CLONING AND CHARACTERIZATION OF BOVINE GROWTH HORMONE (BGH) GENE FROM LOCAL GAUR (SELADANG)

by

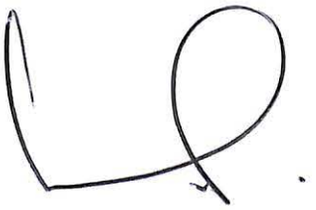
ABDUL RASHID BIN JUSOH

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Health Science (Biomedicine)

March 2005

CERTIFICATE

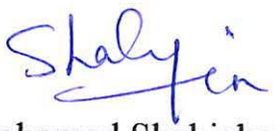
This is certify that dissertation entitled **'Cloning and Characterization of Bovine Growth Hormone (bGH) Gene from Local Gaur (Seladang)'** is the bonafide record of research work done by **ABDUL RASHID BIN JUSOH** during the period from Jun 2004 to March 2005 under our supervision

Signature of Supervisor : 

Name and address of Supervisor : Dr. Shaharum Shamsuddin

: PPSK

Date : 14 March 2005

Signature of Co-Supervisor : 

Name and address of Supervisor : Dr. Mohamad Shahjahan

: PPSK

Date : 14 March 2005

ACKNOWLEDGEMENT

First of all, thank to **God** that given me an opportunities to finish this thesis, without his bless, may be I cannot finish this thesis.

I take this opportunity to express my profound thankfulness to **Dr. Shaharum Shamsuddin** for her graceful acceptance to be my guide and her encouragement throughout this study.

I am graceful to **Dr. Mohamad Shahjahan** for his involvement and encouragement in this study and also useful suggestion during the study.

I am also thankful to the **Dean, PPSK, USM Health Campus, Kubang Kerian kelantan,** for permitting to do research.

I am also very thankful to **Scientific Officer of Molecular Biology Laboratory, PPSK** for permitting and co-operation during my study.

My sincere thank to **Mr. Hadi and Mr. Venugopal Postgraduate Student** that very generous to pass their knowledge and given good guidance during my study.

I am thankful to all **Postgraduate Students** that work in **Molecular Biology Laboratory, PPSK** for their help and co-operation during my study.

I am graceful to **my entire friend** that given moral supports and helped me during my hard time especially when both my computer and printer are failed.

Not forgotten too, to **my family** that understands my situation when I cannot spend much time to them during I do the lab works.

Lastly I would like to thank **all the individuals** mentioned above again for helping me to acquire the basic skills in molecular works and the talent to troubleshoot.

Table of Content

List of Table, Figures and Pictures

List of Table

List of Figure

List of Pictures

Abbreviation

bGH	: Bovine growth hormone
$CaCl_2$	: Calcium chloride
ddH_2O	: Double distilled water
DNTP	: Deoxyribonucleoside triphosphate
EB	: Elution buffer
E. coli	: *Escherichia coli*
EDTA	: Ethylene diamine tetra acetic
EtBr	: Ethidium bromide
GH	: growth hormone
GHRH	: Growth hormone releasing hormone
GHRIH	: Growth hormone releasing inhibit hormone
LB	: Luria bertani broth
LBA	: Luria bertani agar
PCR	: polymerase chain reaction
TE	: Tris-HCl, EDTA
TEN9	: Tris-HCl, EDTA, NaCl

Abstract

Bovine growth hormone gene (bGH) is well know among the scientist all over the world. Various types of bGH gene has been publish from various bovine species. Expression of that sequence has produced a valuable protein that is known as bovine growth hormone. These hormone play important role in regulated the differentiation, growth and metabolism of cattle. Has been proven that, this hormone play important role in increasing the production of milk. In searching full information about that gene, many bGH sequence has been explore in different bovine species

However the sequence of bGH gene from *Bos gaurus hubbacki* (local Malaysian gaur) still not explore yet. To grab these opportunity my supervisor with collaboration of Seladang Conservation Centre has proposed these study. In this study we try to clone and characterize bGH gene from genomic DNA. The genomic DNA was extracted from gaur tissue using phenol chloroform method proposed by Maniatis et al. (1989). The bGH gene has been amplified using polymerase chain reaction. Amplified product then has been further clone into TA Cloning vector. The recombinant plasmid has been transformed into *E.coli* DH5α. The cloned DNA finally has been submitted for sequencing and sequence analysis of the gene has been carried out.

This study reveals that, bGH gene has been successful cloned from genomic DNA of local gaur. The size of the cloned gene is 1208 bp and has high similarity with bGH gene from other bovine species reported in GenBank.

