Application of molecular markers and genomics for diversity, growth and meat quality in Malaysian cattle

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by

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Declaration

I hereby declare that no part of this thesis has been previously submitted to this or any other University as part of the requirements for a higher degree. The content of this thesis is the result of my own work unless otherwise acknowledged in the text or by reference.

The work was conducted in the Department of Biology, University of Leicester during the period January 2003 to January 2006.

Signed..................................................

Saadiah Jamli, December 2006
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By

Saadiah Jamli

Abstract

Molecular markers can be used for identification and selection of animals through genotype. To improve beef production and meat quality, investigations are carried out on genes involved in animal growth and related to meat quality. The overall aims were to investigate genetic variation underlying economically important traits in the Malaysian cattle (*Bos taurus* subspecies *indicus*) breed Brakmas. Awareness of the value of genetic resources has stimulated the study of the genetic diversity of Brakmas cattle. Specifically, the aims are to measure diversity within Brakmas cattle using microsatellite markers, and to study genes regarding growth and meat quality. The genes investigated were growth hormone (*GH*), growth hormone receptor (*GHR*), insulin-like growth factor-I gene (*IGFI*), pituitary-specific transcription factor 1 (*PIT1* or *POU1F1*), calpain, leptin, thyroglobulin (*TG*), diacylglycerol acyltransferase (*DGATI*). Molecular techniques were carried out such as PCR amplification, cloning and sequencing, PCR-RFLP and some fluorescent *in situ* hybridization.

Brakmas display a high microsatellite polymorphism but within the range of less intensively bred cattle. Brakmas do not display evidence for a genetic bottleneck, a decrease in genetic diversity, or loss of alleles, and this indicates that the Brakmas herd is rich reservoir of genetic diversity. This is justification for preservation of the breed because of the productivity and adaptation to tropical climates. Sequence polymorphisms were identified in growth-related genes in the Brakmas population, giving potential markers for growth rate and meat quality. Verification of linkage of production traits with the molecular markers has to be established in Brakmas.

Knowledge of polymorphisms in livestock is important for the manipulation of meat production. Understanding the function of these genes will lead to the development of new genetic markers. It is hoped that knowledge of polymorphism will provide the basis for future selection to enhance beef production performance and for conservation purposes.
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This thesis is dedicated to MARDI. It is also dedicated to my husband, parents, parent in law and sisters for their constant support and encouragement that made this possible. Least but not less, it is for my sons, Irfan and Faris who are too young to understand but gave me the strength to continue throughout my studies.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DGAT1</td>
<td>diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tags</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GHR</td>
<td>growth hormone receptor</td>
</tr>
<tr>
<td>IGF1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base</td>
</tr>
<tr>
<td>MAS</td>
<td>marker assisted selection</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>polymerase chain reaction-restriction fragment length polymorphism</td>
</tr>
<tr>
<td>PIC</td>
<td>polymorphic information content</td>
</tr>
<tr>
<td>PIT1</td>
<td>pituitary-specific transcription factor 1</td>
</tr>
<tr>
<td>QTL</td>
<td>quantitative trait loci</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>SNPs</td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SSRs</td>
<td>simple sequence repeats</td>
</tr>
<tr>
<td>TG</td>
<td>thyroglobulin</td>
</tr>
</tbody>
</table>
TABLE OF CONTENT

Declaration ................................................................. ii
Abstract ................................................................. iii
Table of content ........................................................ iv

Chapter 1: Introduction

1.1 Overview of cattle and breeds......................................... 1
1.2 Overview of current breeding technologies ......................... 4
1.3 Overview of production challenges ................................... 6
1.4 Strategies to improve production ....................................... 7
1.4.1 Selection and improved growth characters ..................... 7
1.4.2 Bovine Genome Project ............................................. 9
1.4.3 Quantitative Trait Loci ............................................... 10
1.4.4 Marker Assisted Selection (MAS) ................................. 11
1.5 Application of molecular markers ..................................... 13
1.5.1 Microsatellites ....................................................... 13
1.5.1.1 Definition and function ............................................ 13
1.5.1.2 Advantages of microsatellites ................................ 15
1.5.2 Single Nucleotide Polymorphisms ............................... 16
1.5.3 Polymerase chain reaction-restriction fragment length polymorphism .............................................. 17
1.5.4 Other molecular markers ............................................ 17
1.5.4.1 Amplified fragment length polymorphism ................. 17
1.5.4.2 Randomly amplified polymorphic DNA .................... 18
1.5.4.3 Restriction fragment length polymorphism ............... 18
1.5.4.4 Denaturing gradient gel electrophoresis .................... 19
1.5.4.5 Single strand conformation polymorphism ............... 19
1.5.5 Rationale use different of markers ............................... 20
1.5.6 Cytogenetic markers ................................................ 20
1.6 Economically important traits ........................................ 24
1.6.1 Growth .................................................................. 24
1.6.1.1 Growth Hormone .................................................. 25
1.6.1.2 Growth hormone receptor ..................................... 27
1.6.1.3 Insulin like Growth Factor-1 .................................. 29
1.6.1.4 Pituitary-specific Transcription Factor 1 ................. 30
1.6.2 Meat quality ........................................................ 32
1.6.2.1 Tenderness .......................................................... 33
1.6.2.1.1 Calpain ............................................................. 35
1.6.2.2 Marbling .............................................................. 37
1.6.2.2.1 Leptin ............................................................... 37
1.6.2.2.2 Thyroglobulin .................................................... 39
1.6.2.2.3 Diacylglycerol O-acyltransferase ....................... 40
1.6.3 Others economical traits .......................................... 41
1.7 Malaysian cattle scenario .............................................. 42
1.7.1 Development of Brakmas ........................................... 43
1.8 Aims ....................................................................... 43

vi
Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Materials</td>
<td>46</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Blood samples</td>
<td>46</td>
</tr>
<tr>
<td>2.2</td>
<td>Methods</td>
<td>46</td>
</tr>
<tr>
<td>2.2.1</td>
<td>DNA</td>
<td>46</td>
</tr>
<tr>
<td>2.2.1.1</td>
<td>DNA extraction</td>
<td>46</td>
</tr>
<tr>
<td>2.2.1.2</td>
<td>Estimation of DNA concentration</td>
<td>46</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Primers</td>
<td>47</td>
</tr>
<tr>
<td>2.2.2.1</td>
<td>Primers from literature</td>
<td>47</td>
</tr>
<tr>
<td>2.2.2.2</td>
<td>Primer design</td>
<td>48</td>
</tr>
<tr>
<td>2.2.3</td>
<td>PCR</td>
<td>48</td>
</tr>
<tr>
<td>2.2.3.1</td>
<td>Standard PCR</td>
<td>48</td>
</tr>
<tr>
<td>2.2.3.2</td>
<td>PCR optimization</td>
<td>49</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Agarose gel electrophoresis</td>
<td>50</td>
</tr>
<tr>
<td>2.2.5</td>
<td>Cloning</td>
<td>51</td>
</tr>
<tr>
<td>2.2.5.1</td>
<td>Preparation of denaturing polyacrylamide gel electrophoresis</td>
<td>50</td>
</tr>
<tr>
<td>2.2.5.2</td>
<td>Silver staining of the polyacrylamide gels</td>
<td>51</td>
</tr>
<tr>
<td>2.2.6</td>
<td>PCR-RFLP</td>
<td>55</td>
</tr>
<tr>
<td>2.2.7</td>
<td>In situ Hybridization</td>
<td>56</td>
</tr>
<tr>
<td>2.2.8.1</td>
<td>Blood collection</td>
<td>56</td>
</tr>
<tr>
<td>2.2.8.2</td>
<td>Preparation of white blood cell culture</td>
<td>56</td>
</tr>
<tr>
<td>2.2.8.3</td>
<td>Blood harvesting</td>
<td>57</td>
</tr>
<tr>
<td>2.2.8.4</td>
<td>Preparation of chromosome spreads</td>
<td>57</td>
</tr>
<tr>
<td>2.2.8.5</td>
<td>Labelling of probe</td>
<td>57</td>
</tr>
<tr>
<td>2.2.8.6</td>
<td>In situ hybridization</td>
<td>59</td>
</tr>
</tbody>
</table>

CHAPTER 3: Analysis of genetic variation in Brakmas cattle

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction and aims</td>
<td>61</td>
</tr>
<tr>
<td>3.2</td>
<td>Materials and methods</td>
<td>62</td>
</tr>
<tr>
<td>3.2.1</td>
<td>DNA samples</td>
<td>62</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Sample size</td>
<td>62</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Microsatellite primers</td>
<td>62</td>
</tr>
<tr>
<td>3.2.4</td>
<td>PCR of microsatellites</td>
<td>63</td>
</tr>
<tr>
<td>3.2.5</td>
<td>Agarose gel electrophoresis</td>
<td>63</td>
</tr>
<tr>
<td>3.2.6</td>
<td>Denaturing polyacrylamide gel electrophoresis of microsatellites products</td>
<td>63</td>
</tr>
<tr>
<td>3.2.7</td>
<td>Scoring and genotype schematics</td>
<td>63</td>
</tr>
<tr>
<td>3.2.8</td>
<td>Microsatellite analysis</td>
<td>68</td>
</tr>
<tr>
<td>3.2.8.1</td>
<td>Allele number</td>
<td>68</td>
</tr>
<tr>
<td>3.2.8.2</td>
<td>Allele frequency</td>
<td>68</td>
</tr>
</tbody>
</table>
Chapter 4: Structural analysis of genes associated with growth

4.1 Introduction and aims ................................................................. 88
4.2 Materials and Methods ................................................................. 89
4.2.1 Primer design and PCR amplification ....................................... 89
4.2.2 Cloning, sequencing and sequence analysis ................................. 89
4.2.3 PCR-RFLP ............................................................................... 89
4.3 Results ...................................................................................... 89
4.3.1 Growth hormone gene ............................................................. 89
4.3.1.1 PCR amplification of primers and cloning of GH gene .......................... 91
4.3.1.2 Sequence analysis ................................................................. 91
4.3.1.2.1 Fragment GH1 ............................................................... 91
4.3.1.2.2 Fragment GH2 ............................................................... 94
4.3.1.2.3 Fragment GH3 ............................................................... 97
4.3.1.2.4 Fragment GH4 ............................................................... 99
4.3.2 Growth hormone promoter gene .............................................. 103
4.3.2.1 PCR amplification of primers and cloning of GHR gene ................. 103
4.3.2.2 Sequence analysis ................................................................. 104
4.3.3 Insulin-like Growth Factor 1 gene .............................................. 106
4.3.3.1 PCR amplification of primers and cloning of IGF1 gene .................. 106
4.3.3.2 Sequence analysis ................................................................. 106
4.3.3.3 In situ hybridization analysis of IGF1-1 clone .............................. 111
4.3.4 Pituitary-specific Transcription factor gene ......................... 113
4.3.4.1 PCR amplification of primers and cloning of PIT1 gene 113
4.3.4.2 Sequence analysis ...................................................... 113
4.3.4.3 Identification of RFLP site and PCR-RFLP ..................... 117
4.4 Discussion ........................................................................ 117
4.4.1 Polymorphisms in growth hormone gene .......................... 118
4.4.2 Polymorphisms in growth hormone receptor gene .......... 119
4.4.3 Polymorphisms in insulin-like growth factor 1 gene ........ 120
4.4.4 Polymorphisms in pituitary-specific transcription factor 1 gene .................................................................. 121
4.5 Implications ...................................................................... 122

Chapter 5: Structural analysis of genes associated with meat quality

5.1 Introduction and aims ...................................................... 124
5.2 Materials and Methods ................................................ 125
5.2.1 Primer design and PCR amplification ......................... 125
5.2.2 Cloning, sequencing and sequence analysis ................ 125
5.2.3 PCR-RFLP ................................................................ 126
5.3 Results .......................................................................... 126
5.3.1 Calpain gene ............................................................... 126
5.3.1.1 Fragment CAE13114 ........................................ 126
5.3.1.1.1 PCR amplification of primers and cloning of CAE13114 fragment of calpain gene ..................... 126
5.3.1.1.2 Sequence analysis ........................................ 128
5.3.1.1.3 Identification and analysis of PCR-RFLP site 133
5.3.1.2 Fragment CAI7E10 ................................................. 133
5.3.1.2.1 PCR amplification of primers and cloning of CAI7E10 fragment of calpain gene .............. 133
5.3.1.2.2 Sequence analysis ........................................ 133
5.3.1.2.3 Identification and analysis of PCR-RFLP site 140
5.3.1.3 Fragment CAE14115 ............................................. 140
5.3.1.3.1 PCR amplification of primers and cloning of CAE14115 fragment of calpain gene ........ 140
5.3.1.3.2 Sequence analysis ....................................... 141
5.3.1.3.3 Identification of RFLP site and PCR-RFLP ...... 145
5.3.2 Leptin gene ................................................................. 146
5.3.2.1 PCR amplification of primers and cloning of exon 2 leptin gene .................................................. 146
5.3.2.2 Sequence analysis .............................................. 146
5.3.2.3 Identification and analysis of PCR-RFLP site ...... 149
5.3.3 Thyroglobulin gene ..................................................... 150
5.3.3.1 PCR amplification of primers and cloning of thyroglobulin gene ............................................. 150
5.3.3.2 Sequence analysis .............................................. 151
5.3.3.3 Identification of RFLP site and PCR-RFLP ....... 151
5.3.4 Diacylglycerol O-transferase ....................................... 155
5.3.4.1 PCR amplification of primers and cloning of
Chapter 6: General discussion: DNA markers and their application in Malaysian Brakmas Cattle

6.1. Diversity of Brakmas cattle
   6.1.1. Importance to measure and conserve genetic diversity
   6.1.2. Diversity of microsatellites
   6.1.3. Diversity in and around coding regions
   6.1.4. Molecular assessment of diversity in Brakmas

6.2. Functional Diversity within genes in Brakmas
   6.2.1. Previously associated SNPs and QTLS
   6.2.2. Novel SNPs – are they anonymous or QTLs?
   6.2.3. Microsatellites for genetic/marker assisted selection
   6.2.4. SNPs in genes: are they useful in Brakmas selection and breeding?

6.3. Nature and origin of diversity in Brakmas
   6.3.1. Microsatellite diversity
   6.3.2. SNP diversity
   6.3.3. Chromosomal variation and possible future analyses

6.4. Conclusion
   6.4.1. SNPs and possible future analyses
   6.4.2. Microsatellites and possible future analyses
   6.4.3. Chromosomal variation and possible future analyses
   6.4.4. Genome project and possible future analyses

Bibliography
Chapter 1: Introduction

1.1 Overview of cattle and breeds

Meat production, quality assurance and food safety are major issues in the beef industry. In fact, meat production, as indicated by animal growth rate and feed conversion, is the main criterion for the cost efficiency and sustainability of the industry, apart from meat quality that is very important from both value to the farmer and the consumer point of view. Various strategies have been implemented to improve production and quality including breeding and selection, feeding strategy, feeding supplementation, antibiotic growth promoters and hormone growth promoters. As for breeding and selection, traditional breeding is based on selection of phenotype and physical characteristics. In early days, breeding has been focussed on multiple uses and functions of cattle including for production of milk, meat, hides and as a draught animal (Lenstra and Bradley, 1999). Until now, more than 800 different cattle breeds have been identified (Lenstra and Bradley, 1999). However, the definition for breed is not specific due to different interpretations (Buchanan and Dolezal, 1999). Sometimes, to differentiate a breed to its crossbred hybrid, the new genotypes are referred to as ‘synthetic breeds’. Synthetic breeds are using the existing indigenous genetic material and adapted genotypes in order to improve new genetic combinations and expanding diversity (Blackburn et al. 1998). However, generally the term ‘breed’ is accepted following Buchanan and Dolezal (1999) from Lush (1994) that quotes from Lloyd-Jones (1915) as below:

A breed is a group of domestic animals, termed such by common consent of the breeders, a term which arose among breeders of livestock, created one might say for their own use, and no one is warranted in assigning to this word a scientific definition and in calling the breeders wrong when they deviated from the formulated definition. It is their word and the breeders’ common usage is what we must accept as the correct definition.

Cattle are classified in the genus *Bos* (Linnaeus, 1758) and the species *Bos taurus* (Linnaeus, 1758). Here, the nomenclature recommended by the
Integrated Taxonomic Information System is adopted (www.itis.gov). Two cattle species were originally recognized by Linnaeus as the species *Bos taurus* (known as domestic or sometimes taurine cattle) and *Bos indicus* L. (1758) (Zebu, humped, or Brahma cattle) and the additional, now extinct, species, often regarded as the wild relative of cattle, *Bos primigenius* Bojanus (1827) (known as wild aurochs or urus). The three species are now placed within the single species *Bos taurus* Linnaeus (1758). The indicus type, sometimes recognized as a subspecies *Bos taurus indicus*, is a humped group whereas *Bos taurus taurus* is humpless. While there are very distinctive genetic differences between the taurine and zebu types (MacHugh *et al.* 1997), it is now clear that these differences are not at the species level, and indeed many breeds have intermediate morphology due to mixed origin and interbreeding (Lenstra and Bradley, 1999). However, the informal names of *Bos taurus* and *Bos indicus* are convenient and will be used to denote the predominantly taurine and zebu types here. Apart of distinct morphology features, *Bos taurus* and *Bos indicus* vary greatly in performance. *Bos taurus* breeds have superior production and global distribution, but *Bos indicus* breeds are better in surviving in dry and warm climates in the tropics (Lenstra and Bradley, 1999) and show many diseases resistances such as towards tsetse transmitted trypanosomiasis (Teale, 1999).

