CHAPTER III

MATERIALS AND METHODS

1. Source of Animals

In order to detect polymorphic sites within the porcine vinculin gene cDNA samples of five animals of the breeds Hamshire, Duroc, German Landrace, Pietrain and an F2 animal of the Berlin-Bonn resource population (based on the cross of Duroc and Berlin Min.iature Pig) were chosen.

2. Chemical and Reagent

1) deionized water
2) pairs of PCR primers complementary to nine overlapping fragments of the porcine vinculin gene (MWG-Biotech)
3) pair of PCR primers complementary to the M13 regions of the cloning vector pGEM (MWG-Biotech)
4) allele specific primers (MWG-Biotech)
5) dNTPs (Roth)
6) *Taq* DNA polymerase (Amersham Pharmacia Biotech)
7) *Taq* DNA polymerase (Roche)

8) Agarose (Biozym)

9) Ethidium bromide (Roth)

10) 6x Loading buffer (Biomol)

11) DNA marker (Biomol)

12) 1xTE buffer

13) 1xTAE buffer

14) 10x amplification buffer (Amersham Pharmacia Biotech, Roche)

15) Phenol (Biomol)

16) Chloroform (Roth)

17) Ethanol (Roth)

18) Sodium acetate (Merck)

19) *E.coli* strain DH5-alpha

20) LB medium

21) Ampicillin (Sigma)

22) Isopropyl-β-D-thiogalactosidase (ITPG) (Roth)

23) 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) (Roth)

24) Trizol reagent solution (Gibco/BRL Lifesience)

25) DTT (Gibco/BRL Lifesience)

26) Reverse transcriptase Superscript (Gibco/BRL Lifesience)

27) SequaGel XR (Biozym)

28) Dimethyl Sulfoxide (DMSO) (Sigma)

29) Diethyl Pyrocarbonate (DEPC) (Roth)

30) APS (Roth)

31) Glycerol (Fluka)

32) Eco57I restriction enzyme and buffer (Fermentas)

33) PCR purification kit (Qiagen)

34) Rneasy Mini Kit (Qiagen)

35) Ligation kit, pGEM^{R}-T Vector SystemI (Promega)

36) High Purified Plasmid Isolation kit (Roche)

37) SequiTherm^{TM} Sequencing kit , EXCEL^{TM}II DNA Sequencing kit LC (Biozym)

3. Experimental Instruments

1) Thermal cycler (MJ Research)

2) Electrophoresis apparatus (BIO-RAD)

3) Gel Documentation System(UVItec)

4) Centrifuge machine (HERMLE)

5) Sterile laminar-flow hood (Heraeus instrument)

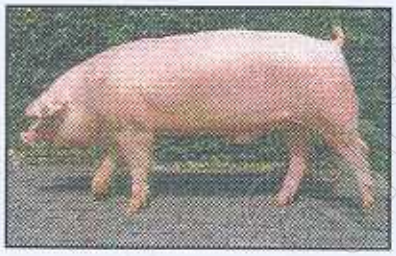
6) LI-COR DNA Sequencer (MWG-Biotech)

4. Methods

The study was divided into 3 parts with the first part dealing with the identification of polymorphisms of cDNA sequence of the porcine vinculin gene by comparative sequencing. In part 2 the vinculin gene was physically mapped and in part 3 protocols were established to perform the genotyping at the polymorphic sites of the vinculin gene to use as genetic marker for the trait eye-muscle area.