Abstrak

Gene yang mengkodkan hormone pertumbuhan species bovine (bGH gene) telah lama dikenali terutamanya di kalangan penyelidik dan cerdik pandai di seluruh dunia. Pelbagai jujukan bGH gene telah dihasilkan daripada pelbagai species bovine. Pengekpresen gene tersebut pula telah menghasilkan sejenis protein yang sangat bernilai yang dikenali sebagai hormon pertumbuhan. Hormon tersebut memainkan peranan yang penting di dalam pengawalaturan proses pembezaan, metabolisma dan pertumbuhan bovine. Telah dibuktikan juga hormon tersebut memainkan peranan yang sangat penting di dalam penghasilan susu.

Bagi tujuan pengumpulan maklumat yang lengkap tentang gene tersebut pelbagai jujukan bGH gene telah diselidiki daripada pelbagai species bovine. Walaubagaimanapun jujukan bGH gene daripada seladang Malaysia (*Bos gaurus hubbaccki*) belum pernah diselidiki. Bagi merebut peluang tersebut, penyelia saya dengan kerjasama Pusat Pemuliharaan Seladang Malaysia telah mencadangkan agar penyelidikan ini dijalankan. Tujuan utama penyelidikan ini dijalankan adalah untuk mengklon bGH gene daripada genom seladang tempatan dan seterusya mencirikan gene tersebut. Genom seladang telah berjaya diekstrak daripada testis seladang tersebut dengan mengunakan kaedah phenol chloroform yang telah disyorkan oleh Maniatis dan rakan- rakan (1989). Jujukan bGH gene telah diekstrak daripada genom tersebut dengan mengunakan 'polymerase chain reaction' (PCR). Seterusya jujukan tersebut telah diklonkan di dalam vector pengklonan TA. Plasmid rekombinan yang terhasil daripada proses pengklonan tersebut kemudianya dipindahkan ke dalam bacteria *E.coli* (DH5α). Jujukan bGH yang telah diklon kemudianya dihantar ke First

Base Laboratory Malaysia untuk tujuan penjujukan. Jujukan nucleotide bagi bGH gene yandg diperolehi, seterusnya dianalisis secara terperinci.

Hasil kajian ini mendapati bahawa jujukan bGH gene daripada seladang tempatan telah berjaya diklon. Saiz jujukan tersebut adalah sebanyak 1208 pasangan bes. Ia juga di dapati mempunyai kesamaan yang tinggi dengan jujukan bGH gene daripada species bovine yang lain.

Chapter 1

Introduction

Gaur

Gaur is one of the most impressive wild cattle, with its muscular build and striking eyes. Adult males are in shiny black with cream color leg and rump patch, meanwhile, young males and females are medium to dark brown with the same marking. The structure of the gaur is massive (shoulder height six feet), with a large hump at shoulder, sturdy legs and a narrow dewlap under the chin and between the front legs. Gaur has huge head with a bulging forehead ridge between the horns, which are approximately 30 inches in the length in the males. Male gaurs can weight up to 2,100 pound and females up to 1,600 pound (Plitz *et al.*, 1992). A mature male gaur, female and calves shown in pictures 1.1 and 1.2.

Below is the scientific classification of gaur that has been adapted from Wilson and Reeder (1993);

Kingdom : Animalia

Phylum : Chordata

Class : Mammalia

Order : Artiodactyla

Family : Bovidae

Subfamily : Bovinae

Genus : Bos

Recently, there were thought to be three subspecies of gaur, they are Indian gaur (*Bos gaurus gaurus*), Indochinese gaur (*Bos gaurus readei*) and Malayan gaur (*Bos gaurus*

hubbaccki). Based on the 1995 Asian Wild Cattle Conservation Assessment and Management Plan (CAMP) they have recognized three subspecies of wild gaur. Which are *Bos gaurus laosiensis* (Myanmar to China), *Bos gaurus hubbaccki* (Thailand, Malaysia) and *Bos gaurus gaurus* (India, Nepal) [Plitz *et al.*, 1992].

Gaur are animal of hill country and dominates along the foothill of the Himalayas including the south hills of the Brahmahputra river, the Highland of central India and Western Ghats of the south, they also found at others countries such as Nepal, Burma and Malaysia (Buccholtz., 1990). However, current distribution of gaur includes the countries such as Bangladesh, Bhutan, Cambodia, China, India, Malaysia (Peninsular Malaysia), Nepal, Thailand and Vietnam. Distribution of the gaur in this world has been shown in pictures 1.3.