*Bos taurus* and *Bos indicus* also have been shown to be greatly different based on analysis of microsatellite diversity (MacHugh *et al.* 1997; Loftus *et al.* 1999) and mitochondrial DNA (Loftus *et al.* 1994). It was revealed that zebu and taurine types diverged 10,000 years ago (Loftus *et al.* 1994). Mitochondrial DNA also had been used to study admixture of ancestral types (Miretti *et al.* 2002; Carvajal-Carmona *et al.* 2003; Miretti *et al.* 2004; Tsuji *et al.* 2004; Henkes *et al.* 2005). Variation and evolution of various cattle breeds in different countries has been studied by various groups using mitochondrial DNA (Loftus *et al.* 1994; Bradley *et al.* 1996; Mannen *et al.* 1998a; Yu *et al.* 1999) and microsatellite analysis (Moazami-Goudarzi *et al.* 1997; MacHugh *et al.* 1998, Peelman *et al.* 1998; Martin-Burriel 1999; Kim *et al.* 2002; Grzybowski and Prusak, 2004a). Variation in both nuclear and mitochondrial DNA also has been associated to carcass traits (Mannen *et al.* 1998b).

Currently, the dominant breeds in most countries are a combination of various breeds that may originate from different countries. Distinctive cattle
breeds have originated from Continental Europe, the British Isles, Asia, North America, Africa, Australia and Oceania (Buchanan and Dolezal, 1999), but major breeds now show a global distribution and their performance has been tested widely. Major breeds from Europe include Belgian Blue, Charolais, Limousin, Danish Red, Holstein-Friesian, Maine-Anjou, Piemontese (UK breed society name; US society is Piedmontese), Polish Red and Simmental. Breeds from the British Isles include Angus, Ayshire, Devon, Dexter, Belted Galloway, Guernsey, Hereford, Jersey, Guernsey, Red Poll, and Shorthorn, and rare or less well recognized breeds such as British Friesien. Breeds from Asia include Bengali, Chinese Yellow, Nellore, Ongole, Sahiwal and Wagyu whereas breeds from Africa are Africander, Boran, Brown Atlas, Butana, N’Dama and Tuli (Buchanan and Dolezal, 1999).

Each breed has its own special features that make identification of one superior breed is impossible. Individual cattle belonging to the different breeds are normally recognized by national or international herd-book Societies which define breed characters and register breeding cattle; some of these have histories dating back 200 years and most now have their own websites. Although animal production (growth or milk) is now usually the main priority, the Bos taurus is dominant world-wide although Bos indicus cattle types offer tough animals that are suitable in harsh conditions. Bos indicus usually have small frame size and lower weight about 200 kg to 400 kg (Bradley and Cunningham, 1999); although some European Bos taurus breeds such as Dexter may be a similar size (http://www.dextercattle.org/index.htm), most are 600kg to 1500kg in size. Compared to Bos indicus, Bos taurus breeds are superior in growth rate and body weight (Bradley and Cunningham, 1999). Examples of superior breed of Bos taurus include Holstein-Friesian, Aberdeen Angus, Belgian Blue, Charolais and an example of Bos indicus is Brahman.

Aberdeen Angus originated in Aberdeenshire, Scotland and is now spread world-wide including America, Canada, Australia and New Zealand. Aberdeen Angus breed cows have average weight of 650 kg whereas the bulls are on average 1000 kg. Originally they had various colours but later on, during the selection programme only black were selected. The most important characteristics of this breed are tasty marbled beef (http://www.cattlenetwork.net/Breeds/Angus.htm). Belgian Blue which is
famous for its double muscling characteristics is originally from Belgium. This breed have blue-roan pied colour. The cow weight is between 700 – 800 kg and the bull’s weight is between 1150 - 1400 kg (http://www.cattlenetwork.net/Breeds/Belgian_Blue.htm). Charolais is originally from France. Morphologically, it is bigger and longer bodied than other breeds, with a white coat with a cream tinge and have pink muzzle. The cows weight is 750 – 1200 kg whereas the bulls are 1200 – 1650 kg (http://www.cattlenetwork.net/Breeds/Charolais.htm)

Brahman is a relatively large breed of *Bos indicus* type and originated in India but is now spread world-wide, especially within hot climate environments. (The breed name is different from ‘Brahma’, a word used as a synonym for the zebu type of cattle previously recognized as *Bos indicus*.) In the USA, Brahman cows weigh at 450 - 600 kg and bulls weigh at 700 – 1000 kg. The colour of Brahman breed is variable: typically light to medium grey but may vary from light grey or red to black. The bulls usually have dark areas on the neck, shoulders and lower thighs. Brahman is unusually thrifty, hardy and adaptable to a wide range of feed and climate. The heat tolerance characteristics in Brahman may be due to several factors such as hair coat, skin pigmentation, loose skin, sweating ability and internal body heat (http://www.ansi.okstate.edu/breeds/cattle/).

In Malaysia, one local indigenous breed is called Kedah-Kelantan (KK). This *bos indicus* is small in size, brown in colour, low in growth rate but highly resistant to local parasitic infection and diseases. Females typically weigh 230 kg whereas male weight 300 kg (see section 1.7 for Malaysian cattle breeds).

### 1.2 Overview of current breeding technologies

Current issues in the beef cattle industry include how to improve meat production. It is a complex combination of multiple factors which includes breed or crossbred hybrids, age, sex, nutritional composition, climate and environmental factors. A study on composition of beef carcasses in UK (Lawrence and Fowler, 2002) can be summarised as follows. In lean cattle, the composition of lean meat, total fat and bone is 66%, 16% and 18%, respectively. In average size cattle, the composition of lean meat, total fat and bone is 59%,
25% and 16%, respectively, whereas in fat cattle the proportion is 50%, 37% and 13%, respectively.

Current breeding is rapidly improved with application of new technology such as in reproductive technologies and molecular technologies. Reproductive technologies include application of artificial insemination, multiple ovulation and embryo transfer, *in vitro* production of embryos, semen and embryo sexing and embryo cloning.

Success in reproductive technologies has involved contributions from various methods. Artificial insemination is the major breeding method used in dairy cattle (Buchenauer, 1999). It has been applied by cattle breeders for over 50 years in order to improve reproductive rate of males (Simm, 1998). An important advantage is that comprehensive genetic progeny testing can be done. Artificial insemination is done by harvesting the semen from elite bulls, diluting it in media including a cryoprotectant, and freezing it before artificial insemination is carried out (Buchenauer, 1999). Artificial insemination is relatively cheap and simple to carry out and is used widely successfully in many countries in the world for commercial herds (Simm, 1998).

Multiple ovulation and embryo transfer (MOET) is a technology that harvests and conserves genetic material from female animals, also by freezing. Due to prediction of potential impact on improvement of beef and dairy cattle up to twofold compared to conventional breeding in mid-1970s, lots of commercial dairy cattle in several countries were starting to apply this technology. However, in mid-1980s was shown that optimistic success rates for MOET were difficult to achieve in field practice (Simm, 1998).

*In vitro* production of embryos is another technology that had been used to improve cattle production. This technique is an invasive technique that requires skilled veterinary input (Simm, 1998). In early development of this technique, the main source of eggs was from ovaries of slaughtered beef heifers fertilised with high merit proven bulls but this resulted in calving difficulties due to large calves at birth (Simm, 1998). However with proper selection of donor and recipient, this technique can maximize the number of offspring from valuable cows as well as improving quality and uniformity of cattle production (First *et al.* 1999; Simm, 1998).
Semen and embryo sexing is favourable in a cattle industry with sex-specific related production (First et al; 1999; Simm, 1998). This includes heifer replacement herds, bull replacement herds, dairy herds and meat production. In the case of meat production, there is tendency to use male cattle because it produces leaner and more meat (Simm, 1998). This technique enables the industry to reduce the cost of production although the limited reliability and costs mean that the methods are only starting to be widely commercialized (e.g. by the US company XY Inc.).

Cloning has a high potential for genetic improvement as it can be used to produce uniform quality animals from the same genotype. Application of conventional methods to produce cattle with favourable traits is difficult due to recombination of unfavourable traits compared to cloned animals. However a reliable and cost-efficient method is required for this new technology, although early prospects include clonal propagation of animals with unique characteristics and transgenic individuals for pharmaceutical production.

Simm (1998) highlighted the current and potential future value of reproductive technologies for both value in genetic improvement programmes and value in dissemination of improvement to commercial tier. For the former, the value is highest in AI technology, followed by MOET and IVF and less in sexing and cloning. However, in value of dissemination of improvement to commercial tier, AI and cloning was identified to have highest value. This is followed by IVF and sexing but MOET is identified as of no or having little value except for very high value animals, and perhaps in genetic resource conservation programmes. Currently, AI is most used technique.

1.3 Overview of production challenges

The cattle production industry faces many challenges which include pressure to increase production, to reduce production cost and to increase the quality and safety of the products. Furthermore, another bigger challenge is to satisfy consumers. Some of the major traits of interest of the consumer include tenderness, flavour, consistency, food safety, nutritional value, palatability, value and appearance of the product. Therefore, farmers need to improve productivity, efficiency and product quality in order to remain competitive.
Maintaining animal genetic diversity in intensive production is a tremendous challenge (Blackburn et al. 1998). This is because there is need to conserved genetic material but at the same time to suit the need for cattle industry which depends more on product consistency and uniformity.

Another challenge is to improve growth and productivity. Crossbreeding between breeds may produce favourable increases in performance in cattle but at the same time may loose some important characteristics. For example, crossbreeding between temperate breeds with tropical breeds may increase growth rate but may cause reduction in heat tolerance and increase disease susceptibility. Another problem such as dystocia also may occur as a result of birth of bigger calves.

Climate is another challenge for the hot and humid countries. Physiologically adapted animals will reduce their feed intake in order to reduce heat produced from the body. This may results in reduced growth rate. Another challenge is in terms of feeding and nutritional value. One of the challenges involves cost of feed ingredients. And the scenario is even worse in countries which depend on import of feed ingredients. High energy content feeds sometimes do not necessarily solve the insufficient feeding and low nutritional values especially in hot climates. Feeding the animals with this type of nutritious feed may cause excessive panting to reduce heat produced by the body. Ordinary grass sometimes is not nutritionally sufficient for optimum growth, so there is a need to grow nutritious grass needing fertilizer and other maintenance, sometimes with supplements to reach a growth maximum, further increasing costs. Grass obviously has a cost, but is usually lower quality land, not easy to till, irrigate etc.

1.4 Strategies to improve production

1.4.1 Selection and improved growth characters
Various alternatives had been carried out in order to improve the production including improved nutrition and other characters, use of pharmaceuticals and breeding. Selective breeding has produced enormous improvement in farm livestock. It has been achieved through selection on phenotype based on
performance and physical characteristics of different generations. Strategies include selection between breeds, selection within breeds and crossbreeding which has been done intensively.

In order to improve further on the production and quality, strategies that had been implemented include use of antibiotic growth promoter and hormonal growth promoter. However, it also causes controversy as it has been considered it may have a detrimental effect on health of human, although not proven. For example, although antibiotic growth promoters given at sub-therapeutic doses can increase growth (Phillip et al. 2004), improve average daily weight gain (Phillip et al. 2004), improve quality of product by reducing fat percentage and protein content (Hughes and Heritage, 2004), increase the feed conversion efficiency (Jensen, 1998; Phillip et al. 2004) and control zoonotic diseases (Hughes and Heritage, 2004), they impose selection pressure between antibiotics to be used in medical and veterinary practice (Hughes and Heritage, 2004), leading to developing resistance (Jensen, 1998; Forano and Flint, 2000; Smalla et al. 2000; Heritage et al. 2001).

Subsequently, as concern of antibiotic resistant in animals might transmitted to human, Sweden banned all food animal growth promoting antibiotics in 1986 (Casewell et al. 2003). In Denmark, avoparcin and virginiamycin were banned in 1995 and 1998 while the European Union banned avoparcin in 1997 before banning bacitracin, spiramycin, tylosin and virginiamycin in 1999 (Casewell et al. 2003). However, the consequences of this ban were increased prevalence of several infections in human in Denmark and subsequently, increased therapeutic antibiotics used in animals (Casewell et al. 2003; Phillip et al. 2004). The issues is still not settled and it is still ongoing debate about antibiotics growth promoter usage (Phillip et al. 2004; Turnidge, 2004)

Another controversial issue is the use of hormonal growth promoters. In cattle, the commercial product was also known as bovine growth hormone (bGH), bovine somatotrophin (bST), recombinant bovine growth hormone (rbGH) and recombinant bovine somatotrophin (rbST). Although it was claimed to be natural, reduce cost, increase milk yield and give safety for consumers (Monsanto Dairy), it was claimed it has detrimental effect on health by
consuming product from rbST treated animals, and on animal welfare by production of much larger amounts of milk.

Administration of rbST improves growth performance (McLaughlin et al. 1993; Govoni et al. 2004), increases milk yield (Armstrong et al. 1995), increases carcass leanness (McLaughlin et al. 1993), decreases fat deposition (Armstrong et al. 1995), improves carcass quality (Govoni et al. 2004), increases weaning weight (Armstrong et al. 1995) and increases feed efficiency (Govoni et al. 2004). However, it was claimed to have effects on human as well as animals. Consumption of milk from rbST treated animals was claimed to increase diabetes and hypertension in human; and mastitis, infertility, lameness and susceptible to diseases (Epstein, 1990) and increase disease incidence (Epstein, 1990).

Although FDA approved the use of rbST, some countries such as Canada refuse to approve rbST. This is based on comprehensive review of two independent committees. Although it had no significant affect on human safety such as carcinogenic effect, increased antibiotic resistance or allergic reaction was found, in animals, it increased the rate of mastitis (25%), infertility (18%) and lameness (50%). Based on this finding, the Canadian Government reject the use of rbST.

Therefore, alternatives to reliance on antibiotic growth promoter, hormonal growth promoter and other chemicals are desirable. Genetic selection is seen as an alternative. By selecting the animals with superior traits, genetic improvement can be done. One of alternatives is by identification of variation in livestock genomes (Womack, 2005) that contribute to production and meat quality traits. This leads to the initiation of the Bovine Genome Project.

1.4.2 Bovine Genome Project

The initiative for study of the Bovine Genome started in the late 1980s with early attempts based on somatic cell genetics and in situ hybridization (Womack and Moll, 1986 in Womack, 2005; Fries et al. 1986) and later on based on molecular markers. Subsequent development of markers resulted in a genetic linkage map based on molecular marker recombination (Berendse et al. 1994; Kappes et al. 1997). Various laboratories were involved in the international effort for generating libraries such as BACs and ESTs. These laboratories include Texas
A&M University; Institut National de la Recherche Agronomique (INRA), France; U.S. Meat Animal Research Center, Clay Center, Nebraska (USDA-ARS); The Roslin Institute, Edinburgh, Scotland, United Kingdom; University of Alberta, Edmonton, Canada; The Institute of Genomic Research (TIGR), Rockville, Maryland; the University of Illinois; AgResearch (New Zealand), and CSIRO (Australia). Sequencing of the bovine genome will eventually lead to identifying genes underlying various traits, and a first draft of the complete sequence was published on the web in 2004 based on 4x coverage sequencing which by 2006 was increased to 7.1 x coverage (Human Genome Sequencing Centre at Baylor College of Medicine http://www.hgsc.bcm.tmc.edu/projects/bovine/).

1.4.3 Quantitative Trait Loci

Quantitative trait loci are regions along a chromosome associated to particular traits (Erickson et al. 2004). Most quantitative traits are controlled by many genes (Simm, 1998) although other works including the same author do not agree and claim that “studies have indicated the presence of a major gene (or genes) for production traits in sheep and other mammalian species” (Walling et al. 2004).

The importance of QTL studies is for identification of genes and markers that can be used in breeding programmes (Spelman and Bovenhuis, 1998; Kuhn et al. 1999; Khatkar et al. 2004). For QTL mapping, a pedigreed population with production traits is required (Katkhari et al. 2004). Several methods for QTL detection such as granddaughter design (GDD) and daughter design (DD) have been used (Khatkar et al. 2004). Differences between designs include population structure and number of generations studied (Bovenhuis et al. 1997). Population structure includes half-sib or full sib as well as use of outbred lines or line-breed crosses (Bovenhuis et al. 1997). Advances in statistical methods and genetic markers such as microsatellites leads to entire genome scan for QTL (Arranz et al. 1998; Velmala et al. 1999; Kuhn et al. 2003; Viitala et al. 2003).

In cattle, several QTLs have been associated to growth (Casas et al. 2000; 2001; Li et al. 2002a; Kneeland et al. 2004) identified at chromosome 2, 6, 14, 19, 21 and 23 (Kneeland et al. 2004) and chromosome 14 (Mizoshita et al. 2004), carcass traits (Casas et al. 2000; 2001; MacNeil and Grosz, 2002;

However, Spelman and Bovenhius (1998) state categorically that “Results from quantitative trait loci studies cannot be readily implemented into breeding schemes through marker assisted selection because of the uncertainty of whether quantitative trait loci identified are real and whether the identified quantitative trait loci are segregating in the breeding population”. Furthermore, QTLs do not identify the actual gene underlying the phenotypic traits but rather a region of DNA that is closely linked with the gene and it requires considerable extra work to find the actual genes involved (Spellman and Bovenhius, 1998; Hayes and Goddard, 2001). Association may come from mutant analysis or from mouse or human homologues (and transgenic model organisms) where more detailed studies as show directly the effect of a gene on a QTL-type character.