Doroc sire and Berlin Miniature Pig dam

F1 dam with F2 piglets



German Landrase sire

German Landrase sire

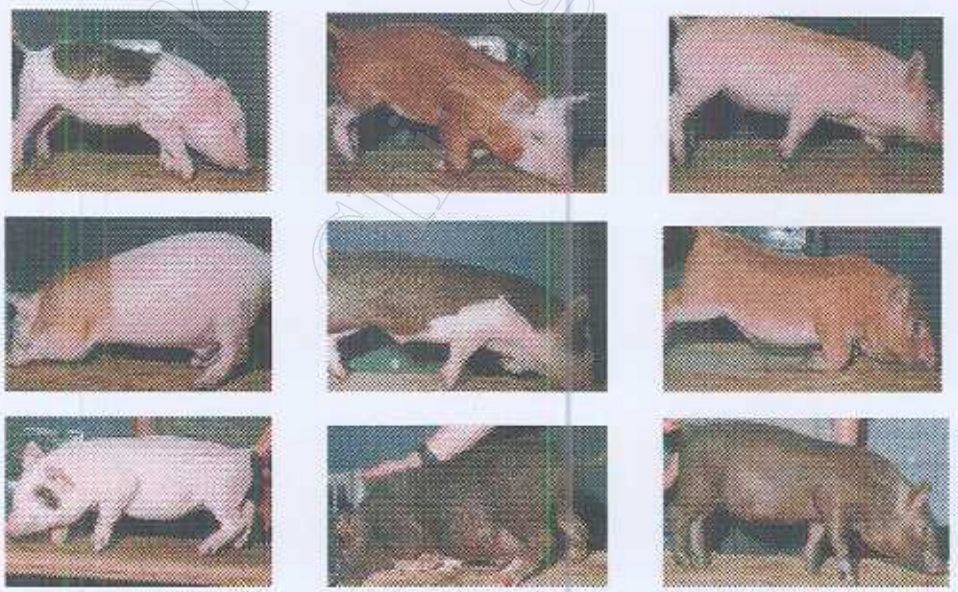


Figure11 Diagram of breeding F2 resource population (Ponsuksili *et al.*, 2000b).

Part I Identification of Polymorphic Sites

1. Preparation of total RNA

RNA from muscle (*M. longissimus dorsi*) was isolated in a procedure comprising of extraction with TRIzol Reagent (Gibco/BRL, Life Technologies, Karlsruhe, Germany), decontamination of trace genomic DNA with DnaseI (Roche, Mannheim, Germany) and purification using the Qiagen Rneasy kit (Qiagen, Hilden, Germany). RNA integrity was evaluated on 1% agarose gels containing formaldehyde and ethidium bromide. The protocol in detail was as follows:

1.1 RNA Isolation from *M. longissimus dorsi* tissue.

1) Homogenize the tissue in liquid N_2 , per 30-100 mg tissue add in 1 ml. Trizol reagent solution, then homogenize further with syringe.
2) Incubate for 5 min. at room temperature.
3) Add 300 µl chloroform and incubate at room temperature for 3 min. for phase separation.
4) Centrifuge at 12,000xg for 15 min. at 4°C.
5) Carefully transfer the aqueous phase to a fresh tube, then add Trizol solution 500 µl. and repeat step 2-4.
6) Add 1 vol. of isopropanol, mix and incubate for 10 min. at room temperature.
7) Centrifuge at 12,000xg for 10 min. at 4°C (pellet should be visible, otherwise centrifuge again at high speed for a long time). Then, carefully

remove the supernatant and wash pellet by centrifuge 5 min. with ice cold 70% ethanol.

8) Remove supernatant and partially dry RNA pellet at room temperature for 15 min.

9) Dissolve the total RNA in an appropriate volume of DEPC water.

10) Dnase digestion.

11) Purification of total RNA (RNeasy Mini Kit).

12) Evaluation of RNA integrity by electrophoresis on 1% agarose gels containing formaldehyde and ethidium bromide.

13) Quantification of total RNA by the absorbance at 260 and 280 nm.