Gaur are browse and grazer, they eat grass and herbs or shrubs such as *Hibiscuss lampas, Grewier aspera, Grew hirsuta, Helicteres isora, Butea porvifloara.* They also browse on leaves and some twigs of tall shrubs, beside that they also eat young Bamboo and some fruit such as *Aegle marmelos, Cassia fistula, Gmelina alborea and Terminalia bellerica* (Buccholtz., 1990). Basically they are diurnal animal that are active at early morning and late afternoon, and resting during the hottest time (Huffman, 1999). In areas that has been distributed habitat, the gaur has assumed a more nocturnal lifestyle. They usually inhabit the woodland forest at altitude of 5000 to 6000 feet (1500 to 1800 meters) above the sea level (Plitz *et al.*, 1992). Gaur generally drinks once a day and they are also seen as regular visitor to salt lick (Huffman, 1999).

Gaur usually moves in herd that contains six to 20 animals and typically consists of a few old bulls, juvenile and adult bull with calves (Plitz *et al.*, 1992). Each herd is lead by a huge bull, with the finest horns, a sleek, dark brown coat and white stocking (Botman *et*

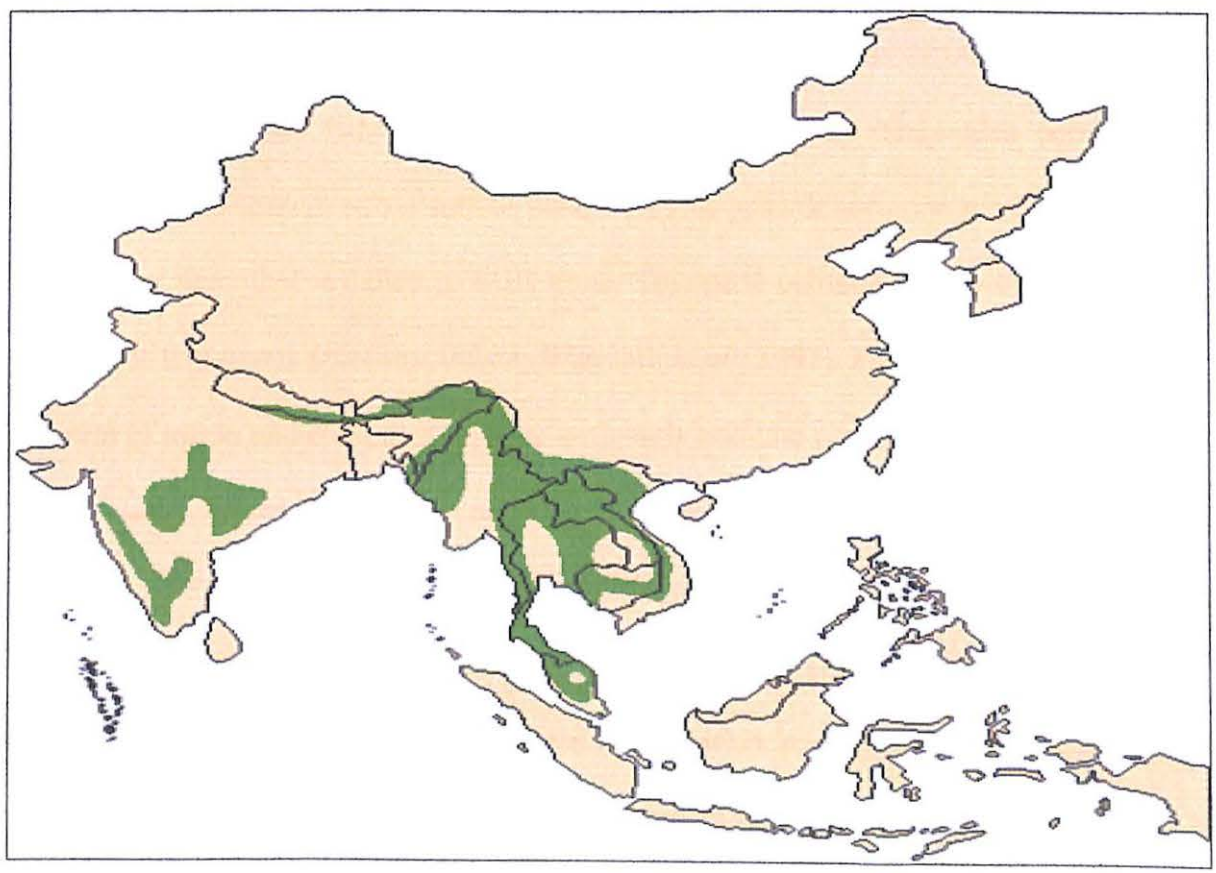
al., 2004). During the breeding season the mature male bull will search for receptive female to breed. Only the dominance bull can freely choose the partner, usually the domination of the male gaur is determined by his strength and superiority among other bulls. Single calves are dropped after a gestation period of nine month in August and September (Buccholtz., 1990, Huffman., 1999).

