che Ju (Korean)	AF276933	Asian indigenous pig
JH	Jin Hua (China)	AB041475	Asian indigenous pig
TC	Tong Cheng (China)	AF276923	Asian indigenous pig
MS	Meishan (China)	D17739	Asian indigenous pig
SM	Satsuma (Japanese)	AB015091	Asian indigenous pig
MC	Mon Cai (Vietnam)	AB041481	Asian indigenous pig
Warthog	Warthog	AB046876	Outgroup

3.5 DNA extraction and qualification

3.5.1 Genomic DNA extraction

Whole genomic DNA was extracted from blood (white blood cells) or ear clip (tissue) samples by the so called salting out method applied from Sambrook *et al.* (1989) and Miller *et al.* (1988). The hair sample (root hair cells) was extracted by using the QIA-amp DNA Blood mini kit (Qiagen, Germany). The DNA extraction protocols were as follows:

DNA extraction protocol from blood samples

1) Centrifuge blood samples (5-10 ml) at 6,000 rpm for 15 min and discard supernatant of plasma.
2) Carefully transfer the buffy coat (white blood cells) to a clean 1.5 ml microcentrifuge tube by a pasteur pipette.
3) Resuspend the buffy coat with 1 ml of millipore water, shake vortex for 20 sec to lysis red blood cells.
4) Add 100 µl of 9% sodium chloride solution to get a physiological condition. Shake by vortex, centrifuge at 12,000 rpm for 10 min. Discard the supernatant (repeat steps (3) and (4) until the pellet is white).
5) Resuspend the pellet with 1 ml of PBS solution, centrifuge at 12,000 rpm for 10 min and discard the supernatant.
6) Resuspend the pellet with 800 µl of digestion buffer, add 10 µl of proteinase K solution (20 mg/ml) and mix by vortex. Add 50 µl of 10% SDS solution and gently mix by hand.
7) Incubate overnight at 55^{o}C in a shaking incubator.
8) Incubate at room temperature for about 5-10 min, and then add 500 µl of 6M sodium chloride solution, incubate again at room temperature for 5-10 min, and then centrifuge at 12,000 rpm for 15 min.
9) Put the supernatant of about 500 µl into a clean 1.5 ml microcentrifuge tube.
10) Add one-tenth volume of 3 M sodium acetate solution and an equal volume of isopropanol. Gently shake the sample until precipitation of DNA.

11) Wash the DNA three times with 80% ethanol (centrifuge at 12,000 rpm for 5 min) and dry at room temperature.
12) Dissolve the DNA with 50-100 µl of TE buffer (until the concentration of DNA) and keep it at 4°C.

DNA extraction protocol from ear clip samples

1) Wash the 1 cm^2 ear clip with 80% ethanol and further with PBS solution. Transfer the sample into a clean 1.5 ml microcentrifuge tube.
2) Add 10 µl of proteinase K solution (20 mg/ml) and 800 µl of digestion buffer, mix by vortex, add 50 µl of 10% SDS solution and gently mix by hand.
3) Incubate overnight at 55°C in a shaking incubator.
4) Incubate at room temperature for about 5-10 min. Add 500 µl of 6 M sodium chloride solution, incubate again at room temperature for 5-10 min, and then centrifuge at 12,000 rpm for 15 min.
5) Put the supernatant of about 500 µl into a clean 1.5 ml microcentrifuge tube.
6) Add one-tenth volume of 3 M sodium acetate solution and an equal volume of isopropanol. Gently shake the sample until precipitation of DNA.
7) Wash the DNA three times with 80% ethanol (centrifuge at 12,000 rpm for 5 min) and dry at room temperature.
8) Dissolve the DNA with 50-100 µl of TE buffer (until the concentration of DNA) and keep it at 4°C.

DNA extraction protocol from hair samples by QIA-amp DNA Blood mini kit

1) Cut the root hairs (about 20 hairs for one sample) and take the sample into a clean 1.5 ml microcentrifuge tube.
2) Add 200 µl Millipore water and add 200 µl AL buffer.
3) Add 50 µl of proteinase K solution (20 mg/ml) and centrifuge briefly.
4) Incubate at 56°C for 3 hr and warm up the AE buffer.
5) Add 200 µl of 96% ethanol to the sample and mix again by hand for 30 sec and centrifuge at 14,000 rpm for 2 min.

6) Transfer the supernatant of sample into the QIAmp spin-column and centrifuge at 8,000 rpm for 2 min and change the collection tube.
7) Add 500 µl of AW1 buffer and centrifuge at 8,000 rpm for 2 min and change the collection tube again.
8) Add 500 µl of AW2 buffer and centrifuge at 8,000 rpm for 2 min.
9) Place the QIAmp spin-column in a clean 1.5 ml microcentrifuge tube, add 100 µl of warm AE buffer, incubate at room temperature for 5 min and then centrifuge at 14,000 rpm for 2 min. The DNA solution will drop into a 1.5 ml microcentrifuge tube.