1.4.4 Marker Assisted Selection (MAS)

One of the ways for genetic improvement of livestock is by using variation in genomic DNA and major genes. Variation between animals with regard to their genetic level for certain trait is caused by differences in DNA genotypes of these animals. Selection of animals with the best genotype results in genetic progress. Until recently, selection was based only on phenotypic and pedigree information of animals. Due to influence of environmental factors on the expression of traits, phenotypes can only provide part of the information on the genetic level of animals. Polymorphism at DNA level can be revealed and detected by molecular markers and particular polymorphisms can be linked to desirable phenotypic traits. In fact these molecular markers provide a cost efficient production and save a lot of time, particularly for traits which may be difficult (disease resistance or carcass quality) or impossible (milking characters in bull genotypes) to assess.
The use of molecular techniques that have been developed in the past decades can increase the amount of information at the genetic level of animals. Because genetic markers are available early in life, the accuracy of selection at young age can be increased and the generation interval can be reduced. MAS (marker assisted selection) has the potential benefit of selection in animals breeding programs. Furthermore, use of molecular marker is much more important as some of the parameters such as carcass composition and fat deposition cannot be measured on live animals. Despite the promise of marker assisted selection using molecular DNA polymorphisms, by 2006 there are relatively few examples where it is used in animal breeding programmes; three carcass quality trait markers have been validated for use in the US by the National Beef Cattle Evaluation Consortium, including markers in the thyrogolbulin, calpain and calpastatin genes (www.nbcec.org; tests offered by Igenity and Bovigen/ GeneSTAR).

Most economically important traits in beef production are influenced by many genes as well as environmental factors. Breeding programs aim at selecting animals with the most favourable set of genes, to produce animals for the next generation. Genetic markers associated with these genes can be used in the marker assisted selection (MAS) to select potential genes. Implementation of MAS in breeding strategies for production traits has started (Spellman and Bovenhius, 1998). MAS is expected to be especially beneficial for traits that cannot be improved efficiently by current breeding such as low heritability traits or traits that cannot be or are difficult to measure or based on post mortem performance. Markers can be used to genotype a population, detect differences among population and even among individuals. The origin of diversity is mostly due to mutations occurring within the DNA regions which occur at different rate in different genomes. Current knowledge and understanding of the genome has opened up the possibilities for direct identification and selection of animals carrying the best genes through selection on genotype. Searching for genes with significant effects in various traits is facilitated by the marker genome map. By 2005, the bovine marker genome map was covered by over 4585 marker loci (Snelling et al. 2005). Marker-based genome maps became an important tool in animal breeding and evaluation of breeding value can be enhanced at known quantitative trait loci or genetic marker loci tightly linked to them.
1.5 Application of molecular markers

Various molecular markers have been used in livestock production for selection programmes (Montaldo, 1998; Vignal et al. 2002; Switonski, 2002) and diversity measurement (Kemp et al. 1995; Canon et al. 2001). Molecular markers are relatively simple to detect and abundant throughout the genome. These markers include microsatellites, single nucleotide polymorphisms (SNPs), polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), amplified fragment length polymorphism (AFLP), rapid amplification polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DDGE) and single strand conformation polymorphism (SSCP). Cytogenetic markers are also valuable for examining chromosome morphology, inheritance of marker chromosomes and changes in karyotype (Chaves et al. 2003).

1.5.1 Microsatellites

Microsatellites have become one of the most widely used molecular markers (Schlotterer, 2000; Canon et al. 2001; Pritchard and Feldman, 1996; see 1.5.1.2).

1.5.1.1 Definition and function

Microsatellites are also known as simple sequence repeat (SSR) (Tautz, 1989) or short tandem repeat (STR) (MacHugh, et al. 1997). A microsatellite is a sequence with repeat regions consisting of two to six nucleotides (Schlotterer, 2000; Tautz, 1989). In microsatellites, the repeat units or sequence consisting two, three, four, five or six bases (di-, tri-, tetra-, penta- or hexanucleotides) which are repeated a variable number of times in tandem, typically ranging from 8 to 80 times.

Microsatellites can be classified based on their composition into two categories, perfect or compound. Perfect microsatellites refer to regions with simple repeat motifs such as AC$_{17}$ as in the microsatellite marker give the code CSRM66 or GT$_{15}$ as in BM1824. On the other hand, compound microsatellites may have a combination of simple units such as (CA)$_{17}$(GT)$_{15}$ or combination of
simple units with non repetitive sequence such as 
(TG)$_2$(TACA)(TA)$_3$(TG)$_2$(TA)(TG)$_2$(TA)$_2$ as in ILSTS005.

Microsatellites tend to occur in non-coding regions of the DNA. Most of 
the articles report microsatellites as having no function (Tautz and Renz, 1984). 
However, recently, there are reviews of the possible function of microsatellites 
(Li et al. 2002b) “These includes in chromatin organization, DNA structure, 
centromere formation, hotspots for recombination, control of DNA replication 
and cell cycle, modulation of mutation rate, control of transcription and gene 
expression, binding of regulatory protein as well as inhibition of translation”.

However, recently microsatellite motifs were reported as being present in 
Expressed Sequence Tags (EST) (Lamoureux et al. 2006; Varshney et al. 2006; 
Zhang et al. 2006). In barley, EST-derived microsatellite loci (EST-SSRs) was 
placed into genetic map (Varshney et al. 2006) and the evidence proved an 
uneven distribution of genes and the segmentation of the barley genome in gene-
rich and gene-poor regions (Varshney et al. 2006)

Microsatellites have been increasingly used as the marker of choice in 
studies of evolution and diversity (MacHugh et al. 1997; Switonski, 2002) for 
their several advantages over the other markers. Microsatellites loci are found in 
large numbers and are relatively evenly spaced throughout the genome. They are 
usually highly polymorphic. Microsatellites had been reported to contribute to 
the identification of genes. A microsatellite (CA)$_n$ polymorphism in the promoter 
region was associated with birth weight and weight gain from birth to yearling in 
populations of Hereford cattle (Moody et al. 1996) and a microsatellite with a 
TG polymorphism in the growth hormone receptor (GHR) promoter region in 
Angus breed (Hale et al. 2000).

A particularly important area of new technological developments in 
microsatellite markers could have a major impact on beef production and 
breeding systems in the near future. They will allow rapid genetic selection of 
animals, based on DNA screening for growth, feed efficiency and carcass merit 
which has already been investigated in other breeds and which have the potential 
for application in selection of elite from local populations. One study revealed a 
putative QTL on bovine chromosome 14 through an association with the 
microsatellite locus CSSM66. The gene encoding thyroglobulin (TG) was 
identified as a positional candidate, based on its close linkage to CSSM66. In this
thesis microsatellites are used to study diversity within the target Malaysian breed.

Genetic diversity using microsatellites in livestock has been reported in various species including chicken (Hillel et al. 2003), sheep (Worley et al. 2004), goat (Swiss goat) (Saitbekova et al. 1999), African buffalo (van Hooft et al. 1999) and Asian water buffalo (Barker et al. 1997). Within cattle, various cattle breeds had been studied, although most diversity studies have focussed on European cattle breeds (Moazami-Goudarzi et al. 1997; MacHugh et al. 1998, Grzybowski and Prusak, 2004a) including Belgian cattle (Peelman et al. 1998), Spanish cattle (Martin-Buriel et al. 1999), Polish Red cattle (Grzybowski and Prusak, 2004b), and also South American breeds with European pedigrees such as Brazilian Argentine Creole cattle (Liron et al. 2004). Genetic diversity of north-east Asian cattle (Kim et al. 2002) also has been published.

Furthermore, microsatellites are used for the development of a marker genome map (Ihara et al. 2004). Various groups have been involved in the construction of the genetic map (Ihara et al. 2004; Berendse et al. 1994; Vaiman et al. 1994; Moore et al. 1994). It had been developed with the use of microsatellite segregation. This facilitates the search of genes for various traits as well as for the animal breeding (Switonski, 2002).

Microsatellites will allow rapid genetic selection of animals, based on DNA screening for growth, feed efficiency and carcass merit which has already been investigated in other breeds and which have the potential for application in selection of elite from local breeds.

1.5.1.2 Advantages of microsatellites
There are some advantages to utilize microsatellites over the other markers: microsatellite loci are found in large numbers and are relatively evenly spaced throughout the genome (Tautz, 1989; Ihara et al. 2004). They are usually highly polymorphic (Ihara et al. 2004) and easily automated with PCR (Canon, 2001). These markers are suitable for measuring genetic diversity within and between breeds and populations (Kemp et al. 1995; Canon et al. 2001; Dettman and Taylor, 2004; Peelman et al. 1998)
1.5.2 Single Nucleotide Polymorphisms

A Single Nucleotide Polymorphism (SNP) is a DNA sequence variation, occurring when a single base changed in DNA sequence. This results in transition or transversion. Transitions refer to base changes that are purine to purine or pyrimidine to pyrimidine exchanges, whereas transversions refer to purine to pyrimidine or pyrimidine to purine exchanges. Allele frequency should occur in at least 1% of the population to be considered a SNP (Vignal et al. 2002). SNPs may occur within coding sequences of genes, noncoding regions of genes (introns) or between genes (intergenic regions). SNPs within a coding sequence change the codon, which may or may not change the amino acid in the protein sequence. In some cases, if changes occur at the third position of the codon, it will result in silent mutations (no amino acid changes).

The study of SNPs is important in livestock breeding programmes as this marker is able to detect single base changes in a DNA sequence. In the bovine genome programme, informatic analysis has identified some 121,531 bovine SNPs in the 8 April 2006 release of the assembly (ftp://ftp.hgsc.bcm.tmc.edu/pub/data/Btaurus/snp/Btau20060804/README). Point mutation had been shown to have significant economic impact on cattle production. Grobet et al. (1997) found that double muscling in Belgian Blue cattle is the result of mutation of the myostatin gene. Subsequently, it is also found that G→A transition at position 938 in Gasconne and Piemontese breed, C→T transition at position 610 in Charolais and Limousine, G→T transition at position 676 in Maine-Anjou and G→T transition in Marchigiana results in loss of function in the myostatin gene (Karim et al. 2000). Furthermore SNPs in other genes such as growth hormone, insulin-like growth factor I, growth hormone receptor as well as leptin had been shown to have effects on various production traits in cattle (Oprzadek et al. 2003a; Hale et al. 2000; Ge et al. 2001; Buchanan et al. 2002).

If not from whole genome programmes, identification of SNPs is done by amplification of specific sequence fragment and sequencing. By comparing the sequences, SNPs could be identified.
1.5.3 Polymerase chain reaction-restriction fragment length polymorphism

This technique carried out by amplification of certain sequences followed by digestion of the amplified DNA with restriction enzymes. This technique is able to identify polymorphism and subsequently can be used for population genotyping (Barendse and Fries, 1999). As mentioned in paragraphs above, prior to PCR-RFLP, alleles that contain recognition sites for a restriction enzyme can be identified from the sequences before being used to genotype the population. The enzyme only cuts where there are specific DNA sequences recognized by the enzymes. The restriction fragments are then separated according to length by agarose gel electrophoresis. This result in different fragment sizes produced and could be used to characterise the differences among samples or population. Therefore this technique can be used to detect any single mutation within the gene fragment.

1.5.4 Other molecular markers

1.5.4.1 Amplified fragment length polymorphism

Amplified fragment length polymorphism is a technique that involves digestion of genomic DNA with restriction enzymes before ligating to the restriction site with specific adaptors (McPherson and Moller, 2000). Primers are designed from the known sequence of the adapter and sequences that do not match to the adaptor primer will not be amplified (Vos et al. 1995). The variable number of bands produced indicates the polymorphisms present. Although the disadvantages includes medium accuracy and not a multi allelic co-dominant (Vignal et al. 2002) but AFLP technique is being used in various studies and consider as a powerful tool in comparing closely related species or genomes (van Bergen et al. 2005; Sharma et al. 2006; Johnsen et al. 2006). In work by Johnsen et al. (2006), *Campylobacter jejuni* was genotyped using AFLP in human and bovine isolates taken from different farm. Campylobacteriosis is important foodborne bacterial disease causing diarrhoea. AFLP technique was able to differentiate three different clusters within bovine isolates as well as in detection of similarity between bovine and human isolates.
1.5.4.2 Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) is a technique of PCR using short primers on genomic DNA (Sambrook et al. 1989). RAPD products are visualized on agarose gels stained with ethidium bromide. The amplification was done on random DNA fragments. RAPD products will generate unique pattern profile. It is usually used in unknown genomic background with no knowledge on DNA sequenced required. Although the method has been found to have low reproducibility and is not usable in high-quality, routine studies (Vignal et al. 2002; hence it is not discussed further here), but RAPDs provide rapid and easy in identifying, valuable epidemiological tools (Singh et al. 2006) and identifying genotype of early sexual maturation (Alves et al. 2005; Acik and Cetinkaya, 2006). In a study by Singh et al. (2006), differentiation among *Mycobacterium tuberculosis* strains were detected using RAPDs. Differentiation among different strains is important in epidemiological studies such as source of infection, outbreak of new strain and relatedness of strains in different patients.

1.5.4.3 Restriction fragment length polymorphism

The technique is one of the first molecular markers and involves digestion of genomic DNA digested with restriction enzymes (Sambrook et al. 1989). Variations in genomic DNA are caused by base pair deletions, insertion and mutations recognised by the restriction enzyme. This will results in a various fragment band length which indicate polymorphisms present. In bovine, genetic variation studies had used RFLP (Georges et al. 1987; Hallerman et al. 1987). However, currently, most use PCR-RFLP since enough enzymes are known to most genes and this avoids use of radioactivity. However, RFLP is still being useful for gene diversity study (Mobius et al. 2006; Marson et al. 2005). In a study by Mobius et al. (2006), luteinising hormone receptor and follicle-stimulating hormone receptor gene in six different breed compositions was characterised using RFLP. This technique is able to detect heterozygosity within the population studied. Genetic variability would aid the selection of animals with economically reproductive traits.
1.5.4.4 Denaturing gradient gel electrophoresis
Denaturing gradient gel electrophoresis (DGGE) is a technique separating the DNA using polyacrylamide gel electrophoresis (Sambrook et al. 1989). Gradient in the denaturants is the basis to detect variation (Sheffield et al. 1989). The conditions of denaturant vary across the gel and double strands melt at different location in the gel, depending upon their sequence (Barendse and Fries, 1999). Currently, although not commonly used, DGGE is used for detection of polymorphism (Sasaki et al. 2005; Obodai and Dodd, 2006; Ercolini et al. 2006). Ercolini et al. (2006) study the effect of microbial spoilage in refrigerated beef under different packaging condition. In this study, *Pseudomonas*, *Enterobacteriaceae*, *Brochothrix thermosphacta* and lactic acid bacteria were monitored in three different modified-atmosphere packaging during 14 days of storage using DGGE analysis. Thirteen different genera and 17 different species were identified using DGGE. This indicates wide diversity of spoilage microbial during beef storage. This method enable establishment of proper storage condition of beef.

1.5.4.5 Single strand conformation polymorphism
Single strand conformation polymorphism is a technique to separate different allele conformations by denaturing gel electrophoresis (Ripoli et al. 2006). Heterozygous alleles can be detected on gels as the single strand DNA with nucleotide changes will have different three-dimensional conformation. As the three dimensional structure is different, the rate DNA travels during electrophoresis will be different although the size of the fragment is same and subsequently different bands will be detected. SSCP has been considered a useful tool in diversity study (Ripoli et al. 2006; Kamenetzky et al. 2005). Ripoli et al. (2006) studied the polymorphism in *DGAT1* gene in 14 cattle breeds using SSCP. SSCP was used to detect CG/AA allele at position 10,433-10,434. The A allele (represent AA and lysine at amino acid 232) and the K allele (represent CG and alanine at amino acid 232). In this study, three SSCP variants were detected, A allele and two alternative conformations from K allele. This indicate that SSCP capable of identifying co-dominant allele. K allele had been associated to low milk fat content and A allele with higher milk fat content.
1.5.5 Rationale use different of markers

Decision of choosing markers depends on the objective of the experiment. All these markers are able to detect polymorphisms. For unknown genomic background DNA with no knowledge on DNA sequenced, AFLP and RFLP can be used (Dodgson et al. 1997). AFLP use short DNA oligonucleotides to amplify at random whereas RFLP use restriction endonuclease. In AFLP techniques large numbers of anonymous loci can be analysed simultaneously (Barendse and Fries, 1999). Identification of SNPs can be done from sequence analysis. Furthermore, identification of single base pair substitution and indel can be done using AFLP, RFLP, PCR-RFLP and SSCP. However, RFLP can only be detected if polymorphisms are present at within the recognition site of a restriction endonuclease. As for microsatellites, although it cannot detect single basepair substitution (except in the primer regions under some conditions), it can detect indels (Vignal et al. 2002). Analysis of AFLP, PCR-RFLP, SSCP, DDGE and microsatellites are dependent upon PCR. As for DDGE, as mentioned above, variation detected is based on a gradient of denaturants. However, for identification different allele conformations, SSCP technique should be used.