2. Frist strand cDNA synthesis

First-strand cDNA template was synthesized by mixing 1 µg. Dnased-RNA and $(dT)_{11}$-primer. The reactions were heated for 5 min. at 65 °C and placed on ice to cool. Then the mixed of 1xRT buffer, Reverse Transcriptase, dNTP and dithiothreitol (DTT) were added and incubated for 1 h at 37°C in a final volume of 20 µl. After heating inactivation for 5 min. at 70°C, the cDNA was diluted 1:5 and stored at -20°C for the subsequent PCR reaction. To confirm the successful decontamin.ation of genomic DNA, RNA and cDNA were used as a template in a PCR reaction using intron spanning primers of the ß actin gene. The protocol was as follows:

1) Use 1 µg. of total Dnased RNA in total reaction volume of 10 µl.

2) Add 1 µl. of 20 µM oligo$(dT)_{11}$

3) Incubate for 5 min. at 70°C then chill on ice for 2 min.

4) Add:

- 5xRT-buffer(GIBCO/BRL, Life Technologies)	4	µl.
- dNTP (2.5 mM each)	2	µl.
- 0.1 M DTT(GIBCO/BRL, Life Technologies)	2	µl.
- 200U SuperScript RT (Gibco/BRL, Life Technologies)	1	µl.

5) Incubate at 42°C for 1 hr 30 min., then at 72°C for 5 min.