3.5.2 DNA qualification

The absorbance of the DNA solution was measured to determine the amount and quality of the DNA from the optical density (O.D.) using the ND-1000 automed spectrophotometer version 3.1 (Nanodrop, Germany). The ratio of absorbance at 260 and 280 nm is used to assess purity of DNA, a ratio of about 1.8 is generally accepted as pure for DNA. Ratios lower than 1.75 indicate that significant amounts of proteins remained in the prepared sample. Samples were generally stored at 4ºC until PCR reactions were finished and then frozen at -20ºC for long-term storage. Repeated freezing and thawing of samples was avoided.

3.6 Mitochondrial DNA sequence analyses

3.6.1 MtDNA D-loop region amplification

Polymerase chain reaction (PCR) was used to amplify D-loop region or control region (Figure 2.5). PCR reactions were performed on a variety of different thermocyclers. PURE *Taq* Ready-To-Go PCR Beads® (Amersham Biosciences Europe, Germany) were used. These freeze-dried beads contain 200 µM dNTP, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 2.5 units *Taq* polymerase and PCR buffer. 1 µl of each primer (100 pMol), 1 µl DNA (50-100 ng) and 22 µl PCR grade water were added to reach a final reaction volume of 25 µl. The PCR amplification reaction for the mtDNA D-loop region fragment was done using the following program:

Initial denaturation	1 cycle	4 min	at 94.0ºC
Amplification	35 cycles	1 min	at 94.0ºC
		1 min	at 60.0ºC
		1 min	at 72.0ºC
Final extension	1 cycle	5 min	at 72.0ºC

PCR reactions were applied as introduced by Kim *et al.* (2002a). The primers used to amplify the mtDNA D-loop region were "D-loop 4F" as forward primer and "D-loop 4R" as reverse primers originated in the region of threonine tRNA and phenylalanine tRNA respectively (Figure 2.5). The primers were equal to the primers mitL44 and mitH45 as described by Okumura *et al.* (2001). For direct DNA sequencing both primers were tailed with the universal M13uni primers (5'-CCTACCATCAGCA CCCAAAG-3') or the universal M13rev primer (5'-TCCAGTGCCTTGCTTTAGTA-3') (Table 3.3).

Table 3.3 PCR primers of the mtDNA D-loop region.

Primer	Sequence	T_m (ºC)
D-loop 4F	5'-GTAAAACGACGGCCAGTTCCTACCATCAGCACCCAAAG-3'	60
D-loop 4R	5'-CAGGAAACAGCTATGACCTCCAGTGCCTTGCTTTAGTA-3'	60

After PCR, 5 µl of the PCR product were electrophoresed. Electrophores of PCR products in 1x TBE buffer were carried out on 1% (w/v) agarose gels containing ethidium bromide. PCR products were visualised by the UV-transilluminator and the gel documentation system. Before the specific PCR product could be used for sequencing it is necessary to purify it from residual reaction components such as primers, unincorporated nucleotides, enzymes, salts, mineral oil and nonspecific amplification products. For purification the QIA-quick PCR Purification Kit was used following the manufacturer's protocol.

3.6.2 DNA Sequencing

The PCR product was sequenced by a modified and automated dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). The reaction used the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with 8 sequencing primers (Primer walking strategy) (Table 3.4). The thermal cycle sequencing reaction was run on the Biometra T-Gradient with the following temperature parameters:

Initial denaturation	1 cycle	30 sec at 96.0ºC
Amplification	29 cycles	10 sec at 96.0ºC
		5 sec at the specific T_m
		4 min at 60.0ºC

Products were precipitated and cleaned before being analysed on the automated DNA sequencer. In this analysis fluorescent labeled DNA samples are injected into a capillary filled with a stationary polymer. When a voltage is applied the labelled fragments are separated and pass an analysing "window". This window is a glass cuvette, where a laser activates the dyes and causes them to fluoresce. Fluorescent radiation is then detected by a charged coupled device camera and converted into an electronic signal, which is transmitted to a computer workstation. The result is an electrophoregram, which is saved as an ABI-Trace data-file (Applied Biosystem, 2001).

Table 3.4 Primers used for the primer walking strategy.