In term of inheritance, AFLP are able to detect two dominant alleles; RFLP, PCR-RFLP, SSCP and SNP are able to detect two co-dominant alleles whereas for multiallelic co-dominant, RFLP and SSCP can be used (Vignal et al. 2002). Technically, development effort is high in SNP, RFLP and PCR-RFLP; medium for SSCP; low for AFLP (Vignal et al. 2002). In term of speed of assay, Dodgson et al. (1997) report that RFLP is low compare to AFLP, SNP and microsatellites. Genome distribution in AFLP, RFLP, SNP and microsatellites are ubiquitous.

1.5.6 Cytogenetic markers

Cattle have 60 chromosomes (2n=60): 58 are autosomal chromosomes and two are sex chromosomes (Gustavsson, 1969; Gallagher and Womack, 1992). The autosomal chromosomes are acrocentric and sex chromosomes are submetacentric (Gustavsson, 1969; Chaves et al. 2000). Within sex chromosomes, the large and small submetacentric represents the X and Y chromosome, respectively (Schifferli et al. 2003). Karyotyping of cattle is
difficult as all autosomes are acrocentric and have small size differences and therefore require banding methods (Gautier et al. 2001) but basically the arrangement is based on the length of the chromosomes; first in the autosomal chromosomes and followed by the sex chromosomes. Standardization of cattle karyotyping is done by International System for Cytogenetic Nomenclature of Domestic Animals (ISCNDA) (Popescu et al. 1996). The first international nomenclature of bovine karyotypes was established in 1976 using GTG-bands (G-bands, trypsin using Giemsa); the second nomenclature was in 1990 using GTG- and RBA/RGB-banding (R-bands, 5-bromo-2'-deoxyuridine using acridine orange/R-bands, BrdU using Giemsa) technique and the third nomenclature in 1995 which correlates GTG-band, RBA/RGB-band and 31 marker genes (Fries and Popescu, 1999). Now fluorescent methods are widely used for band visualization – for example for R-bands using acridine orange for RBA bands or propidium iodide for RBP bands, and quinacrine or Hoechst 33258 for Q bands (Popescu et al. 1998).

Cytogenetics has a strategic function in chromosome mapping including construction of linkage maps, verifying the position of candidate genes and markers in positional cloning projects, and information on the location of loci relative to evolutionary conserved regions (Fries and Popescu, 1999). Mapping of cattle chromosomes is done by in situ hybridization or somatic cell hybrid cross to standard karyotypes and to linkage maps for cattle (Fries and Popescu, 1999). Currently, a comprehensive radiation hybrid map comprises 5593 loci (Itoh et al. 2005). Furthermore, there are 1800 synteny markers and 2500 linkage markers in bovine genome (Womack, 2005). Synteny markers refer to markers of all loci on one chromosome. Synteny marker is the first gene map of cattle (Womack and Moll, 1986; Womack, 2005) and designated as U1 to U29 in 28 autosome and X chromosome (Fries and Popescu, 1993). Linkage map was started in the early 1990s as a result of development of microsatellite markers and expanded for mapping loci (Womack, 2005). Radiation hybrid is a technique of physical mapping using numerous small fragments of chromosome produced by fusion with lethally irradiated cell. RH panels allow rapid mapping of sequence-tagged site (Strachan and Read, 2004)

Various chromosome abnormalities can be detected by cytogenetics. Chromosome abnormalities, both numerical and structural are very often
associated with reproductive problems and developmental disorders and in reproductive system; cattle chromosome abnormality is usually associated to fertility problems and reproductive failure (Chaves et al. 2003). Examples of cattle chromosome abnormalities are Robertsonian translocation and sex-chromosome trisomies. Robertsonian translocation such as t(1;29) result in reduction of fertility performance (Berland et al. 1988) whereas sex-chromosome trisomies whether XXX, XXY or XYY lead to sterility or subfertility (Fries and Popescu 1999). In cattle, most chromosomal abnormalities are considered to be Robertsonian translocations (Mastromonaco et al. 2004). In Robertsonian translocations, 42 different translocations had been reported in various breeds with different effect on performance in different countries (Fries and Popescu, 1999). The same authors also showed that among the breeds that had been associated to Robertsonian translocation are Holstein Friesian, Friesian, Limousine, Simmental, Japanese Black and Barrosa. New reciprocal translocations are still being detected: one was found in the Montbéliarde breed in France (Ducos et al. 2000) and the t(1;29) was found in the Creole breed in Argentina (Schifferli et al. 2003).

Due to important economic effects caused by the abnormality detected in livestock, a control policy has been made in order to prevent diffusion of chromosomal abnormalities (Ducos et al. 2000) such as artificial insemination that may introduces chromosome abnormalities in a herd (Nel et al. 1988). The only official routine cytogenetic controls carried out up to now in bovines is to detect Robertsonian translocation t(1;29) in certain breeds. Schifferli et al. (2003) also emphasise that programmes for genetic and reproductive improvement incorporate chromosomal analysis of bulls and cows with a high genetic value for natural and artificial insemination. Individuals with chromosomal abnormalities could then be eliminated helping to assure high fertility rates of the breeds.

Effects of chromosomal abnormality are different between different breeds and translocations. For example, reciprocal translocation (12;17)(q22;q14) in Montbéliarde breed was detected in a group of animals that exhibit fertility lower than 30% (Ducos et al. 2000) whereas in another study, t(1;29) causes 5-10% reduction of fertility (Dyrendhal and Gustavsson, 1979 in Fries and Popescu (1999). Another example of differences observed between
breeds is on frequency and distribution of t(1;29) in Barrosas, Maroneses and Mirandesas breed in Portugal, which are 65%, 40.2% and 1.6% respectively (Rangel-Figueiredo and Iannuzzi, 1993). Cytogenetics also is useful in study of chromosomal diversity. Phylogenetic relationship carried out includes using satellites DNA (Chaves et al. 2005), in situ MspI (Chaves et al. 2000) and complex satellites in t(1;29) (Chaves et al. 2003). Furthermore, cytogenetics is able to detect gene duplication as well as evolution of genes (Geraldes and Ferrand, 2006). It is believed that during evolution, chromosome number increases by centric fission; arm number by pericentric inversion; but temporary reduction of chromosomes numbers occur by centric fusion (Hoshiba and Imai, 1993 in Stanimirovic et al. 2005). The screening and assessment of reproductive potential of captive and domestic breeds was also done using cytogenetics (Mastromonaco et al. 2004)

Cytogenetics also had been used in evaluation of efficiency in sperm sorting by technique of flow cytometry (Piumi et al. 2001). This was used in order to manipulate and determine the sex-ratio after sperm cell sorting as in livestock, there is interest to produce animals of one sex, where male progeny is preferable for beef breeding and female is desired for milk production (Piumi et al. 2001). The basis of flow cytometry sorting of spermatozoa is that DNA content is significantly larger in X-chromosome spermatozoa due to the size of chromosome. Recently, the same group of authors designed a specific probe for X and Y chromosome in order to assess the sex chromosome content of bovine spermatozoa.

Furthermore, for species with complex karyotypes where identification of chromosome by flow cytometry cannot be done, microdissection can be used (Kubickova et al. 2002, Pinton et al. 2003). Microdissection produces painting probes specific for each chromosome. Microdissection can be used to identify chromosomal rearrangements that had escaped detection during using standard chromosome banding techniques (Pinton et al. 2003).

The only disadvantages of application of cytogenetic markers are that they are laborious (McNiel et al. 2006).
1.6 Economically important traits

Economically important traits in beef production are always associated to the meat produced. In order to achieve higher meat production, the growth aspect is important. Furthermore, as the meat quality is important from the consumer point of view, meat quality aspect is also always being emphasized in beef production.

1.6.1 Growth

Growth is an economically important trait in livestock. It is a complex concept with no specific definition (Lawrence and Fowler, 2002). However, growth in farm animals is more associated to the animal weight output compared to feed input as this reflects the meat produced. Growth also is not as a contribution of one particular gene but as a result of interaction of various genes (Zhou et al. 2005). In understanding the contributing factor in growth, a lot of studies on interactions of various genes were carried out apart from the study of the effect of each of these genes to the production traits.

Various studies on association of various factors on growth have been carried out. These include effects of breed, age, sex, feeding strategies, feeding system, feed resources, intake, formulation and composition. Components such as aspect of lean meat composition, total fat, meat and bone ratio, subcutaneous and intermuscular fat ratio were studied.

Currently, with the advances in molecular techniques, more research focuses on DNA polymorphism. Polymorphisms has been associated to growth and four important genes that have been identified are the growth hormone (GH) gene and growth hormone receptor gene (GHR), insulin-like growth factor 1 gene (IGF1) and pituitary-specific transcription factor gene (PIT1).

Growth hormone gene action stimulates anabolic processes such as cell proliferation, skeletal growth and protein synthesis by modulating the expression of many other genes. PIT1 stimulates both the development of these cells and production of growth hormone. Growth hormone action on target cells depends on growth hormone receptor. The growth hormone receptor mediated growth hormone action on target cells by transducing the growth hormone stimulating signal across target cell membrane and inducing transcription of many genes.
including IGF1. Therefore, genes encoding growth hormone, growth hormone receptor, IGF1 and PITI are viewed as potential candidate markers for selection in cattle.

Currently, studies carried out are on polymorphism on each of those genes and their association with growth (Lucy et al. 1993; Ge et al. 2001; Oprzadek et al. 2003a), feed conversion (Oprzadek et al. 2003b), meat production (Di Stasio et al. 2003; Te Pas, 2001), carcasses quality (Hale, 2000; Oprzadek et al. 2003b; Lagonigro et al. 2003; Kuryl et al. 2003), leaner carcass (Buchanan et al. 2002) and fat deposition (Wheeler et al. 1994; Buchanan et al. 2002; Kuryl et al. 2003; Buchanan et al. 2003).

1.6.1.1 Growth Hormone

Growth hormone (GH), also called somatotropin or somatropin (US), plays a central role in growth and metabolism in cattle by binding to growth hormone receptor (GHR) and stimulating production of insulin-like growth factor 1 (IGF1). Growth hormone is released from anterior pituitary (Loevendahl, 2004) and is a pituitary hormone that affects growth, lactation and reproduction (Lucy et al. 2001). Bovine growth hormone (GH) gene is a single copy gene that spans 1,800 bp and consists of five exons and four introns (Sorensen et al. 2002; Kim et al. 2003; Loevendahl, 2004). This gene has been associated with chromosome region 19q26 in bovine genome (Heideger et al. 1990 in Dybus, 2002a). Effects of polymorphism have been studied in various species especially in dairy cattle. Many reports showed significant association of various polymorphisms of GH gene in milk and meat traits in cattle. It has been documented that several polymorphisms at various locations had contributed to production performance.

A Leu/Val (LV) polymorphism in bGH gene is the best known allelic variant (Zwierzchowski et al. 2002). This sequence polymorphism, caused by a nucleotide change in the Leu codon to Val codon at position 127 of GH in exon 5, was first found by Lucy et al. (1991). Lucy reported that the LL genotype of bGH is associated with higher milk production in Holstein cows. In Danish Holstein cattle, higher milk production, higher fat content but lower protein content also been reported (Grochowska and Zwierzchowski 2000 in Zwierzchowski et al. 2002)). A different trend was observed in Polish Friesian
in which LV shows higher milk and protein content but LL genotypes have higher fat content. However, in Polish Black and White cows LL genotype had higher milk production, fat and protein content compared to LV heterozygotes (Dybus, 2002a). Also it had been reported that in Danish Jersey, VV genotype was observed to have all the favourable traits (Grochowske and Zwierzchowski (2000) in Zwierzchowski et al. (2002)). Zwierzchowski et al. (2002) reported that such conflicting results may due to analyses of different groups of animals. Switonski (2002) also reported that phenotypic effects of the GH gene polymorphisms on growth are not concordant.

The LL and LV genotype has been associated with higher meat deposition than VV genotype (Oprzadek et al. 2003a). LL genotypes also have significant effect on carcass gains compared to LV and VV genotypes (Schlee et al. 1994 in Dybus, 2002; Chrenek et al. 1998 in Dybus, 2002). In another study, LV heterozygotes were observed to have higher body weight and feed intake (Oprzadek et al. 2003b).

Another polymorphisms reported within the GH gene is a MspI polymorphism. Dybus (2002b) studied MspI polymorphism site in intron 3, of growth hormone gene in Holstein-Friesian cattle. The MspI polymorphism produces fragments of 224 and 105 bp and known as $GH^{+/+}$ genotype 329, 224 and 105 for the $GH^{+/-}$ genotype and single fragment of 329 bp (no digestion) for genotype $GH^{-/-}$. In that study, it was found that cattle with $GH^{+/+}$ allele produce more milk compared to $GH^{-/-}$. However $GH^{+/-}$ cattle have higher fat content than $GH^{+/+}$ cattle. This result is in agreement with results shown by Zhou et al. (2005) who also looked at effects of the polymorphism in different lactation stages. In another report (de Mattos et al. 2004), it was reported that $GH$-MspI(-) have higher fat content. This finding indicates that $GH$-MspI allele can be used as a marker for selection for higher milk production or alternatively selection for milk fat content.

Effect of MspI site polymorphism of bGH on meat traits had been studied (Di Stasio et al. 2003). In this study, the traits analysed include growth at different stage, size of different parameter as well as different meat conformation apart of meat quality characteristics analyses. However, it was found that no significant association of these traits to the $GH$-MspI polymorphism which
indicating that the variability detected is not related to the polymorphism detected.

A Dral polymorphism at regulatory region had been studied by Kim et al. (2004). Dral polymorphism is a single nucleotide mutation (C/T) at position -120 of bGH gene. Dral polymorphisms produce two fragments of 455 and 210 bp (allele A) and three fragments of 308, 210 and 147 bp (allele B). In this study, it was shown that BB genotype has significant effects on weight gain at 3 months old and carcass quality. However, Kim et al. (2004) observed no significant effect on carcass traits such as backfat thickness, longissimus muscle area and marbling score.

Although GH has been recommended to be a marker for growth, some authors recommended further investigation before being used in a selection programme (Knorr et al. 1997; Switonski, 2002)

1.6.1.2 Growth hormone receptor

Growth hormone receptor (GHR) has a major role in regulation of growth hormone action (Moisio et al. 1998). Growth hormone exerts its effects on growth and metabolism by interacting with specific receptors on the surface of the target cells (Di Stasio et al. 2005). GHR is a transmembrane protein found in a variety of tissue (Moisio et al. 1998) and highest level in liver (Lucy et al. 1998; Zhou and Jiang, 2005). As growth hormone receptor has a major role in the regulation of growth hormone action, it has been proposed as a candidate gene associated to milk production traits (Moisio et al. 1998) and meat production in cattle (Di Stasio et al. 2005). Mutations of GHR gene has been demonstrated as the cause of laron syndrome and idiopathic short stature (Rosenbloom and Guevara-Aguirre 1998 in Di Stasio et al. 2005) and in chickens, the mutation in GHR gene results in dwarf phenotype (Tixier-Boichard, 2002 in Di Stasio et al. 2005).

Transcription of GHR gene in cattle is initiated by three promoters from exons 1 and known as exon 1A, 1B and 1C (Zhou and Jiang, 2005).

Polymorphism in TG-repeat microsatellites in the bovine growth hormone had been associated to growth performance in cattle (Hale et al. 2000). It was reported that a shorter allele with 11 TG-repeat had been shown to have lower growth rate compared to animals with longer TG-repeat of between 16-20 TG-repeat (Hale et al. 2000). An 11 TG-repeat allele commonly occurred in Bos indicus (Lucy et al. 1998; Hale et al. 2000) whereas 16–20 TG-repeat allele commonly occurred in Bos taurus (Lucy et al. 1998). The Bos indicus breeds studied were Brahman and Nellore whereas Bos taurus breeds were Hereford, Limousine and Angus. Hale et al. (2000) reported that the 11 TG-repeat microsatellites allele in Angus have decreased growth of 17 kg and 23 kg at weaning and slaughter, respectively. However, these polymorphisms do not appear to have an effect on birth weight (Hale et al. 2000; Zhou et al. 2005). Therefore, this polymorphism has a potential to be used as a marker for growth in breeding programme.

Growth hormone receptor also had been suggested as a candidate gene for meat quality traits (Di Stasio et al. 2005). Polymorphisms at position 257 which involve transition A to G induce amino substitution Ser/Gly in exon 10. However, study in Angus breed (Ge et al. 2003) and Piedmontese breed (Di Stasio et al. 2005) on this polymorphism do not show any association with traits in growth and meat quality.

Another polymorphism within the 5'-noncoding region of bovine growth hormone receptor had been studied by Maj et al. (2006) in order to associate weight gain, feed intake and conversion and carcass parameters to these polymorphisms. Four polymorphisms studied were single nucleotide polymorphisms recognized by restriction enzymes AluI, AccI, Fnu4HI and NsiI. The results showed that individual SNPs had no effect on growth rates, feed intake and feed conversion. However, significant effects on carcass traits were observed in animals with genotype (-/-) at AluI site and the animals were superior in carcass weight, carcass dressing percentage, weight of valuable cuts and carcass lean weight. Genotype (+/+ ) at NsiI were superior in all the carcass traits studied. Combined genotype (+/+ ) at AluI and heterozygous (+/-) at AccI, Fnu4HI and NsiI were superior for growth rate, feed intake and carcass
composition. From this study, Maj et al. (2006) proved that combined genotypes can be used as well to determine association of polymorphism with production traits.