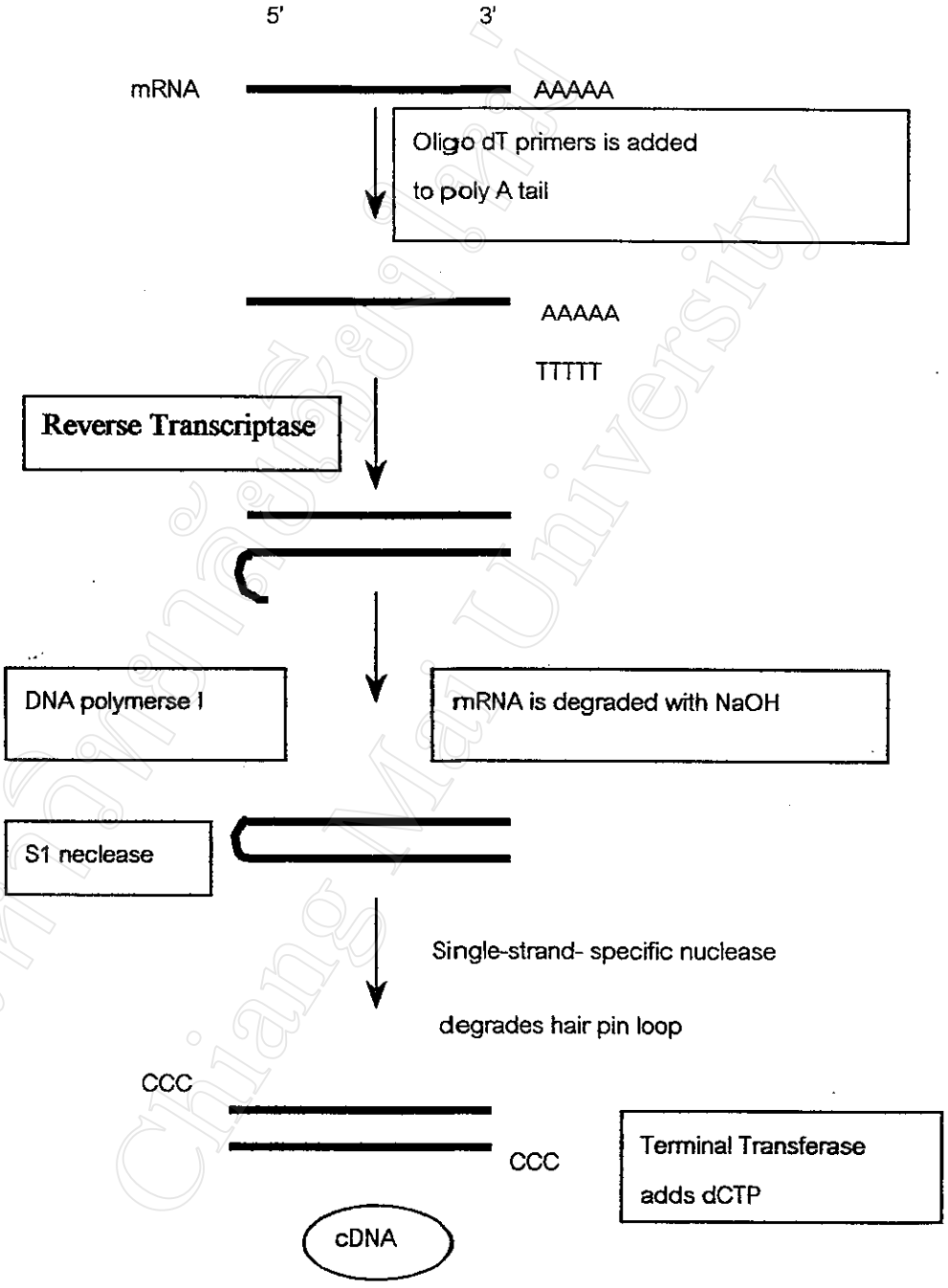


Figure 12 Priciple of RT-PCR (Lewin., 1994).

3. Design of specific forward and reverse primers and amplification of specific DNA fragment by PCR.

The cDNA sequence of the porcine vinculin gene has been identified by Ponsuksili et al. (2000, accession no AF16517). In order to produce overlapping PCR-products that are screened for polymorphims by comparative sequencing 18 oligonucleotide primers – nine pairs of primers - were designed to cover over 5172 base pairs of the cDNA sequence of the porcine vinculin gene. Primers specific to the cloned DNA sequences were derived using the primer design software from http://www.williamstone.com. The primer were diluted by adding deionised water(ddH_2O) to obtain a working solutions of 10 pmol/µl. that were in reactions composed like follows:

1) PCR mixture prepared in a 0.2 ml microcentrifuge tube on ice.

- forward primer (10 pmol/µl.)	0.75	µl.
- reverse primer (10 pmol/µl.)	0.75	µl.
- cDNA (50 ng/µl.)	2.00	µl.
- 10x amplification buffer (Boehringer[1])	3.00	µl.
- dNTP (2.5 mM each)	0.75	µl.
- *Taq* DNA polymerase (Boehringer[1])	0.15	µl.
- add ddH2O to final volume of	30	µl.

[1] Expand™ High Fidelity PCR System results in a 3-fold increased fidelity of DNA synthesis ($8.5*10^{-6}$ error rate) compare to *Tag* DNA polymerase ($2.6*10^{-5}$ error rate) (Boehringer Mannheim Cat:No.1732641).

2) Place the tubes in a thermal cycler and perform PCR,

- Predenaturation	95°C	3	min.
- Denaturation	95°C	30	sec.
- Annealing	60°C	30	sec.
- Extension	70°C	1	min.
- Final extension	72°C	5	min.

Cycling 34 cycles of denaturation, annealing and extension time.

Notes:

1) The denaturation step was done rapidly at 95°C with hot start.

2) Primer annealing depends on the melting temperature of the primers. Extention occurs at 70-72°C for most template (Kolmodin and William., nodate).

3) Touch down PCR was performed when Tm of forward and reverse primer were different more than 2°C to select a broad range of annealing temperatures that begins above the estimated Tm and end below it.

4. Purification of DNA fragments from agarose gel by phenol-chloroform extraction.

The PCR products were run in 0.8% ethidium bromid stained agarose gels at 100 volt. Pieces of the agarose gels containing the desired DNA-fragments were sliced out and chilled at –70°C for at least 20 min. The pieces of chilled gel were crushed using pipette tips and further reduced to small pieces by adding 500 µl. 1xTE buffer and mixing with a syringe. 500 µl. phenol-chloroform were added, the contents in the tube were mixed by syringe until an emulsion forms. The mixture was

centrifuged at 12,000 g for 15 min. at room temperature. The aqueous phase was transfered to another tube, 500 µl. of chloroform were added, mixed gently and centrifuged for 15 min. The supernatant was transferred to a new tube. The DNA was recovered by ethanol precipitation at –70°C for 2 hours to overnight and subsequent centrifugation at 4°C by high speed for 30 min. The DNA pellets were diluted in 7-10 µl. of ddH2O and stored at –20°C until use.