Malaysian Gaur (*Bos gaurus hubbaccki*)

In Malaysia gaur is widely known as "seladang", it is Malaysia's second largest animal after the elephant (www.kempen.gov.my/coci.html). Currently, the population of seladang is found in large forest areas in the states of Perak, Pahang, Kelantan and Terengganu (www.virtualmalaysia.com/destination/state.html). Seladang is among endangered species, day by day their population decreasing. It is estimated that there are only 350 to 500 individual left in peninsular Malaysia (www.journeymalaysia.com/mr-jenderak.html). Gaur species are in shrinkage by the increase destruction at their normal habitat and increase in poaching. Seladang has been classified under the wildlife protection act 1972 as vulnerable species (www.journeymalaysia.com/mr-jenderak.html).

Picture1.1: A mature male gaur

Picture 1.2: Female gaur and his calve



Picture 1.3: Distribution of the gaur in this world (mark with green color) adapted from Corbet and Hill (2002).

Bovine growth hormone

Bovine growth hormone or know as bovine somatotropin (BST) present naturally in dairies cattle. This hormone is produce by somatotroph cell of the anterior pituitary, that why it is called as bovine somatotropin (Nial *et al.*, 1971). It is a peptide hormone that regulates differentiation, growth and metabolism of the cattle. This hormone will affect the grow rate, body composition, health, milk production and aging processes of cattle. The most important function of the bGH is increasing production of milk (Kalter *et al.*, 1985). This hormone has structurally, evolutionary and functionally related to the chorionic somatotropin and prolactin hormone. When compared with human growth hormone, they are nearly similar, where both hormones contain proteins about 22,000 Dalton and their amino acid is about 191 residues (Woychik *et al.*, 1982). They also contain single polypeptide and contain two disulfide bonds. Bovine growth hormone has been coded by a single copy gene that is called as bGH gene. This gene contains 1.8 kb of nucleotide and consists of five axons and fours intron (Woychik *et al.*, 1982). Figure 1.2, shown schematic diagram of intron and exon in bGH gene with early and late protein translation.

Based to the figure 1.1, it shown the amino acid sequence for bovine growth hormone gene, that figure has been adapted from Wallis *et al.*, 1973. Amino acids sequence of bovine growth hormone consists of four alpha helices packages closed together. The closeness gives the protein a somewhat square appearance. The adjacent alpha helices are antiparallel with somewhat tilted angle between each of the adjacent pair making the four alpha helices look like a left handed twisted handle (Carlacci *et al.*, 1991). The disulfide bridges are formed between cysteine residues with both connecting a loop to a helix. The first disulfide brides connecting the hooking middle of the long loop (located at amino acid number 53) with the 4th helix (located at amino acids 164) which pulls the long

loop onto the surface of the bundle. Meanwhile the second disulfide bridges hooks the small loop of the C-terminal segment (located at amino acid 189) to the 4th helix(located at amino acids 181) so it force the C- terminal segment to bend toward the helix bundle (Havel *et al.*, 1989). Those bridges play important role as structural stability of the bovine growth hormone.

Some interesting heterogeneity has been detected in the sequence of the bGH at amino acid terminus and at residues 127. At the N terminus, two N- terminal residues are detected, in approximately equal amount. Specific analysis of the N- terminal sequence revealed that this was due to the presence of extra alanyl residues on about half of the polypeptides chains (R. E. Fellow *et al.*, 1972). However heterogeneity that occurs at residues 127 are more popular among the researcher, where a lot of papers are have been published. At that residue both leucine and valine are always found during sequence determination in a ratio about 2:1 (Santome *et al.*, 1973). That heterogeneity has been shown to be the result of an allelic polymorphism in the population of cow from which the growth hormones were prepared (Seavey *et al.*, 1971). When growth hormone prepared from bovine posed beef pituitary was examined, it was found that some bovine just have leucine or valine or both at residues 127.