1.6.1.3 Insulin like Growth Factor-1

Insulin like growth factor-1 (IGFI) plays an important physiological role in the growth and development (Ge et al. 2001). IGFI is a polypeptide that is secreted primarily in the liver as well as peripheral tissue. The production of IGFI is by stimulus of growth hormone and the function is includes regulation and promotion of cell growth and development. Serum IGFI concentration is associated with growth traits in many livestock species (Ge et al. 2001). IGFI gene has been localised on chromosome 5 (Ge et al. 1997).

Several polymorphisms had been reported with the IGFI gene. Polymorphism detected at 512 bp 5’ to the first codon of the first exon of the bovine IGFI has been associated to growth performance in Angus cattle (Ge et al. 2001). Transition of T to C within IGFI gene indicates presence of B allele. BB allele had been associated to higher weight gain (Ge et al. 2001) and first 20 days after weaning. Calves with BB genotype gain 4.69 kg higher than AA genotype whereas the heterozygous AB genotype calves gain 4.78 kg higher than AA genotype (Ge et al. 2001). However, in different study (Li et al. 2004a) which was also on the Angus breed, no association with growth traits were observed for this polymorphism site.

Another polymorphism had been studied within 5’ regulatory region of bovine IGFI (Curi et al. 2005) in order to associate this polymorphism with growth and carcass traits. A polymorphic CA repeat in the 5’ flanking region of IGFI gene has been identified in cattle (Kirpatrick 1992; Ge et al. 2001) and was reported to be associated with weaning and yearling weights (Moody et al. 1994 in Ge et al. 2001) and birth weight (Moody et al. 1996 in Ge et al. 2001) in beef cattle. Four alleles were found within IGFI microsatellites which are 225, 227, 229 and 231 bp in size. It was found that the results were different between breeds. Nellore breed were observed to have 225 and 229 allele; Canchim breed with allele 229; ½ Simmental group with allele 225, 229 and 231 whereas ½ Angus group have higher frequency in allele 229 compared to allele 225, 227 and
However, although different breeds show obviously different alleles, no association with any traits were observed.

1.6.1.4 Pituitary-specific Transcription Factor 1

Pituitary-specific transcription factor 1 (PIT1) is a positive regulatory factor of growth hormone, prolactin and thyrotropin β-subunit in the mammalian pituitary (Zwierzchowski et al. 2002). The PIT1 gene has been assigned to cattle chromosome 1 (Moody et al. 1995; Woolard et al. 1994). PIT1 gene mutation has been identified as the cause of genetic disorders resulting from multiple hormonal deficiencies in both human and rodents (Wollard et al. 1994; Renaville et al. 1997a; Zhao et al. 2004). Deficiency of PIT1 reduces the expression of GH, because of a decrease in the proliferation of the cell lines producing GH (Di Stasio et al. 2002). PIT1 has been described as the critical cell-specific transcription factor responsible for activating expression of the prolactin (PRL) and GH genes in the anterior pituitary gland (de Mattos et al. 2004). As PIT1 plays important role in growth, PIT1 may be a candidate gene for a MAS programme. Wollard et al. (1994) propose use of PIT1 as a candidate gene for improving growth efficiency in cattle. PIT1 was also chosen as a candidate gene to investigate its association with growth, carcass traits and lactation performance in cattle (Moody et al. 1995).

Wollard et al. (1994) were the first authors who described the polymorphism within bovine PIT1 gene using RFLP method using Hinfl nuclease (Zwierzchowski et al. 2002). A point mutation, A→G in exon 6, affecting a Hinfl restriction sites, was observed with the detection of two alleles A and B (Wollard et al. 1994). Allele A is the undigested allele (451 bp) whereas allele B generating 244 and 207 bp fragments.

A Hinfl polymorphism in exon 6 was reported to be associated with body composition and milk yield in dairy cattle and associated with early-age body weight in beef cattle. Renaville et al. (1997a) showed that both genotypes with allele A at the PIT1 locus positively affected all milk production traits studied in Italian Holstein-Friesian. The AB genotype was superior for the milk yield and for daily yield of all milk components, while AA was shown to positively affect their concentrations. Furthermore, allele A was found to be superior for milk and
for daily yield of all milk components, while AA was shown to positively affect their concentrations. Furthermore, allele A was found to be superior for milk and protein yields and inferior for fat percentage in dairy cattle (Renaville et al. 1997a). Zwierzchowski et al. (2002) also reported that allele A of the PIT1 locus positively affected milk production traits and PIT1 gene identified to be a stronger factor compared to growth hormone gene on milk production.

Another study was done on Gyr breed (de Mattos et al. 2004). In this study, PIT1-HinfI polymorphism has two allele, PIT1 Hinfl (+) which generate four fragments with size of 660, 385, 270 and 40 bp and PIT1 Hinfl (-) generate three fragments with size of 660, 425 and 270. In this study, it was found that PIT1 gene variants have effects on milk production, fat and protein content. The heterozygous allele PIT1 Hinfl (+/-) cattle were superior for milk production compared to PIT1 Hinfl (+/+) allele. De Mattos (2004) also believe that PIT1 gene is a strong candidate for dairy cattle. However no association between PIT1-Hinfl polymorphism and milk production traits was reported in Poland Black-and-White cow (Dybus et al. 2004)

In beef cattle, the PIT1-Hinfl polymorphism is associated with early-age body weight. Allele B was associated with higher body weight at 7 months of age in Belgian Blue bulls (Renaville et al. 1997a). Oprzadek et al. (2003b) reported that PIT1 has an effect on carcass dimensions. The AA homozygotes were found to have a higher chest circumference, chest depth and circumference around but allele BB homozygotes had higher round width. However, Zhao et al. (2004) reported that PIT1-Hinfl polymorphisms appear have no affect growth traits in Angus cattle. Zwierzchowski et al. 2001 in Switonski (2002) reported no relationship between variants of PIT1 gene and meat productions in beef cattle. In Piedmontese cattle, PIT1-Hinfl polymorphism was also reported no association between this polymorphisms and growth and meat production traits (Di Stasio et al. 2002).

Another polymorphism detected within PIT1 gene was as PIT1-Hinfl, PIT1-NlaIII and PIT1-BstNI. Analysis of Hinfl and NlaIII polymorphisms in intron 3 and BstNI in intron 4 in Angus breed showed no significant association between these polymorphisms and growth and carcass traits (Zhao et al. 2004). SNP detected in intron 5 also showed no association to growth and carcass traits in Angus breed (Zhao et al. 2004).
that these markers seem to have different effects in different populations (Zhao et al. 2004).

1.6.2 Meat quality

Meat quality comes from multiple factors including tenderness (Koohmaraie, 1996), palatability (Page et al. 2002), flavour (Buchanan et al. 2002), water holding capacity (Koohmaraie and Geesink 2006), juiciness (Buchanan et al. 2002), colour (Koohmaraie and Geesink 2006) and nutritional value (Koohmaraie and Geesink 2006). Another major concern from the consumer point of view is fat content (Page et al. 2002), although high fat is associated with good flavour and tenderness (Nelson et al. 2004). Meat quality is considered as a very important criterion from a consumer point of view. The top three meat quality factors were reported as tenderness, palatability, and uniformity and consistency. However, producers have to deal with the issues of low overall uniformity and consistency, inadequate tenderness, and low overall palatability.

Apart from physiological factors, variation in meat quality is due to genetic structure of the cattle (Koohmaraie, 1996). Several studies have documented a genetic basis for differences in beef tenderness and intramuscular fat content (Shackelford et al. 1994; Wulf et al. 1996). Therefore genetic factor involvement has to be considered in order to improve the quality in beef production. Interactions and contributions of several genes will produce different meat quality traits. These traits somehow also result from interaction of several other genes that are related to growth in livestock. To improve meat quality of the population, selection and breeding is important. With current development in marker assisted selection (MAS), breeding based on MAS is possible and promising. By applying MAS, animals can be tested for their genetic potential early in their development and independently from their gender. So far, selection has been based on observable phenotypes but the limitation is when the phenotype is expressed at a later age.
1.6.2.1 Tenderness

Meat tenderness is one of the major concerns from the consumer point of view (Brooks et al. 2000). Variation in meat tenderness has a significant impact on consumer satisfaction (Page et al. 2002) but tenderness has numerous contributions from different factors along the meat production chain. Variation in beef tenderness is the effect of various factors including breed (Johnson et al. 1990a; Koohmaraie, 1996; Riley et al. 2002; Kolczak et al. 2003; Riley et al. 2003a), age (Kolczak et al. 2003), feed resources (Andrae et al. 2001; Kuber et al. 2004; Schoonmaker et al. 2004), sex (Splan et al. 1998; Nephawe et al. 2004;), postmortem temperature and rate of glycolysis (Jerez et al. 2003), μ- and m-calpain activity (Riley et al. 2003b), insoluble collagen (Riley et al. 2003a), marbling (Nelson et al. 2004), type of muscles (King et al. 2003), cooking temperature (George-Evins et al. 2004) and cooking method (Thompson, 2002). Furthermore, pH also has effect on meat tenderness. High pH muscle is more tender than low pH muscle but high pH meat is darker and this has a lower market value (Jerez et al. 2003).

Breed also had been reported to have influences in meat tenderness. Genetics has a significant contribution to the variation in tenderness within and between breeds (Koohmaraie, 1996). Study on effects of different breed hybrids including the B. indicus breed Brahman showed that Warner-Bratzler sheer (WBS) force increased as a percentage of Brahman increases (Johnson et al. 1990a). This indicates that increase in Brahman percentage in breeding will produce less tender meat (Johnson et al. 1990a). The underlying cause of would be in muscle fibre component and proteolytic enzyme activity (Johnson et al. 1990b). Whipple et al. (1990a, 1990b) indicate that less tender logissimus muscle in Bos indicus is due to reduced post-mortem proteolysis of myofibrillar protein which is associated with higher activity of calcium dependent protease inhibitor. However, another study on effect of different phenotypes showed that all phenotypes could be managed to produce tender beef (Hilton et al. 2004). Although there is increase of percentage of Brahman breeding, it did not affect flavour, muscle and incidence of off flavour.

Myofibrillar proteolysis for intracellular calcium–dependent protease, u-calpain and m-calpain enhanced meat tenderness (Koohmaraie et al. 1992 as in Montgomery et al. 2002; Huff–Lonergan et al. 1996). This increase muscle
calcium may improve beef tenderness (Montgomery et al. 2002) and differences in meat tenderness among breed types may due to contribution of differences in proteolytic enzyme activities (Johnson et al. 1990b). Vitamin D3 plays an important role in maintaining blood concentration of calcium (Montgomery et al. 2002).

Feeding supplementation approaches have been shown to improve meat tenderness. Feeding supplemental of 25-hydroxyvitamin D3 (Wertz et al. 2004; Foote et al. 2004; Ridel Sell et al. 2004) shows increased in longissimus dorsi and semi membranous steak tenderness. However, Rider Sell et al. (2004) shows that supplementing Angus crossbreed cull beef cows had little effect on longissimus and semitendinosus muscle.

Various post-mortem technologies have been developed to enhance the tenderness of meat products. Aging is one of the factors that had effect on tenderness. Tenderness increased as aging time increases (Raes et al. 2003; Kolczak et al. 2003a; Ilian et al. 2004; Monson et al. 2004). In the case of aging cull cows, 14d post-mortem aging is required for longissimus muscle but this aging period does not affect semitendinosus muscle (in practice mostly used for processing and minced/ground meat).

Another strategy that can be used to enhance tenderness is biochemical manipulation of glycogen breakdown (Jerez et al. 2003). Inhibition of glycolysis will prevent lactic acid formation and therefore will maintain high pH (Jerez et al. 2003; Wulf et al. 2002). These will result in increased tenderness as well as water holding capacity. Jerez et al. (2003) shows that administration of sodium citrate improves beef tenderness and at the same time does not alter the colour although at high pH. This will be accepted by consumers and increases values of products. Alternative treatments have been widely considered for increasing tenderness – traditional use of papaya, pineapple, kiwi fruits, or horseradish sauces include enzymes (papain in papaya) which affect tenderness has now been expanded and can be used commercially (http://www.enzymedevelopment.com/html/applications/protein.html).

Tenderness reflects myofibrillar structure. It may be due to influence of sarcomere length and post-mortem proteolysis (Koohmarie et al. 1996). Different muscles at different locations which have different functions may result in differences in tenderness. These differences are due to collagen content as well
as differences in muscle contraction during rigor mortis (King et al. 2003). Rapidly chilled muscle has higher pH values (King et al. 2003). Furthermore, pre-rigor, temperature and pH decline will cause cold shortening and subsequently produce tough meat. Interaction between muscle and chilling treatment was observed for sarcomere length. Rapid chilling causes drastic shortening and will result in toughening of the muscle (King et al. 2003). Rapid chilling also results in lower ultimate temperature and subsequent decreased in temperature will lower the proteolytic activity of \( \mu \)-calpain and finally will cause slowing in the rate of post-mortem tenderization (King et al. 2003). This information is useful in order for the producer to decide which breed will maximise their profit in their production system.

However, searching for all best traits in one breed is difficult as multiple factors influence the meat tenderness. In fact, as said by Wheeler et al. (2001) - "No single breeds is excellent in all traits that are important to beef production". Wheeler et al. (2001) had shown that Belgian Blue and Piemontese (Piedmontese) have a desirable combination of high yield grade and longissimus muscle whereas Hereford and Angus have desirable quality grade and longissimus muscle but Brahman is excellent in heat tolerance.

Although various strategies could be applied to improve meat tenderness, selection based on genetic performance will enable producers to produce superior animals, economically. Selection based on marker assisted selection will result in identification of superior animals at lower cost beside improving food safety and enhancing food quality. Cattle with different genetic potential for marbling and tenderness can quickly be identified and managed to maximize their value (Page et al. 2002). The Calpain gene has been identified as candidate gene for tenderness.

### 1.6.2.1.1 Calpain

Calpain genes are proposed to affect meat quality (Morgan et al. 1993) by initiation of muscle protein degradation. Calpain is cytoplasmic cystein protease which is \( \text{Ca}^{2+} \) dependent (Juszczuk-Kubiak et al. 2004). The micromolar calcium-activated neutral protease (CAPN1) is the main enzyme for degradation of myofibrillar proteins under post-mortem conditions (Koohmaraie, 1992; Koohmaraie 1994; Koohmaraie 1996). \( \mu \)-calpain (CAPN1) is the isoform of
calpain which requires calcium at micromolar concentration for activity (Juszczuk-Kubiak et al. 2004) and may affect variation in tenderization (Geesink and Koohmaraie, 1999). Calpain gene has effect on proteolytic pathway of myofibrillar protein (Ouali et al. 1992) is located on chromosome 29 consist 22 exons (Smith et al. 2000). QTL for tenderness was found segregating in BTA29 (Page et al. 2002). However, a QTL that influences beef longissimus tenderness was also found located 28cM from the centromeric marker on BTA15 (Keele et al. 1999).

Study on CANP1 gene revealed 38 SNPs; two SNPs were found within exon and 36 SNPs were found within introns (Page et al. 2002). The same authors found that glycine rather than alanine at position 316 and isoleucine rather than valine at position 530 were associated with decreased meat tenderness. Glycine to alanine substitution is as a result of transition A—>G in exon 9 and valine to isoleucine substitution due to transversion of G—>C in exon 14.

Study on CANP1 gene polymorphism within exon 9 was also had been carried out in Irish cattle (Hamill et al. 2006). These authors also believe in the potential of incorporation this polymorphism into marker assisted breeding program although currently allele A which represent amino acid Gly present at low frequency. Another study within CAPNI done in Brahman cattle also shows that these SNPs have effect on tenderness. However, allele frequencies detected within Brahman cattle is different than reported in Bos taurus population which indicate that molecular marker develop in Bos indicus require development of additional markers (Casas et al. 2005).

In another study within μ-calpain gene, a SNP within intron 14 was detected (Juszczuk-Kubiak et al. 2004). Transition of C—>T creates FokI restriction site detected with PCR-RFLP analysis. Association between CAPNI-FokI gene polymorphism and meat production was studied in Polish Red and Polish Black-and White cattle (Juszczuk-Kubiak et al. 2004). It was found that animals with genotype TT have higher favourable lean share in valuable cuts than CC animals.
1.6.2.2 Marbling

Marbling refers to intramuscular fat and is an important trait in meat quality. Higher marbling will improve the palatability and tenderness of meat (Crouse et al. 1984 as in Sasaki et al. 2005). Marbling also contributes to the juiciness and flavor of cooked meat (Wood et al. 1999).

Formation of marbling is influenced by various factors including breed (Chambaz et al. 2002), feeding regime (Schoonmaker et al. 2004), length of fattening period (Chambaz et al. 2002) and body weight (Chambaz et al. 2002). It was also found that feeding of grain to cattle produced meat with higher marbling score compared to forage feeding (Schoonmaker et al. 2004). Apart from feed resources, the amount of energy also affecting partitioning of fat deposition in animals (Schoonmaker et al. 2004). Early-weaning fattening system increases marbling but excessive fat also becomes present (Schoonmaker et al. 2004). Although restricting intake during different growing phase may result in leanness of carcass but it also will result in decrease weight gain as well as market value (Schoonmaker et al. 2004).

Marbling is a complex trait affected by various biological pathways of different genes (Pannier et al. 2006). Interaction between genes and production environment may have effect on fat metabolism. However, only few genes has been associated and identified as candidate genes for marbling. These genes are leptin, thyroglobulin (TG) and diacylglycerol O-acyltransferase (DGAT1). Leptin has been proposed as candidate gene for marbling as it influences intramuscular fat in animal (Wheeler et al. 1994). Another recent finding that had been associated to marbling is work on QTL map. A QTL regarding marbling had been mapped to the centromeric region of bovine chromosome 14 and gene encoding thyroglobulin (TG) and DGAT1 are being proposed as the positional and functional candidate gene (Thaller et al. 2003a).