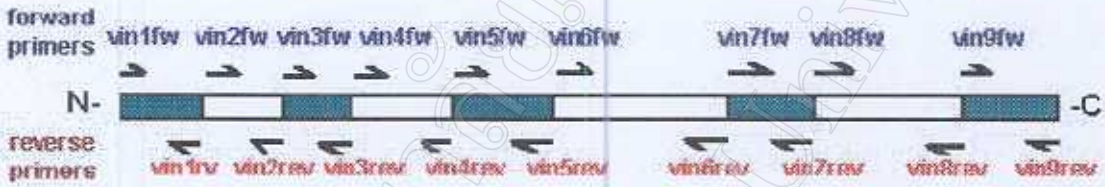


Figure 13 18 Oligonucleotide primer designed clover over 5,172 bp of vinculin gene.

5. Cloning of DNA fragments

The DNA fragments recovered from the agarose gels were cloned into a plasmid vector for further analysis. Therefore, the following ligation reaction was set up on ice using the pGEM ^{R}-T Vector System Kit, Promega:

- vector DNA	1	µl.
- insert DNA	3	µl.
- 2x ligase buffer	5	µl.
- T4 DNA ligase	1	µl.

The ligation reactions were incubated in thermocycler at 16-20°C for 2 hrs. or alternatively for higher efficiency at 4°C overnight. The competent cells for

6. Insert amplification

PCR reactions were performed to amplify the inserted DNA sequence between the M13 promotor sites of the plasmid vector. The reactions were as follows:

- M13 forward primer (10 ng/μl.)
- M13 reverse primer (10 ng/μl.)
- 10x amplification buffer (Pharmacia)
- colony lysate
- dNTP (2.5 mM each)
- *Tag* DNA polymerase (Pharmacia)
- add dd H2O to final volume 30 μl.

Aliquots of the products of the insert-amplification PCR were evaluated by electrophoreses in 1% agarose gels. Colonies that provided insert-amplification PCR products of the desired size were selected for further analyses.

7. Isolation and purification of plasmid DNA

In order to obtain template DNA for subsequent sequence analyses plasmid DNAs of those white colonies with inserts of the expected size as shown by insert amplification were isolated using the High Pure Plasmid Isolation Kit (Roche) following the manufacturers recommendations:

1) Inoculate a single colony containing the plasmid DNA of interest which was tested by M13 insert-amplification PCR in 5 ml. of LB medium with 50 μl/ml. ampicillin , culture the bacteria at 37°C overnight with shaking at 120 rpm.

2) Transfer the overnight culture into 2 ml. microcentrifuge tube and centrifuge at 6,000 rpm for 10 min. Then, carefully aspirate the supernatant. Add more culture bacteria into microcentrifuge tube and centrifuge again to get up to 10 µg. plasmid DNA from 2-4 ml. bacterial suspension.

3) After having pelleted bacterial cells, resuspend the pellet in 350 µl. suspension buffer/RNase and mix well.

4) Add 350 µl. lysis buffer, mix gently and incubate for 5 min. at room temperature. Add 450 µl. chilled binding buffer, mix gently then incubate for 5 min. on ice. The solution becomes cloudy and flocky.

5) Centrifuge for 10 min. at maximum speed.

6) Combine the High Pure filter tube and the collection tube. Pipette the supernatant into the upper reservoir and centrifuge for 60 sec. at maximum speed.

7) Discard the flow through and add 500 µl. washing bufferII with no guanidine hydrochloride (for DH5-alpha strain with low nuclease activity), centrifuge for 60 min.