Primer	Sequence	T_m (ºC)
M13uni	5'-CCTACCATCAGCACCCAAAG-3'	53.7
M13rev	5'-TCCAGTGCCTTGCTTTAGTA-3'	53.7
D-loop AF	5'-TACCATGCCGCGTGAAACCA-3'	59.4
D-loop 1R	5'-TGGGCGATTTTAGGTGAGAT-3'	55.3
D-loop 2F	5'- ACGACAATCCAAACAAGGTG-3'	55.3
D-loop 3R	5'-GGGGGTTTGAATGAGCTAATAA-3'	56.5
D-loop 5F	5'-CGCGCATATAAGCAGGTAAA-3'	55.3
D-loop 6R	5'-CGTGCATATAAGCAGGTAAA-3'	59.4

3.6.3 Primer walking

Because the length of the PCR product exceeded the length of a single sequencing run, sequence data of the first run can be used to prepare the primer for a second run into the region of interest. This process, called primer walking, can be repeated many times to sequence extensive tracts of DNA (Figure 3.2). The major advantages of primer walking are that no subcloning is required, and the location and direction of each sequencing run is known. Primer walking minimizes the degree of redundancy needed to obtain the final sequence, and avoids assembly problems caused by repetitive DNA.

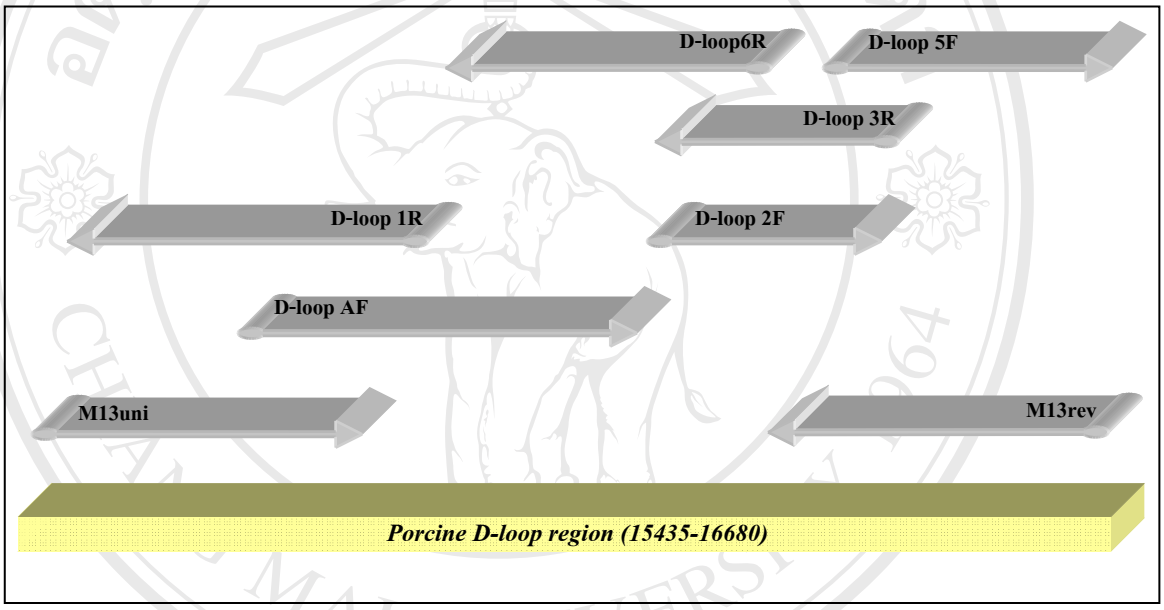

Figure 3.2 Primer walking strategy (the bar represents the segment of target DNA).

3.6.4 Sequence editing and alignment

ABI traces were aligned and manually checked using the program SeqMan. Sequences were double checked with the growing alignment and especially haplotypes occurring only once were double checked. The identity of sequences was verified using BLAST search as implemented in GenBank. All sequences of the mtDNA D-loop region were aligned using ClustalW (Thomson *et al.*, 1994) and alignment parameters were default. For the phylogenetic analyses unresolved sites, coded as N in the alignments were allowed, only completely resolved sequences without any ambiguous sites were used.

3.7 Phylogenetic analysis

The phylogenetic analysis was performed using the PHYLIP software version 3.6a (Felsenstein, 1995). The mean number of substitution per nucleotide site was calculated using the Kimura 2-parameter model (Kimura, 1980) after elimination of nucleotide gaps. From the estimated distance matrix, phylogenetic trees were created using the Neighbor-jointing method (Nei *et al.*, 1983; Saito and Nei, 1987; Takesaki and Nei, 1996), the corresponding regional sequence of the Warthog (*Phacochoerus aethiopicus*) from GenBank (Accession No. AB046876) was used as an outgroup. The bootstrap method (Felsenstein, 1985) was used to determine the confidence interval of each phylogeny from 1000 bootstrap repetition. Nucleotide diversities and net nucleotide differences were calculated to estimate intra- and inter-breed genetic variations of the domestic pig breeds.