1.6.2.2.1 Leptin

Leptin is a hormone product of the obese gene that acts on central and peripheral tissue (Moschos et al. 2002). It is secreted in various cells and organ includes adipose tissue, placenta, skeletal tissue and pituitary. In ruminant animals, leptin is secreted predominantly by adipocytes (Buchanan et al. 2002; Liefers, 2003).
and recently found during late pregnancy (Macajova et al. 2003; Liefers et al. 2004).

It helps in regulating appetite and metabolism. Important economic factors for animal production which may influenced by leptin include feed conversion efficiency and intramuscular fat, which considered to improve meat quality (Wheeler et al. 1994) although currently there is pressure for leaner meat (Lagonigro et al. 2003). Leptin also has a role in growth, reproduction, appetite regulation (Matteri, 2001) and feed intake (Macajova et al. 2003). Leptin serum has been associated with carcass traits such as ribeye fat thickness, growth rate, carcass composition and marbling (Geary et al. 2003) and increased milk yield and milk protein content (Buchanan et al. 2003). Leptin has been proposed as a candidate gene for evaluation of genetic polymorphism that could affect carcass fat content in cattle.

Adipose tissue deposition occurs once animals reach mature size, when growth occurs in the form of adipose tissue deposition (Corah, et al. 1995; Stephenson et al. 2001). However, the rate of adipose tissue growth varies depending on location. In growing cattle, subcutaneous fat hypertrophy occurs faster than intermuscular and intramuscular (Kempster, 1980). Hypertrophy of adipose tissue is the major fat deposition involved in finishing animals to market weight.

The bovine leptin gene has been mapped to chromosome 4 (Pfister-Genkow et al. 1997; Pomp et al. 1997) and several highly polymorphic microsatellites markers have been reported within the same chromosomal region (Wilkins and Davey, 1997). Variants of the BM1500 microsatellites, located 3.6 kb downstream of the gene have been associated with fat characteristics in beef bulls (Fitzsimmons et al. 1998). Additionally, several SNPs have been reported in both introns and exons of the leptin gene (Lein et al. 1997; Konfortov et al. 1999; Buchanan et al. 2002) but these have not been tested for association with altered leptin function.

Allelic variation in the leptin has been associated with increased fat deposition in beef cattle (Buchanan et al. 2002; Buchanan et al. 2003). Several SNP have been reported in bovine and associated various traits. SNP in exon 2 has been associated to feed intake (Lagonigro et al. 2003), leaner carcass (Buchanan et al. 2002), intramuscular fat (Wheeler et al. 1994) and feed
conversion efficiency (Wheeler et al. 1994). It is found that a transition of C to T that encoded an amino acid change of an arginine to a cysteine was identified at exon 2 of the leptin gene and been associated with fat deposition (Buchanan et al. 2003). T allele was associated with fatter carcasses and the C allele with leaner carcasses (Buchanan et al. 2002). Furthermore, Kononoff et al. (2005) reported that animals with CC genotype have higher carcass weight than TT genotype.

Recently, an effect on polymorphisms at bovine leptin promoter has been reported (Nkrumah et al. 2005). The effect of polymorphisms includes increased backfat thickness, higher marbling score, higher feed intake and growth rate as well as higher slaughter weight. Mutation on bovine leptin promoter also has been reported in dairy (Leifers et al. 2005). They discovered 20 SNPs in the 1.6kb region and out of 20, 14 SNP is associated with leptin concentration during late pregnancy. However, in that study, it was not influencing leptin concentration during lactation. Three SNPs have been associated with fertility, energy balance and protein yield. Further research requires understanding and better knowledge in energy regulation and adipose tissue development and may enable manipulation of meat production in livestock (Laganigro et al. 2003).

1.6.2.2.2 Thyroglobulin

Thyroglobulin (TG) is the glycoprotein precursor of the thyroid hormone (Mendive et al. 2001). In human, thyroglobulin contains at least 37 exons which cover at least 300 kb of genomic DNA and (Baas et al. 1986). The number of exons in bovine is unknown but the EMBL database shows that up to exon 16 has been sequenced.

Quantitative trait loci for marbling have been mapped to the centromeric region of chromosome 14 (Thaller et al. 2003b). Previously, a putative QTL on this chromosome was proposed through an association with the microsatellite locus CSSM66 (Barendse, 1999). Subsequently, the gene encoding thyroglobulin gene (TG) was identified as the candidate gene based on its close linkage to CSSM66 as well as because its product is the precursor of hormones that affect lipid metabolism.

TG was reported can be used to predict fat deposition in muscle tissue as well as assessment of milk fat content (Barendse, 1999). The polymorphisms
were detected within 5' region of TG gene. Analysis of C/T polymorphism of TG sequence was done by PCR-RFLP using enzyme PsuI. Allele C, which also known as allele '2' generate three fragment of 295, 178 and 75 bp, whereas T allele which also known as allele '3' produce two fragments of 473 and 75 bp. Allele 3 in the 5'-region of TG was associated with enhanced marbling (Barendse, 1999).

Effect of polymorphism within 5' of TG in German Holstein and Charolais was carried out by Thaller et al. (2003b). The traits investigated for the association of this polymorphism was fat content of m. semitendinosus and m. longissimus dorsi. It was found that TG has significant effect on m. longissimus dorsi in both breeds.

However, a study on association of TG on polymorphism with carcass composition in Brahman cattle revealed that TG is associated with fat thickness but not with marbling score (Casas et al. 2005). However, allele frequencies detected within Brahman cattle is different than reported in Bos taurus population. This indicate that molecular marker developed for marbling in Bos indicus require further development of additional markers (Casas et al. 2005).

1.6.2.2.3 Diacylglycerol O-acyltransferase
Gene encoding the enzyme diacylglycerol O-acyltransferase (DGAT1) was been mapped within the region of marbling QTL as in TG. It consists of 17 exons. DGAT1 has been associated to the fat content of milk (Winter et al. 2002; Kaupe et al. 2004). It was hypothesized that a lysine residue at position 232 of the DGAT1 protein, as is found in all non-bovine mammalian species studied so far, could confer more efficient binding of acyl-coenzyme A than an alanine residue at this position (Winter et al, 2002). It was postulated that at DGAT1, lysine variant is result in increasing fat yield, fat and protein percentage whereas alanine variant result in increasing protein and milk yield (Winter et al. 2002). Analysis of lysine/alanine polymorphism of DGAT1 sequence was done by PCR-RFLP using enzyme CfrI. Alanine variant generated two fragments of 203 and 208 bp, whereas no digestion occured in the lysine variant.

Recently, the effect of DGAT1 on fat related traits has been studied (Thaller et al. 2003a). In this study, the lysine allele was identified to contribute to intramuscular fat (Thaller et al. 2003a). Effect of lysine/alanine polymorphism
with \textit{DGAT1} in German Holstein and Charolais was carried out (Thaller \textit{et al.} 2003b). Investigation on fat content of m. semitendinosus and m. longissimus dorsi shows that \textit{DGAT1} is suitable as candidate genes for marbling in both breeds. In this study, it was found that marbling is higher in German Holstein cattle compared to Charolais cattle as indicated by the frequencies of lysine. Homozygous lysine/lysine of \textit{DGAT1} in German Holstein had significantly higher marbling in m. semitendinosus and m. longissimus dorsi than heterozygous lysine/alanine or homozygous alanine/alanine (Thaller \textit{et al.} 2003b).

It was also found that beef breeds have a tendency to have the alanine allele compared to dairy cattle whereas dairy cattle have both low and high frequencies of the allele lysine (Kaupe \textit{et al.} 2004). However, study on association of \textit{DGAT1} polymorphism with carcass composition in Brakmas cattle revealed that there is no association of \textit{DGAT1} polymorphism with carcass composition such as marbling score and tenderness (Casas \textit{et al.} 2005).

\textbf{1.6.3 Other economic traits}

Other economically important traits include biotic stress resistance, abiotic stress resistance, milk production traits as well as fertility traits. Abiotic stress refers to non-living factors or environmental factors such as extreme temperature and nutritional condition. Continuous abiotic stress can result in reduced performance and it was a major constraint in animal production. For example, animals exposed to high temperature will reduce feed intake in order to reduce heat production and subsequently this will result in low production performance.

Biotic stress, on the other hand refers to stress induced by biological organisms such as bacterial, viral and fungal infections. These organisms usually will affect health performance of the animals and subsequently reduce the production performance. Disease control options includes vector control, animal movement control and quarantine, disease eradication, vaccination, drug treatment, proper management practices and selection of disease-resistant animals. Some of the diseases that had been studied and are associated to disease resistance are brucellosis, trypanosomiasis, bovine leucosis and helminthiasis (Teale, 1999). However disease resistance is not absolute but depends on other
environmental factors such as nutritional status and level of challenge. In broad terms, under tropical conditions the *Bos indicus* type of cattle have increased resistance to biotic and abiotic stresses met in such climates.

Other important economic traits are related to milk production. Various studies had been made on milk production, milk fat and protein content as well as other milk composition (Bawden and Nicholas, 1999). Milk production also had been associated to lactation number, age during lactation, calving interval. The contribution factor to the milk production includes nutritional factor, climate as well as genetic background.

Fertility trait is another important economic trait. Various studies had been carried out on genetics: puberty, gestation length and conception rate (Kirkpatrick, 1999). Fertility trait is a contribution factor of nutritional level as well genetic background of the animals.

### 1.7 Malaysian cattle scenario

The beef industry in Malaysia, like that in other countries, is also facing huge challenges. Apart from low cattle population, local cattle also have low growth rate. A high proportion of the beef is imported for local consumption, mostly from India, Australia and New Zealand. Production of beef in Malaysia is at 19% self-sufficiency ([link](http://faostat.fao.org/site/336/DesktopDefault.aspx?PageID=336) and [link](http://agrolink.moa.mv/iph/dvs/statistics/stsara-all.html)). Production of beef in Malaysia increased from approximately 15,000 to 26,000 tonnes from 1990 to 2004, although the consumption nearly doubled from 64,000 to 114,000 tonnes in the same period ([link](http://faostat.fao.org/site/336/DesktopDefault.aspx?PageID=336)). In the early 1970s, huge importation of animals from abroad of high quality performance was carried out in order to enhance the population of the cattle. To a certain extent, improvement had taken place. For example Brakmas, a crossbred between local Malaysia cattle Kedah-Kelantan and Brakman had produced an increment of up to 35% in body weight gain.

Optimization of resources in beef industry leads to beef production under oil palm plantations (Figure 1.1). Malaysia has more than three million hectares of oil palm plantation. With these huge plantation areas and the amount of
vegetation found under the oil palms, integrated rearing of beef cattle is found economically viable and has been identified as an innovative system. Brakmas had been developed by Malaysian Agriculture Research and Development Institute (MARDI) and fits well under the integrated cattle/oil palm production system.

1.7.1 Development of Brakmas

Brakmas is the crossbred resulting from many generation of continuous selection and breeding of Brahman bulls from Texas, USA and local indigenous Kedah-Kelantan beef cattle. Brakmas is medium in size, white greyish in colour and has resistance to infestations by local and external parasites. Generally, Brakmas produce an increment of up to 35% in body weight gain compared to Kedah-Kelantan. Brakmas have an average birth weight, weaning weight, yearling weight and mature weight of 20.2 kg, 97.3 kg, 119.0 kg and 316 kg, respectively. In oil palm plantations, Brakmas achieved body weight of 180 kg at one year of age with an average daily weight gain of 0.5 kg.

To further improve the performance, the focus will be into economically important traits. And with current knowledge and understanding of the genome, there are possibilities for direct identification and selection of animals carrying the best genes through selection on genotype. To improve the beef production and meat quality of beef, investigations will need to be carried out on those genes involved in the control of animal growth as well as those genes related to meat quality.

1.8 Aims

To improve the production and meat quality of beef, investigations will be carried out on those genes involved in the control of animal growth as well as those of genes related to meat quality. The focus will be to identify and characterise genetic variation underlying economically important traits in Brakmas cattle, finally aiming to characterise the roles of these identified genetic polymorphisms on growth and meat quality and important economic traits in Brakmas. Also, awareness of the value of genetic resources has stimulated the study of the genetic diversity of native cattle. Little information is available
Figure 1.1 (A) Kedah Kelantan cow (B) Brahman bull (C) Brakmas cattle (D) Brakmas cattle grazing on pasture; note dryness and red, lateritic tropical soils. (E) Brakmas cattle grazing under oil palm plantation (F) Processing of oil palm leaf into feed ingredient for cattle.
concerning the genetic diversity of cattle in Malaysia. Despite the introgression of several European breeds into Malaysian cattle for upgrading native breeds, it is also important to preserve local breed’s genetic resources.

The overall aims of this present work are to investigate the genetic variation in Brakmas cattle which can be linked to economic traits using a series of DNA-based markers. The results of these experiments will lead to a measurement of DNA-level variation within Brakmas cattle which will be helpful in determination of appropriate breeding targets as well as for improvement strategies. The polymorphism detected will allow us to determine underlying quality characters present within the different breeds and individuals that are analysed, and allow evidence-based decisions about upgrading (introgression of new characters) or selection for quality traits to be implemented in the breeds. Finally, fundamental results will allow us to make decision in future breeding programmes. For this purpose, a wide range of molecular and cytological techniques was used. Molecular techniques comprised PCR amplification, cloning and sequencing, PCR-RFLP. Some investigations at cytological level were performed by fluorescent in situ hybridization.

More specifically, the aims of the work were:

(1) to measure diversity within Brakmas cattle using microsatellites as indicator which will be presented in Chapter 3.

(2) to determine the underlying genes regarding to growth, and the genes under investigation are growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factor-I gene (IGFI) and pituitary-specific transcription factor (PIT1 or POU1F1) see in Chapter 4.

(3) to determine the underlying genes regarding to meat quality and the genes under investigation that contribute to meat quality are calpain, leptin, thyroglobulin (TG), diacylglycerol acyltransferase (DGAT1) see Chapter 5.

Knowledge of effects of polymorphism in livestock is important for the manipulation of meat production in livestock. It is hoped that knowledge and understanding of the nature of polymorphism will provide the basis for future selection. Understanding of the function of genes will lead to the development of new genetic markers to enhance beef production performance. Baselines for diversity in the breed can be set and used to assess any loss of diversity within the population and for conservation.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Blood samples
A total of 37 blood samples of Brakmas (crossbred of Kedah-Kelantan (KK) and Brahman bulls) were obtained from Malaysian Agriculture Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. The blood samples were collected at MARDI station in Bukit Ridan, Pahang and were transported to laboratories in MARDI Serdang. DNA was isolated and transported in microcentrifuge tubes in dried form to University of Leicester, Leicester, United Kingdom. Blood and blood products were carried and imported under the conditions of the DEFRA Animal Health Act 1981 and Importation of Animal Products and Poultry Products Order 1980 under Import Licence No TAY/03/707 issued 28 May 2003. Animal codes are as in Table 2.1

2.2 Methods

2.2.1 DNA

2.2.1.1 DNA extraction
As mentioned above, DNA samples were extracted using a Promega Wizard® Genomic DNA Purification System according to the manufacturer’s instructions. Precipitated DNA samples were labelled appropriately and transported in dried form. Table 1 shows the code of animals used in the studies.

2.2.1.2 Estimation of DNA concentration
The concentration of DNA was estimated by running on a gel together with a ladder of known size and concentration. The DNA was mix with 0.5 μl loading buffer (6X stock containing 0.25% bromophenol blue, 0.25% xylene cyanol FF and 50% glycerol in H2O) and run in a 0.8% agarose gel (0.8 g agarose in 100 ml TAE buffer; 50X stock of TAE buffer containing 121 g of Tris-base, 28.6 ml glacial acetic acid, 50 ml of 0.5 M EDTA and adjusted to pH 8) containing 0.5 μl of ethidium bromide (10mg/ml) at 80-100 V for 45 min. The gel was
photographed under UV. Concentrations were also measured by spectrophotometry.

Table 2.1. Animal code of animals used in experiments

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2.2.2 Primers

2.2.2.1 Primers from literature

Primers for amplification by PCR for genes were selected from various published articles that related to the genes of interest. Primers for growth hormone receptor gene, insulin-like growth factor 1 gene and pituitary-specific transcription factor 1 gene were in chapter 4 (Table 4.1). Primers for meat quality include three pairs
of primers to amplify three fragments within calpain genes, and one pair each for
the leptin gene, thyroglobulin gene and diacylglycerol O-transferase 1 gene were
as in Table 5.2.

2.2.2.2 Primer design
Primers for growth hormone gene fragments were designed from Genbank
accession M57764 using online primer design software, Primer3
(http://www.genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi). The fragment
sizes were designed to be 400 bp to 700 bp and this produced four sets of primers
within the growth hormone gene.