8) Discard the solution in collection tube and insert the filter tube in a clean 2 ml. microcentrifuge tube. Add 25 µl. elution buffer or ddH2O and centrifuge for 60 min.

9) Determine the plasmid yield by 1% agarose gel electrophoresis.

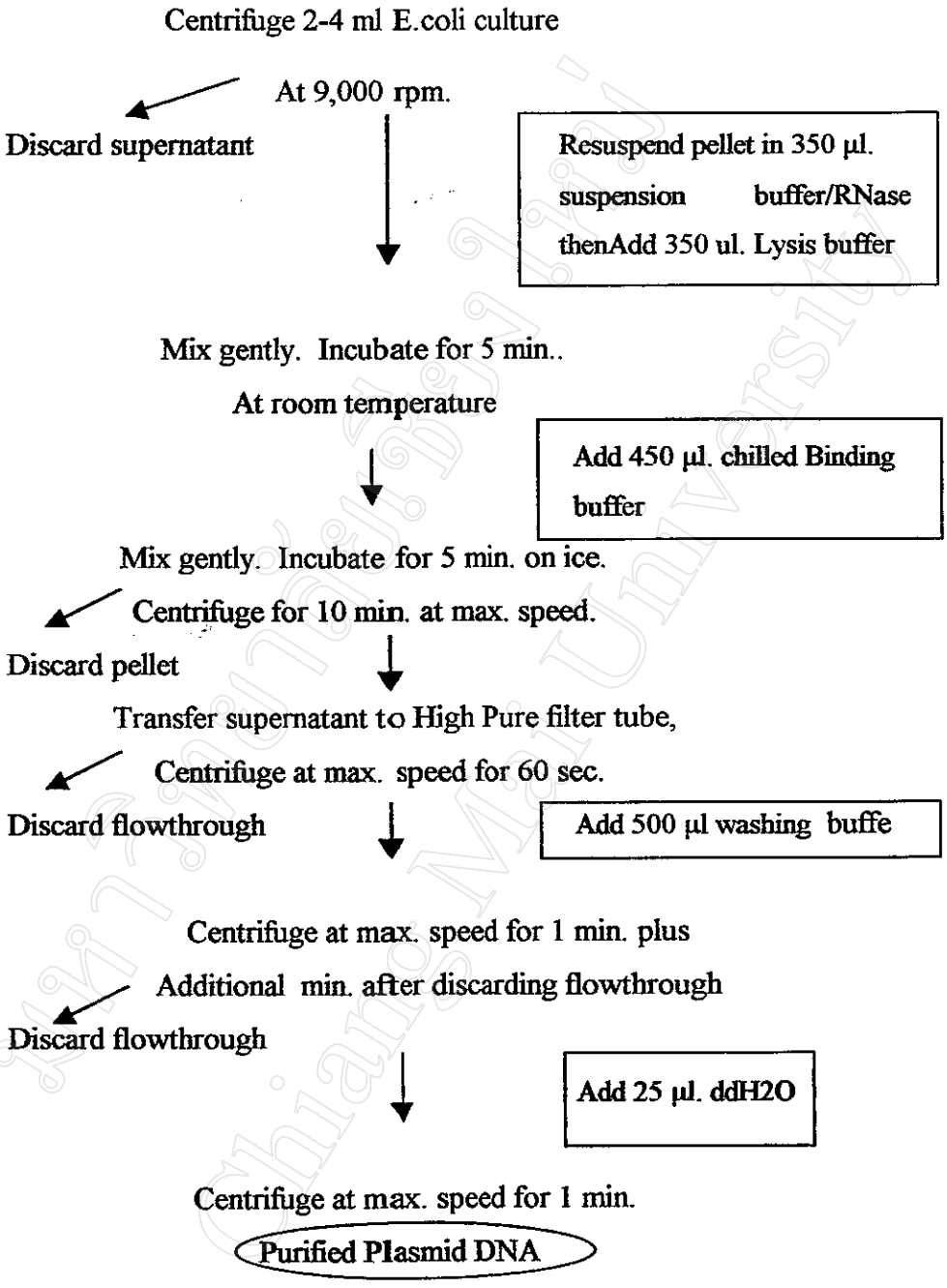


Figure 14 Plasmid Isolation by High Pure Plasmid Isolation Kit (Modified from Roche Molecular Biochemicals.,1999).