Productions of growth hormone are regulated by growth hormone releasing hormone (GHRH) and growth hormone releasing inhibit hormone (GHRIH) that also produce at anterior pituitary (Lovendhal *et al.*, 1994). The mechanism of the bGH in production of milk still not clear, where no receptor sites have been detected in the mammary tissue (Bauman et al., 1985). The action of bGH in mammals tissue may be indirect, because during the production of these hormone it also enhance production of

somatomedin. This hormone then helps to channel the nutrient through the blood to the cells involve in production of milk (Bauman *et al.*, 1985).

The first studies using bGH derived from recombinant bacteria was led by Dr. Dale E. Bauman of Cornell university in 1982. He found that an increased in milk yield similar to what have been obtained with pituitary or naturally occurring bGH. On other study, Robert J *et al* (1985) found that injecting recombinant bGH into the lactating cows can increase 10-40 % of milk yield. The successful of that study has attractive many companies to commercialize that hormone. As a result recombinant bovine growth hormone (rBGH) has been manufactured by Monsanto Company and has been sold to dairy farmers under the name 'Posilac'. The use of that hormone has been approved by Food and Drugs Assosiation, USA in 1993 and has been in use since 1994 (Baubane, 2000).

However few years later, cow injected with rBGH showed various complications such as udder infection (mastitis), severe reproductive problem, digestive disorder, foot and leg ailment and persistent sores and laceration (Baubane. 2000). On other hand the product that derived from cow injected with rBGH are harmful to humans. Based on the report that made by USA Consumer Union, milk from rBGH injected cow contains substantial higher amount of potent cancer promoter and high level of pus, bacteria and antibiotic (Baubane. 2000). Meanwhile, in Canada and Europe has been reported that consuming rBGH derived product have caused mad cows diseases or bovine spongiform encephalopathy (Baubane. 2000). Due to many complications of rBGH products to human and animal, most of the industrialized country except Unite State has banned the commercialization of rBGH.

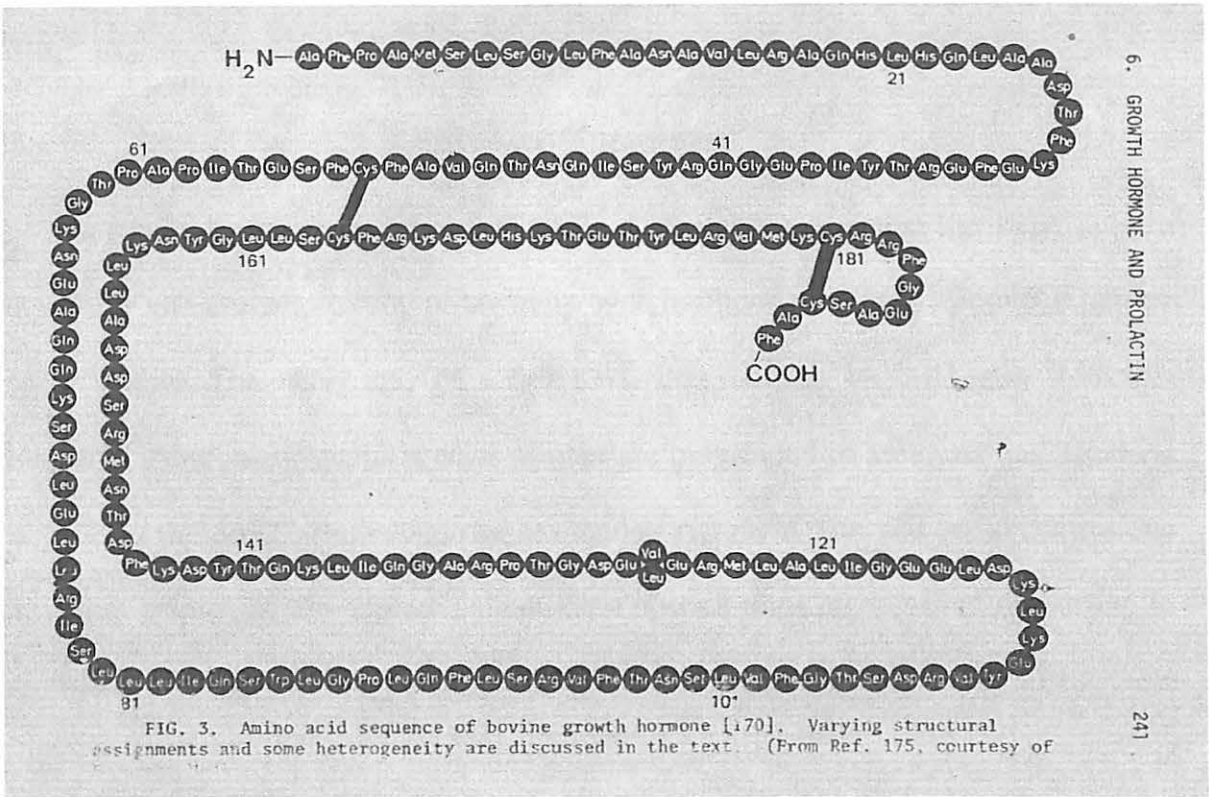


Figure 1.1: An amino acid sequence of bovine growth hormone adapted from Wallis *et al.*, 1973.