2.2.3 PCR
2.2.3.1 Standard PCR
Polymerase chain reaction (PCR) reactions for gene amplification were
performed in 20 μl reactions containing (final concentrations): 1X PCR buffer
(10X buffer contain 10mM Tris-HCl (pH 9.0), 50mM KCl and 0.1% Trixton®X-100 from Promega), 1.5 mM MgCl₂, 160 μM of dNTPs (Roche), 0.3 μM of each
primer (Sigma), 0.5 U of Taq DNA Polymerase (Promega) and 0.7 μl of template
DNA with a concentration range from 10-30 ng/μl. Cycling conditions for primer
pairs were denaturation at 94°C for 3 minutes; 30 cycles of 94°C for 1 minute,
annealing temperature for 1 minute (as in Table 4.1 and 5.1 for growth related
genes and meat quality relates genes, respectively) and extension at 72°C for 1
minute, followed by final extension at 72°C for 5 minutes. PCR reactions were
performed in a TGradient Thermocycler (Biometra).

After PCR amplification, PCR products were separated by 1% agarose
gel electrophoresis in 1X TAE running buffer. PCR products were visualised by
staining with ethidium bromide and the most prominent bands at the expected
size were recovered from gel by using QIAquick Gel Extraction Kit (Qiagen)
according to the manufacturer’s instructions.

For microsatellites, PCR amplification was carried out as follows:
Reactions were performed using 6-10 ng/μl of template DNA in 10 μl reaction
volumes containing 1X PCR buffer (10X buffer contain 160 mM (NH₄)₂SO₄, 670
mM Tris-HCl (pH 8.8) and 0.1% Tween 20 from Bioline), 1.5 mM MgCl₂, 160 μM of each of dNTP (Roche) and 0.3 μM of each primer (Sigma) and 0.5 U of Taq polymerase (Bioline) in a T-Gradient Thermocycler (Biometra). The amplification was performed by an initial 3 minutes denaturation at 95°C, followed by 30-35 cycles of denaturation for 30 seconds at 94°C, primer annealing for 30 seconds at 55-69°C (Table 3.1) and an extension for 45 seconds at 72°C and a final extension at 72°C for 5 minutes. For confirmation of proper amplification of the microsatellites, a few of different bovine accessions were tested initially by agarose electrophoresis before carrying out polyacrylamide gel electrophoresis. 10 μl of these reactions were electrophoresed on a mixture of 1% high resolution agarose gel and 1.5% high gel strength agarose in 1X TAE buffer with 0.5 μg/ml of ethidium bromide. 25 bp ladders were used to provide sequence ladders for initial allele size reference.

2.2.3.2 PCR optimization
PCR reaction for DGAT1 used additives of 5% dimethyl sulfoxide (DMSO) for equal amplification of the alleles. PCR reactions were performed in 20 μl reactions containing: 1X PCR buffer (10X buffer contain 10mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1% Triton®X-100 from Promega), 1.5 mM MgCl₂, 160 μM of dNTPs (Roche), 0.3 μM of each primer (Sigma), 1 μl DMSO, 0.5 U of Taq DNA Polymerase (Roche) and 0.7 μl of template DNA with a concentration ranges from 10-30 ng/μl. Cycling conditions for primer pairs were 94°C for 3 minutes; 30 cycles of 94°C for 1 minute, annealing temperature at 60°C for 1 minute and extension at 72°C for 1 minute, followed by final extension at 72°C for 5 minutes. After PCR amplification, PCR products were separated by 1% agarose gel electrophoresis as mentioned in 2.2.3.1 above.
2.2.4 Agarose gel electrophoresis
After PCR amplification, PCR products were separated by 1% agarose gel electrophoresis in 1X TAE running buffer. To prepare agarose gels, 100 ml of 1X TAE buffer was used to suspend 1.0 g of agarose (Fisher Scientific DNA Grade) and the mixture was microwaved for 2 minutes until the agarose was completely dissolved. After cooling, 0.5 µl of 10 mg/ml ethidium bromide was added to the solution and mixed, poured into the gel casting mould, loading comb inserted and left to solidify. PCR products were mixed with 6X loading dye buffer and separated by 1% agarose gel electrophoresis in 1X TAE running buffer at 5-8 V/cm. Products were visualized by staining and the size of the products was checked by comparison with Hyperladder II (Bioline) as markers of length and concentration.

2.2.5 Denaturing polyacrylamide gel electrophoresis
For microsatellites with larger allele size differences, bands can be separated in agarose gels, but in the case of small allele size differences, detection between alleles can only be made by separation in polyacrylamide gels. Detection of microsatellites using denaturing 6% polyacrylamide gels is preferred as denaturing the samples produces single-stranded DNA and results in clear band separation.

2.2.5.1 Preparation of denaturing polyacrylamide gel electrophoresis
Gels were prepared using urea (14.4 g), 40% acrylamide:bis (19:1) solution (4.5 ml), 10X TBE buffer (3.0 ml), and topped up to 30 ml with distilled water. 15 µL of TEMED and 150 µL of 10% ammonium persulfate (APS) (15 mg and 150 µL dH₂O) and were added to the solution right before casting.

A BioRad Sequi-Gen® (38 x 50 cm) plate was used to run gels. The bigger and the small glass plate were properly cleaned of any debris, with detergent and rinsed with water before being dried. Alcohol was applied to both plates and let dry. Then, the bigger plate was treated with 5 µl of bind silane on one side while the smaller plate was treated with 15 µl of repellent solution. The silane treatment allows the gel to stick to and the repel to release from the glass plate after electrophoresis.
The gels were left to polymerize at least 1 hour at room temperature. The gels were pre-run for 30 minutes in 1x TBE buffer before loading the samples to warm up the gel to about 50°C. 3 μL PCR products were added with an equal volume of loading dye. Denaturation of the samples was done for 5 minutes at 95°C in PCR machine immediately before loading and placed on ice while loading into the gel. The denaturing polyacrylamide gel electrophoresis was run for 75 minutes at 50°C, 110 W (BioRad PowerPac 3000). After electrophoresis, the plates were separated and the bigger plate with gel attached to it was treated in silver stain.

2.2.5.2 Silver staining of the polyacrylamide gels
The gel plate was placed in a tray containing fixer (200 ml glacial acetic acid in 1.8 liters distilled water) on shaker under the fume hood for 30 minutes. Then the fixer was poured off and saved for later. The gel was rinsed in water before placing it in fresh water on a shaker for 10-15 minutes. The gel was rinsed again with water and placed in silver stain (containing 12 ml 1N silver nitrate solution in 2 liters distilled water and 3 ml formaldehyde was added), left on shaker for 30 minutes. Then gel was removed from the silver stain. Immediately before developing, 300 μl sodium thiosulphate (0.1N) and 3 ml formaldehyde (40%) was added to developer solution (containing 60 g of sodium carbonate dissolved in 2000 ml distilled water). The developer solution was dipped into the tray that contained silver stain. Meanwhile the gel was placed in water for 10 seconds before placing into the tray containing developer. Once bands developed, the reaction was stopped with the fixer saved earlier and finally the gel was rinsed in water for 20 minutes and left to dry. The dried plate was scanned directly using a high resolution scanner, and the image saved in .psd format.

2.2.6 Cloning
2.2.6.1 Recovery of DNA from gels or gel extraction
PCR products were extracted as a band from a gel. 60 μl PCR reactions were loaded onto 1.0 % gel (1.4 g in 140 ml TAE buffer). Each band from the most prominent bands at expected size were cut from the gel using a sterile scalpel blade and placed into a 1.5 ml microcentrifuge tubes. The DNA was extracted
from the agarose using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. The DNA concentration and length of the PCR fragments was checked with 5 µl of the product by 1% agarose gel electrophoresis in 1X TAE running buffer with Hyperladder II (Bioline) containing bands of known length and concentration.

2.2.6.2 Ligation of DNA fragments

PCR fragments were ligated into pGEM-T plasmids by using the kit pGEM® T easy vector system I (Promega) according to manufacturer's instructions with minor modifications. The ligation was performed in 10µl reaction containing 5µl of 2X Rapid Ligation Buffer (60mM Tris-HCl, (pH 7.8), 20 mM MgCl₂, 20 mM DTT, 2 mM ATP and 10% PEG); 1 µl of the pGEM-T plasmid; 1 µl T4 DNA ligase (in 10mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA and 50% glycerol) and 3 µl PCR product. The reaction was incubated for 1 hour at room temperature and then overnight at 4°C to maximize the number of recombinant plasmids.

2.2.6.3 Preparation of competent cells

Cells from the frozen glycerol stock of E. coli DH5α were streaked onto 10 ml antibiotic free LB (Luria Broth) liquid medium (containing 1.0 g tryptone, 0.5 g yeast extract 1 g NaCl, topped up to 100 ml with dH₂O, adjusted to pH 7.0) and incubated and grown overnight at 37°C in a 220 rpm shaker. A plastic loop was dipped in the overnight culture and streaked onto antibiotic free LB agar plate and grown in oven at 37°C overnight. One colony was transferred to a 10 ml antibiotic free LB and incubated overnight at 37°C in 220 rpm shaker. 5 ml of the overnight culture was transferred to a flask containing 50 ml antibiotic free SOB (Super Optimal Broth) medium (containing 2.0 g tryptone, 0.5 g yeast extract, 0.05 g NaCl, 1 ml 250 mM KCl and topped up to 100 ml dH₂O, adjusted to pH 7.0) and incubated at 37°C for 60-90 minutes. The culture was measured until the optical density at 600 nm was 0.6 (OD₆₀₀=0.6). The cell was pelleted by centrifugation at 3000 rpm at 4°C for 10 minutes. The supernatant was then carefully poured off. The pellets were re-suspended gently in 25 ml ice-cold 50mM CaCl₂ and set to rest on ice for 30 minutes. The cells were centrifuged again at 3000 rpm at 4°C for 10 minutes. The supernatant was poured off and the
cells were re-suspended gently in 2.1 ml ice-cold 50mM CaCl₂ and placed on ice. Then 900 µl of ice-cold 50% glycerol was added and mixed. The cells were aliquot 100 µl onto micro-centrifuge tube and fast frozen in liquid nitrogen before storage in a -80°C freezer.

Figure 2.1 pGEM®-T easy vector circle map and sequence reference points. pGEM®-T easy vector used in cloning of the genes in all the studies. M13 forward sequencing primer binding site located at position 2956-2972 and M13 reverse sequencing primer binding site located at 176-192.

2.2.6.4 Transformation of competent cells
The ligation reaction was transformed into the competent cells by heat shock. The ligation mixture of 1 µl was added to a half thawed tube of 100 µl competent cells and mixed briefly and was incubated for 30 minutes on ice. The tubes were then placed in a 42°C water bath for 90 seconds and placed on ice straight away for 2 minutes. 900 µl SOB was added to the tube and incubated in a 180 rpm shaker for 90 minutes at 37°C. Then the culture was pelleted by centrifugation for 5 minutes at 5000 rpm. 900 µl supernatant was sucked out by pipette gently.
The balance were mix by pipette and spread onto an agar plate containing 80μg/ml X-gal (5 bromo-4-chloro-3-indolyl-β-D-galactosidase), 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 100 mg/ml ampicillin and leave overnight in 37°C oven for 12-16 hours.

2.2.6.5 Selection and storage of recombinant clones

White colonies were picked using micropipette yellow tips and dipped in 10 ml LB medium containing 10 μl ampicillin and incubated a further 16-20 hours at 37°C in 230rpm shaker. The white colony was chosen as this was the successful transformants. The pGEM-T plasmid carries lacZ gene coding for a β-galactosidase that digests the chromogenic X-gal producing blue colonies, but if a DNA fragment is inserted into the pGEM-T plasmid it disrupts the lacZ gene and thus the resulting white colonies are an indication of successful transformed bacteria with recombinant plasmid. The plasmids were isolated from the overnight cultures by a mini plasmid preparation kit from Promega (Wizard® Plus Minipreps DNA Purification System) following the manufacturer’s instruction, with slight modification, using all 10 ml (100μg/μl) of the overnight culture instead of 1-3 ml as suggested in the protocol. To confirmed the presence of the insert in the pGEM-T plasmids, restriction digestion of plasmid DNA using EcoR1 enzyme or PCR using M13 primers were carried out. Restriction digests were carried out in a mixture containing 1.5 μl buffer (NEB), 0.5 μl enzyme EcoR1 (NEB), 2 μl miniprep DNA and 11 μl dH2O for 4 hours at 37°C before the digestion product were run on 1% agarose gel electrophoresis. If confirmation was by PCR, PCR on 0.5 μl of the overnight LB culture carried out using M13 primers in 15 μl mixture containing 1X PCR buffer (10X buffer contain 160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8) and 0.1% Tween 20 from Bioline), 1.5 mM MgCl₂, 0.16 mM of dNTPs (Roche), 0.2 μM of each M13 primer and 0.5 U of Taq DNA Polymerase (Bioline). PCR cycling conditions were: 94°C for 3 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 7 minutes. The PCR products (5 μl) were run on 1% agarose gel electrophoresis with Hyperladder II (Bioline) for checking length of insert and concentration.
2.2.6.6 Sequencing and EMBL-EBI Database submission

Thyroglobulin gene clones were sequenced at the PNACL Sequencing Facility of Leicester University. Clones for the other genes were sequenced by a commercial service at the John Innes Centre (JIC), Norwich.

For sequencing sent to the Protein and Nucleic Acid Chemistry Laboratory (PNACL, Leicester University), the recovered plasmid of about 100-110 ng was supplied in 8 μl Sigma water in 0.5 ml micro-centrifuge tubes. Sequencing was done using M13 forward and reverse primers using an ABI 377 automated sequencer. For sequencing sent to the John Innes Centre (JIC), Norwich 10 μl plasmid was supplied with 10 pmol of related primers. The sequence with the chromatogram was submitted in computer format was then viewed and edited using Chromas program (www.technelysium.com.au/Chromas.html). The sequences of the genes clones were aligned with the ClustalW using default settings. Forward and reverse primers were included within the sequence and were aligned with GenBank accessions (Table 4.1 and 5.1). For alignments, the forward primer was used as a start site. The Brakmas sequences were submitted to EMBL-EBI Database with the accession numbers AM263422 to AM263425 and AM266809 to AM266811.

2.2.7 PCR-RFLP

Restriction enzymes reported in published articles which have been associated to growth were used to screen the PCR products from Brakmas cattle population in this study. Upon identification of restriction enzyme site polymorphisms for the fragment amplified, the population was genotyped by PCR-RFLP. Digestion was performed using New England Biolab enzymes in 10 μl reaction containing: 1X buffer (NEB), 0.5U enzyme, 100-200ng PCR product and 1x BSA in certain restriction enzymes. The mixture was incubated at 37°C or 60°C depending on restriction enzyme, overnight. Table 2.2 shows the details of buffer and incubation temperature required for the enzyme digestion. Digested PCR products were separated by 2% agarose gel electrophoresis in 1X TAE running buffer and were visualised by staining with ethidium bromide before photography.
Table 2.2. Site of restriction enzyme, source of enzyme, buffer and incubation temperature for restriction enzyme under study.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Site</th>
<th>Source</th>
<th>Buffer</th>
<th>BSA</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>HinfI</td>
<td>G^ANTC</td>
<td><em>Haemophilus influenzae</em> Rf</td>
<td>NEB2</td>
<td>-</td>
<td>37°C</td>
</tr>
<tr>
<td>PvuI</td>
<td>CGAT^CG</td>
<td><em>Proteus vulgaris</em></td>
<td>NEB3</td>
<td>BSA</td>
<td>37°C</td>
</tr>
<tr>
<td>FokI</td>
<td>GGATG(N)9^</td>
<td><em>Flavobacterium okeanokoites</em></td>
<td>NEB4</td>
<td>-</td>
<td>37°C</td>
</tr>
<tr>
<td>HpyCH4V</td>
<td>TG^CA</td>
<td><em>Helicobacter pylori</em> CH4</td>
<td>NEB4</td>
<td>-</td>
<td>37°C</td>
</tr>
<tr>
<td>Clal</td>
<td>AT^CGAT</td>
<td><em>Caryophanobacter latum</em> L</td>
<td>NEB4</td>
<td>BSA</td>
<td>37°C</td>
</tr>
<tr>
<td>BstYI</td>
<td>Pu^GATCPy</td>
<td><em>Bacillus stearothermophilus</em></td>
<td>NEB2</td>
<td>-</td>
<td>60°C</td>
</tr>
<tr>
<td>EaeI</td>
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<td><em>Enterobacter aerogenes</em></td>
<td>NEB1</td>
<td>-</td>
<td>37°C</td>
</tr>
</tbody>
</table>

1Restriction site; 2Incubation temperature

2.2.8 **In situ Hybridization**

2.2.8.1 **Blood collection**

Blood was collected in heparin vacuum tubes and kept in dark at room temperature. The blood was immediately transported to United Kingdom and to maintain the quality, the blood was ensured to arrive within 3 days after collection.