2) Preparation of sequencing gel (SequaGel^{R} XR)

2.1 Clean the glass plates (40*40 cm.) thoroughly. Rinse with ethanol and wipe dry .

2.2 Apply Glass Free^{TM} (Cat.#EC-621) to one glass plate to ensure the gel will release from one plate after electrophoresis .

2.3 Add 30 ml of SequaGel XR monomer Concentrated and 7.5 ml. SequaGel complete Buffer in a beaker. Then, add 400 µl. of DMSO, mix gently.

2.4 Add freshly prepared 10% APS (Cat.#EC504) 300 µl., swirl gently to mix and quickly cast the gel. Insert the comb and allow to polymerise one to two hours. Be careful the air bubbles in the gel and leaking of the gel mixture from the bottom or side edges of the gel apparatus because ether of this two problems can cause failure or poor quality of DNA sequencing.

3) Prerun the gel for 30-45 min. before loading the samples (LI-COR DNA Sequencer). For the large gel use 55-60 Watts. The gel temperature should be between 45-50°C.

4) Denature the DNA Samples at 95°C for 5 min. and then leave the samples in ice before loading.

5) After prerun, rinse the well with running buffer. Reinsert the sharkstooth comb so the teeth just touch the gel and load the samples and run electrophoresis at constant power (40 watt) and 50°C.

6) Evaluate the sequence using the BaseImagIR software.