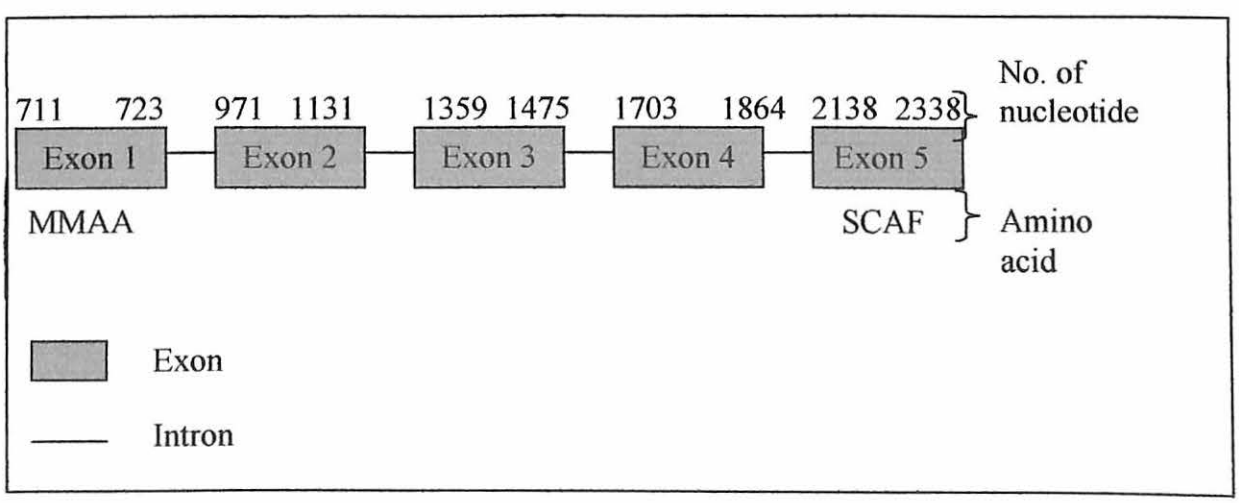


Figure 1.2: Schematic diagram of intron and exon in bGH gene with early and late protein translation

Chapter 2

Review of literature

Survey of literature revealed that, still no available paper that has been publish about cloning and characterization of bovine growth hormone gene(bGH) from *Bos gaurus hubbacki* species. This study may be a first study that focusing on bGH gene from that species. As I mention early, this species of gaur are only found in Malaysia and Thailand forest (Plitz *et al.*, 1992). Both countries has made strict regulation and act to control that species from extinct, so any research about these species must be approved by Senate. In addition lack of money and expertise in that field also caused lack of study about these species.

Lack of paper that related to my study gives some difficulty in searching the journal. Therefore, many papers that have been publish about bGH but very few paper that very close related with my study. However I decided to write this literature review based to the a few paper that I think have some relation with my study.

Wochick *et al.*, (1982) has done a cloning and characterization of a bovine growth hormone gene, in his study, they has cloned and sequence the bGH from genomic DNA. They also made comparison between the sequence of bGH gene with sequence of rat and human growth hormone that already available in gene bank. As a result they found that, the sequence for bovine growth hormone is approximately 1800 bp and containing four intervening sequence. They also found the differentiation of 5'and 3' flanking region and untranslated region of bovine, human and rat growth hormone revealed many areas of highly conserved sequence. Especially noteworthy was the observation that all three genes

had a 38 nucleotide homologous sequence within their 5' flanking region located about 100 bp up stream from transcription initiation sites.

On other study, Miller *et al.* (1980) has cloned DNA complementary to mRNA coding for bGH. Double stranded DNA complementary to bovine pituitary mRNA was inserted into *Pst1* site of plasmid pBR322 by dC-dG tailing technique and amplified in *E.coli*$_x$*1776*. A recombinant plasmid containing bGH cDNA was identified by hybridization to cloned rat growth hormone cDNA. Then, they compared the nucleotides sequence for bovine, rat and human growth pre hormone. They found that nucleotide sequence of bGH mRNA is 83.9% homologous with rat GH mRNA and 76.5% homologous with human GH mRNA. Meanwhile the respective amino sequences homologous are 83.5% and 66.85.

Here, I also made some review about the journal that study about polymorphism that occurs in coding sequence of bovine growth hormone. There are many polymorphisms that happen in that sequence, but the most popular among the scientist are polymorphism that occurs at amino residues 127. Lucy *et al.* (1991) has done study to determine polymorphism that occurs on translation region in bGH gene. They found that, Leu/Val polymorphism occurs at five exon of bGH gene. These polymorphism has been proven to produce two variant of growth hormone that is different via presence either valine or leucine at amino residue 127.

On other hand, Schlee *et al.* (1994) has done study about the production of bGH among German black and white (Holstein) cattles with Lue/Leu, Leu/Val and Val/Val genotypes. They found that German black and White (Holstein) cattle with Leu/Leu genotypes produce more growth hormone than cattle with Leu/Val and Val/Val genotypes. In other study that made by Grochwoska *et al.* (2001) they found that Polish Friesian calves with Leu/Leu genotypes released less GH that Leu/Val and Val/Val genotypes, so the result

is contrast with Schlee study. Such differences may come from two reasons, firstly calves in both study are induce with different stimulant. Schlee *et al.* (1940) has induced the calves with growth hormone releasing hormone (GHRH) meanwhile, Grochwoska *et al.* (2001) used thyrotropin releasing hormone. Second reason, may be caused by the presence a link gene effect between the GH genotypes and genes affecting GH release, whereby there was a positive association within one breed but a negative effect within another.

Chapter 3

Objective

1. To isolated genomic DNA of local gaur using phenol based conventional method.
2. To optimize the PCR parameter for bGH gene.
3. To amplify enzymatically the bGH gene from genomic DNA of local gaur
4. To clone and characterize genomic bGH gene from local gaur

Chapter 4

Material and Methods

4.1 Material and reagent preparation

Tissue collection

To clone bovine growth hormone gene, the sample of DNA is isolated from *Bos Gaurus hubbacki* testis, which was getting from Seladang Conservation Center (Wildlife Department), Jenderak Selatan, Jerantut, Pahang Darul Makmur. The tissue was immediately frozen in liquid nitrogen and stored at – 80°C until extraction of DNA was performed.

Bacterial stocks

The strain of *Escherichia coli (E. coli)* were employed in this study was *DH5α*, which obtained from Dr. Shaharum stock culture.

Reagent and Kit

Reagent and kit used in this study are listed below.

TOPO TA cloning Kits (Invitrogen, USA), QIAquick® PCR Purification Kits (Qiagen, USA), QIAprep® Spin Miniprep Kit (Qiagen, USA), Lambda Hind III- DNA molecular weight marker II (Fermentas , USA), 100bp DNA molecular weight marker (Fermentas , USA), 1Kb DNA molecular weight marker (Fermentas , USA), 10X Loading Buffer (Fermentas , USA), 6X Loading Dye (Fermentas , USA), EcoR1 and Hind III restriction endonuclease (Fermentas , USA).

Solution, Buffer and Media

Double distilled water (ddH_2O), Bacto Tryptone (Promega,USA), Yeast extract (Promega, USA), Bacteriological agar (Sigma, USA), Glycerol, Sodium Hydroxide (E. Merck, Germany), Ethanol (E. Merck, Germany), Ethylene Diamine Tetra Acetic Acid[EDTA] (Sigma, USA), Potassium Chloride (Becton Dickson, UK), Sodium Chloride (E. Merck, Germany), Tris Base (Promega,USA), Tris-Cl (Promega, USA), Etdium Bromide (Sigma,USA), Agarose Powder (Promega, USA), Glacial Acetic Acid (Analar® BDH, UK), Methanol(E. Merck, Germany), Proteinase K, DNAse- Free RNAse, Isophropanol, Glycine, Mercapthoethanol, Acrylamide (Promega, USA),