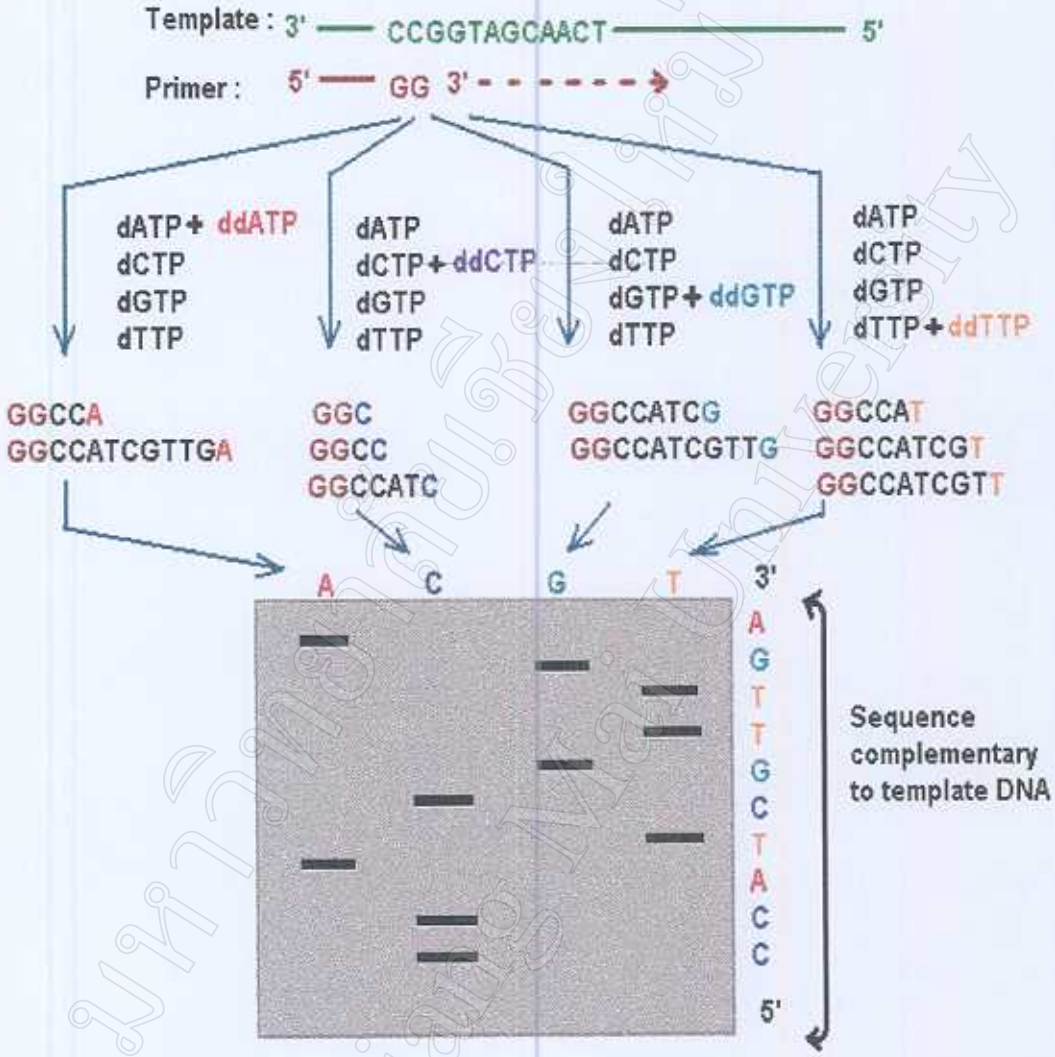


Figure 15 Principle of DNA sequencing, Sanger method (Sambrook *et al.*,1990).

Part II Mapping and Establishment of Protocols Suitable for PCR-based Genotyping the Polymorphic Sites.

The ImpRHpanel was used for physical mapping of the vinculin gene. Therefore, the region of the 3′-untranslated region of the vinculin genes flanked by the primers vin8up and vin8down was amplified by PCR using DNA of the 118 clones of the ImpRH panel and hamster, mouse and pig genomic DNAs as template in reactions composed like:

1) Each ImpRH clone was amplified in 20 µl. reaction volume containing,

- Hybrid DNA(ImpRHpanel)	2.5	µl.
- 10X buffer (Pharmacia)	2.0	µl.
- dNTPs(2.5 mmole each)	0.5	µl.
- Vin 8 Forward Primer(10 pmole)	0.5	µl.
- Vin 8 Reverse Primer (10 pmole)	0.5	µl.
- *Tag* DNApolymerase(Pharmacia)	0.2	µl.
- ddH_2O	13.8	µl.

Amplification was carried out using the condition touch down PCR:

95° 3' ,(95° 1' ,63° -0.5°/cycle 30'' ,70° 1')*10 ,(95° 1' ,58° 30' ,70° 1')*34 ,72° 5'

Control consisted of hamster genomic DNA, porcine genomic DNA and a negative control reaction with out DNA.

2) Electrophoresis by agarose gels.

PCR products for each markers were electrophoresed using 1.5% agarose gels at 110 volt for 20-30 min. Gels were photographed.

3) Scoring and analysis.

Each marker was scored from the photos. Loci were scored either as present (1) or absent (0) for each hybrid. The INRA Toulouse web site was used to analyze this RH data, avaiable at http://imprh.toulouse .inra.fr/.

Part III Restriction Fragment Length Polymorphism (RFLP) Method.

RFLP is a technique in which breeds may be differentiated by analysis of patterns derived from cleavage of their DNA. If the breeds differ in the distance between sites of cleavage of a particular restriction endonuclease (Hill., nodate ; Restriction Fragment Length Polymorphism., 2000). The length of the fragments produced will differ when the DNA is digested with a restriction enzyme and resulting fragments are separated according to moleculare size using agarose gel electrophoresis (Whipple.,1998 ; Recombinant DNA Labs., 2000). The similarity of pattern generated can be used to differentiate species from one another.