Equipment

Bunsen Burner, Syringe 50 ml (Becton Dickson Labware, USA), 0.2 micron membrane filter (Becton Dickson Labware, USA), Beaker (50ml,100ml,500ml, 1000ml), Schott Bottles (250ml,500ml,1000ml), Bacteriological plate,Pipettes (5000µl,1000µl,200µl,100µl,10µl, 2µl), Pipettes tips, 50ml falcon tubes (Becton Dickson Labware, USA), 15ml falcon tubes (Becton Dickson Labware, USA), Waterbath (HETOMIX-TBVS, Scandinavia), Agarose gel apparatus (C.B.S. Scientific Co, California), Electrophoresis power supply (Amarshan Pharmacia Biotech, USA), UV transluminator (Spectrolinee, Model TC-321A,USA), Digital Image Analyzer (Amarshan Pharmacia Biotech, USA), Thermocycler, Mini centrifuge (National Labnet Co, Woodbridge), Perkin Elmer GeneAmp® PCR System 2400 (Applied Biosystem, USA), 20°C refrigerator, -80°C refrigerator, Vortex L24 (Labincobv, Nertherland).

Reagent Preparation

1. 70% Ethanol

   Prepared by dissolving 70 ml ethanol in 30 ml deionised water

2. Luria Bertani Broth and Agar

   LB agars were prepared by dissolving10g NaCl, 10g Bacto Tryptone and 5g yeast extract in 750ml of deionised water. Adjust the pH to 7.5 with NaOH and top up with deionised water to 1000 ml or 1 liter. For LB broth, dissolving 15 g of bacteriological agar per liter of deionised water. Both broth and agar were autoclaved to sterile it and stored at 4°C.

3. Glycerol

   80% glycerol was obtained from the stock glycerol available.

4. 10X TAE

   Prepared by dissolving 48.4g Tris Base, 11.42ml Acetic Acid Glacial and 20 ml 0.5 M EDTA in 1000ml of deionised water.

5. TEN 9

   Prepared by dissolving 2 ml 0.05 M Tris-Cl, 8ml 0.1 M EDTA and 8ml 0.2 M NaCl in 40ml deionised water.

6. 5M NaOH

   Prepared by dissolving 40g of NaOH tablet in 200 ml deionised water.

7. 0.5M EDTA

   Prepared by dissolving 37g EDTA in 100 ml deionised water.

8. 1M $CaCl_2$

   Prepared by dissolving 36.75g of $CaCl_2$ in 200 ml deionised water.

9. 1M Tris-Cl

Prepared by dissolving 15.76g of Tris-Cl in 100 ml deionised water.

10. 1M Tris Base

Prepared by dissolving 12.11g of Tris Base in 100 ml deionised water.

11. Ampicilin

Prepared stock solution of ampicilin by dissolving 500mg sodium salt of ampicilin in 10ml ddH_2O. The resulting ampicilin has concentration of 50mg/ml. Filter the sterile solution of stock ampicilin by using 0.2 micron membrane filter. Ampicilin plate was prepared at a final concentration of 100ug/ml that is 1ul per ml of agar. Allow autoclave media to cool to 48°C before adding ampicilin to a final concentration 100 ug/ml. Ampicilin plate were stored up to 2 weeks at 4°C.

12. Kanamycin

Prepared of 25 mg/ml from stock solution (50 mg/ml) by dissolving 20ml stock solution in 80 ml distilled water.

Preparation of Competent cell and Transformation

1. Transformation buffer

Made up of 50mM of $CaCl_2$ and 10mM Tris-Cl, pH 8.0. Transformation buffer were prepared by dissolving 25ml of 1M $CaCl_2$ and 5 ml of 1M Tris-Cl in 450ml deionised water. Adjust the pH with NaOH and top up to 500 ml.

Preparation of electrophoresis reagent

1. 10X Tris Acetate buffer (TAE)

Prepared by dissolving 48.4 g Tris Base, 28.5 ml glacial acetic acid and 20 ml 0.5M EDTA, pH 8.0 in 1000 ml deionised water. The solution was autoclaved at 121^{0}C for

about 15 minutes and stored at room temperatures. 1X TAE was prepared from stock solution (10X TAE) buffer by dissolving 100ml stock solution in 900 ml distilled water.

2. DNA loading buffer for agarose gel electrophoresis

   Prepared by mixing 20 g sucrose, 0.12 g orange G and 50 ml deoionised water.

3. Ethidium Bromide (EtBr)

   10 mg/ml EtBr were prepared by dissolving 0.1 g EtBr powder in 10 ml deionised water. The solution was kept in dark bottle which wrapped with aluminium foil and stored at room temperature.

4. Preparation of 1% agarose gel

   1% gel was prepared by dissolving 1.0 g agarose in 100 ml TAE or dissolving 1.4 g agarose in 140 ml TAE buffer.

4.2 Experimental Procedure