2.2.8.2 **Preparation of white blood cell culture**

White blood cell cultures were prepared by adding 0.5 ml blood into 7.5 ml culture medium containing 12 ml foetal serum, 1 ml penicillin-streptomycin (10,000 U penicillin and 10 mg streptomycin in 1 ml distilled water), 40 µl lectin, 40 µl phytohemagglutinin (PHA) and top up with RPMI 1640 medium until 100 ml. The culture was incubated at 37°C in carbon dioxide incubator for about 72 hours with a loose cap.
2.2.8.3 Blood harvesting

10 μl colcemide was added to the 8 ml cultures and left at 37°C in a carbon dioxide incubator for 50 minutes. The culture medium was transferred to a Falcon tube and the cells were centrifuged at 1400 rpm for 10 minutes before the supernatant was discarded and the cells were carefully treated with 10 ml pre-warmed at 37°C hypotonic solution of KCl 0.075 M. The cells were centrifuge again at 1400 rpm and treated again with 10 ml pre-warmed at 37°C hypotonic solution of KCl 0.075 M. Then the culture was incubated at 37°C for 17 minutes. The cells were centrifuged at 1600 rpm for 10 minutes before the supernatant was discarded. Fixatives of 3 methanol:1 glacial acetic acid were added; drop by drop at the beginning until reaches about 1 ml and top up until 12 ml. The cells were again centrifuged at 1600 rpm for 10 minutes and the supernatant was discarded. Fixatives were added to about 10 ml and left for 30 minutes. Finally the cells were centrifuged at 1750 rpm for 10 minutes and the supernatant was discarded before cells were stored at -4°C as required for a few months;

2.2.8.4 Preparation of chromosome spreads

For chromosome preparation, slides were prepared by dipping the slides into chromic acid for at least 3 hours. Then the slides were put under running water for 5 minutes before rinsing twice with distilled water. Subsequently, the slides were dried in oven at 60°C before dipping in ethanol for at least 10 minutes and wiping with tissue paper until dry.

Chromosome dropping was done by dropping the sample at the height of about 4 cm and manually blowing to spread the drop before evaluating in phase contrast under the microscope. The slides were left to dry before storage in a container containing silica to ensure the slides are maintained in dry condition and kept in -20°C until used.

2.2.8.5 Labelling of probe

Labelling was performed with the Random Primers DNA Labelling System (Invitrogen Life Technologies) for inserts from plasmid according to the manufacturer’s instructions with slight modification. Approximately 100 ng of DNA was dissolved in 26μl of dH2O and the DNA was denatured in the microcentrifuge tube by heating for 5 minutes in boiling water and rapidly cooled
on ice. Then, the mixture containing 20μl Random Primer Buffer Mixture 5 μl dNTP mix (10X dNTP mix) that contained 1 μl biotin-dUTP were added and mixed briefly. 1 μl Klenow Fragment was added immediately before the mixture was incubated at 25°C overnight. 5.5 μl of 3M sodium acetate was added to stop the reaction and 110 μl of 95% ice cold ethanol was applied and kept at -20°C overnight or at least 4 hours. The next day, the mixture was spun at 12,000 rpm for 20 minutes at 4°C. The supernatant was discarded before adding 500μl of 70% ice cold ethanol, spinning at 12,000 rpm for 5 minutes at 4°C and finally the supernatant was discarded again. The pellet was air-dried at room temperature or at 37°C for 10 minutes. 20 μl of EB buffer (Qiagen) was used to dilute the pellet and left on ice for 30 minutes before storage at -20°C.

Efficiency of probes was checked using a dot-blot test. The procedure was as in Schwarzacher and Heslop-Harrison (2000). A charged nylon membrane was soaked in buffer 1 containing 100 mM Tris-HCl (pH 7.5), 15 mM NaCl for 5 minutes and dried in between filter paper. About 0.5 μl of labelled DNA probes was micro-pipette onto the membrane and left to adsorb and partly dried for 5-10 minutes. Then the membrane was soaked in 4 ml buffer 1 for 1 minute before soaking on a gently shaking shaker for 30 minutes in buffer 2 containing blocking reagent (Roche) 0.5% (w/v) in buffer 1. Then the buffer was poured off before antibody-AP mixture (anti-biotin conjugated to alkaline phosphatase (Roche) in 1:1000 dilution for a final concentration of 0.75 unit ml⁻¹) was distributed over the membrane and covered with plastic sheet and incubated gently on shaker at 37°C for 30 minutes. Then the membrane was washed in buffer1 for 15 minutes before washing another 2 minutes in buffer 3 containing 100 mM Tris-Hcl, pH 9.5, 100 mM NaCl and 50 mM MgCl₂. Then the membrane was poured with detection reagents containing 22.5 μl of NBT solution (4-nitroblue tetrazolium chloride at final concentration of 75 mg ml⁻¹ in 70% dimethylformamide) and 17.5 μl of BCIP (5-bromo-4-chloro-2-indolylphosphate at final concentration of 50 mg ml⁻¹ in 70% dimethylformamide) and left in the dark to develop fully for 5-10 minutes. Finally, the membrane was washed in water and air-dried. In a successfully labelled probe, the dot appeared in a colour range between dark brown to dark purple.
2.2.8.6 *In situ* hybridization

*In situ* hybridization was performed by starting with pre-treatment. Pre-treatment is important in order to remove RNA and protein that may increase the background during binding of probe and detection reagents. Slides were re-fixed in 3:1 ethanol: acetic acid for 10 min before washed twice in 96% ethanol for 10 min and air-dried. 200 μl RNase was applied to slides, covered with a plastic coverslip and incubated for 1 hr at 37°C in a moist chamber. The cover slips were removed carefully and then washed in 2X SSC (containing 175.3g NaCl and 88.2g Na\textsubscript{3}C\textsubscript{6}H\textsubscript{5}O\textsubscript{7}.2H\textsubscript{2}O (trisodium citrate) topped up with dH\textsubscript{2}O to one litre with pH of 7 and autoclaved) twice for 5 minutes before incubating in 10 mM HCl for another 5 min. Excess fluids were removed and 200 μl pepsin were added to each slide, covered with plastic coverslips and incubated for 10 min at 37°C in a moist chamber. Slides were then placed in distilled H\textsubscript{2}O for 1 min and washed twice with 2X SSC for 5 minutes. Then the slides were placed in paraformaldehyde fixative (containing 4g of paraformaldehyde in 80 ml H\textsubscript{2}O which was heated to 60°C and was added with strong NaOH to clear the solution and adjust to pH 8 with 1N H\textsubscript{2}SO\textsubscript{4}, H\textsubscript{2}O before top up to 100 ml) under the flow hood for 10 minutes and rinsed in SSC twice for 5 minutes. The slides then were dehydrated in an alcohol series of 70%, 90% and 96% ethanol for 2 min each and air dried.

Hybridization mixture is prepared depending on the stringency required. Higher stringency allows only higher homology sequences to be detected. Hybridization mixture with a total of 50 ml was prepared consisting of 25 μl 100% of formamide, 2.5 μl of 20X SSC, 10 μl of 10% dextran sulphate, 1 μl of 1μg/ml salmon sperm DNA, 0.62 μl of 10 mM EDTA, 0.63 μl of 10% sodium dodecyl sulfate, 3 μl of probe and 7.5 μl dH\textsubscript{2}O. When the hybridization mixture has formamide of 50% and 1X SSC, the hybridization mixture was at 82% stringency. The hybridization mixture (probe mixture) was denatured at 80°C for 10 minutes. Then the probe mixture was added to the slide and covered with a plastic cover slip and transferred to ThermoHybaid HyPro-20 thermal cycle. The slide were denatured at 78°C for 7 minutes with vibration effect as vibration increased the chances for the probes to be to the target chromosome sites. The temperature was let to drop slowly to temperature of 37°C overnight.
Post hybridization washes were performed to remove excess hybridization mixture and unbound probes. Higher stringency than hybridization stringency allowed non-specifically or weakly bound probe to be removed in order to reduce the background signal. Firstly, the slides were placed in 2X SSC at 35-42°C to float off the coverslips from the previous step during hybridization. The slides were washed with fresh 2X SSC at 42°C for 2 minutes. Then the slides were washed with high stringency wash containing 20% formamide and 0.1X SSC at 42°C for 5 minutes twice followed by lower stringency washes containing 10% formamide and 0.1X SSC 42°C for 5 minutes twice. Finally the slides were washed in 20% and 2X SSC at 42°C for 5 minutes twice.

For fluorescent detection, slides were placed in detection buffer of 4X SSC containing 0.2% Tween 20 for 5 minutes. 200 μl bovine serum albumin (BSA – EM grade) block containing 5% BSA in detection buffer was applied on slide and covered with plastic coverslip before incubated at 37°C for 5-30 minutes in a humid chamber. The BSA block was drained away before detection solution was applied. The detection solution of about 30-40 μl containing streptavidin conjugated to Alexa 594 (Molecular Probe) in BSA block was applied onto the slides, covered with coverslip and incubated for 1 hour at 37°C in a humid chamber. After that, the coverslip was removed and the slides were washed in detection buffer for 20 minutes at 42°C three times. Then the slides were counterstained with 100 μl DAPI solution (4’6-diamidino-2-phenylindole) containing 4 μg/ml DAPI in McIlvaine’s buffer, covered again with plastic coverslip and incubated at room temperature and in the dark for 10 minutes. Finally, the slides were mounted with antifade solution (Citiflou) to prevent fading of fluorescence during slide observation.

Slides were examined under a Zeiss Axioplan epifluorescence microscope with Zeiss filter using x16 and X100 oil immersion lenses. Images were captured with a CCD camera and analysed using Adobe Photoshop software.
CHAPTER 3: Analysis of genetic variation in Brakmas cattle

3.1 Introduction and aims

Maintaining and conserving genetic variation is important in order to improve our livestock in the future and to maintain production in the face of continuing and arguably increasing threats from abiotic (environmental including climate change) and biotic (disease) stresses. Loss of variation will restrict the options available to meet future unknown requirements (Moazami et al. 1997). However, as consumers demand a better product, with uniformity or consistency in product quality and generally decreasing price, farmers need to improve both productivity and product quality to remain competitive. However, production of uniform herd populations will result in reduced genetic diversity and sometimes leads to collection of a limited gene pool and subsequently inbreeding. Therefore it is important to maintain genetic diversity. In any programme to maintain genetic diversity, it is critical that the diversity is quantified, and molecular markers for DNA variation have become the most important method to do this in the last decade.

To measure diversity, microsatellites are widely used. Microsatellites have been used for genetic diversity study, pedigree evaluation and genetic mapping (Metta et al. 2004). Microsatellites have been identified as a good molecular marker for characterisation of cattle breeds for many purposes (Blot et al. 1998; Metta et al. 2004). Apart being used to distinguished breeds, microsatellites also can be used in individual animal identification, as two identical genotypes at all loci are almost never found (MacHugh et al. 1997).

Here, the objectives to study genetic diversity in Brakmas are

1. To characterise Brakmas
2. To look at the pattern of genetic variation within Brakmas population
3. To look for any evidence of loss of genetic variation or reduced genetic diversity
4. To look for evidence of breed uniformity or, in contrast, hybridity
To set a baseline for genetic diversity studies at the breed is developed in the future.

It is hoped that the analysis of genetic diversity in Brakmas cattle will help in characterising Brakmas and their diversity in relation to global cattle breeds.

3.2 Materials and methods

3.2.1 DNA samples

See chapter 2

3.2.2 Sample size

It is important to assess the suitability of sample size and the number of genetic markers to be screened. In this study, the sample size is only 37 animals. McHugh (1997) studied the effect of increasing sample size on the measurement error. It was found that, for a given allele frequency, the standard error decreases dramatically as sample size is increased from a small number of individuals. However the rate of decrease levels off after approximately 30 individuals and starts to approach zero asymptotically. This suggests that in order to balance workload and accuracy, sample sizes of between 30 and 60 would be optimal. So, in this experiment, sample size is likely to be sufficient.

3.2.3 Microsatellite primers

For genetic diversity study in Brakmas, 20 microsatellites were used. Out of these, 17 markers were chosen as recommended by the ISAG (International Society for Animal Genetics) as in Secondary Guidelines for the Development of National Farm Animal Genetic Resources Management Plan – Measurement of Domestic Animal Diversity (MoDAD) and as in http://www.projects.roslin.ac.uk/cdiv/markers.html. Primers for the other three microsatellites (BP8, BM4005 and BMC4216) was used as published (Bishop et al. 1994). Primer sequences of these microsatellites, chromosome location as well as annealing temperature used in the experiment are as in Table 3.1.
3.2.4 PCR of microsatellites
See chapter 2

3.2.5 Agarose gel electrophoresis
For confirmation of proper amplification of the microsatellites, a few different bovine accessions were tested initially before carrying out polyacrylamide gel electrophoresis.

10 µl of these reactions were electrophoresed on a mixture of 1% high resolution agarose gel and 1.5% high gel strength agarose in 1x TAE buffer with 0.5 µg/ml of ethidium bromide. 25 bp ladders were used to provide sequence ladders for initial allele size reference.

3.2.6 Denaturing polyacrylamide gel electrophoresis of microsatellites products
The PCR products were analyzed on 6% denaturing polyacrylamide gels and silver staining of the gels was done follows a series of steps done manually as described in chapter 2 (Materials and Methods). The plate was air-dried before scanning directly using a high resolution scanner, and saved the image in .psd format.

3.2.7 Scoring and genotype schematics
Figures 3.1 and 3.2 show scanned photographs of gels of the microsatellite loci typed and the range of alleles observed at each locus is as described under these photographs. Genotypes were scored independently and the absolute sizes of the alleles were determined in relation to a 25 bp DNA size ladder (Bioline). The allele size increases consistently with number of repeat.
<table>
<thead>
<tr>
<th>Microsatellites</th>
<th>Chr</th>
<th>Amplification primers</th>
<th>Reference</th>
<th>Repeat motif</th>
<th>Reference</th>
<th>Ann T</th>
<th>Cycle</th>
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</thead>
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<tr>
<td>1. BP28</td>
<td>29a</td>
<td>AGGTGCAGGTGAGAGGGG</td>
<td>Bishop et al. 1994</td>
<td>(GT)_{13}</td>
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<tr>
<td></td>
<td></td>
<td>CCTCCACAACACCATCCTTC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2. BM1824</td>
<td>1</td>
<td>GAGCAAGGTGTCCCTCAATC</td>
<td>FAO</td>
<td>(GT)_{15}</td>
<td><a href="http://www.thearkdb.org">http://www.thearkdb.org</a></td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATTCTCAAGTCTTCCTTG</td>
<td></td>
<td></td>
<td>Alexander et al. 1995</td>
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<td>3. BM4005</td>
<td>29a</td>
<td>AGTCCATGGGACCACAAAAG</td>
<td>Bishop et al. 1994</td>
<td>(TG)_{17}</td>
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<td>4. BMC4216</td>
<td>29a</td>
<td>TGAGGAAAAGGGAGATGG</td>
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<td></td>
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<td>5. CSRM60</td>
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<td>(AC)_{17}</td>
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</table>

\(^a\)Originally assigned at BTA29 by Bishop et al. (1994) but assigned at BTA25 as in http://www.projects.roslin.ac.uk/comrad/markers/bta25.html
Table 3.1 (continued) Microsatellites loci used to study genetic variation in Brakmas.

<table>
<thead>
<tr>
<th>Microsatellites</th>
<th>Chr</th>
<th>Amplification primers</th>
<th>Reference</th>
<th>Repeat motif</th>
<th>Reference</th>
<th>Ann</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>8. ETH10</td>
<td>5</td>
<td>GTTCAGGACTGGGCCCTGCTAACA CCTCCAGCCCACTTTCTCTTCT</td>
<td>FAO</td>
<td>(AC)\textsubscript{20}</td>
<td>Embl Z22739</td>
<td>55</td>
<td>33</td>
</tr>
<tr>
<td>9. ETH152</td>
<td>5</td>
<td>TACTCGTAGGGCAGGCTGCTG GAGACCTCAGGGTTTGATGCAG</td>
<td>FAO</td>
<td>(CA)\textsubscript{17}</td>
<td>Embl Z14040</td>
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<td>35</td>
</tr>
<tr>
<td>10. HEL9</td>
<td>8</td>
<td>CCCATTCAGTCTTCAGAGGT CACATCCATGTTCTCACC</td>
<td>FAO</td>
<td>(TG)\textsubscript{7}</td>
<td>Embl AF236380</td>
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<td>30</td>
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<tr>
<td>11. ILSTS005</td>
<td>10</td>
<td>GGAAGCAATGAAATCTATAGCC TGTCTGTGAGTTGTAAGC</td>
<td>FAO</td>
<td>(TG)\textsubscript{4}(TACA)(TA)\textsubscript{5} (TG)\textsubscript{9}(TA)(TG)\textsubscript{2}(TA)\textsubscript{7}</td>
<td>Embl L23481</td>
<td>58</td>
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<tr>
<td>12. ILSTS006</td>
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<td>TGTCTGTATTTCTGTGTGG ACACGGAAGCGATCIAACG</td>
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<td>(GT)\textsubscript{23}</td>
<td>Embl L23482, Kemp et al. 1995</td>
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<td>13. ILSTS011</td>
<td>14</td>
<td>GCTTGCTACATGGAAAGTGCC CAAAATGCAGAGCCCTACC</td>
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<td>(CA)\textsubscript{11}</td>
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<tr>
<td>14. ILSTS030</td>
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<td>CTGCAGTTCTGCATATGTGG CTTAGACAACAGGGTTTGG</td>
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<td>(CA)\textsubscript{13}</td>
<td>Kemp et al. 1995</td>
<td>55</td>
<td>30</td>
</tr>
</tbody>
</table>
Table 3.1 (continued) Microsatellites loci used to study genetic variation in Brakmas.

<table>
<thead>
<tr>
<th>Microsatellites</th>
<th>Chr</th>
<th>Amplification primers</th>
<th>Reference</th>
<th>Repeat motif</th>
<th>Reference</th>
<th>Ann T</th>
<th>Cycle</th>
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<tr>
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<td>3</td>
<td>GAGTAGAGCTACAAGATAAAACTTC</td>
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<td>(AC)$_{21}$</td>
<td>Ciampolini et al.</td>
<td>57</td>
<td>30</td>
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<tr>
<td></td>
<td></td>
<td>TAACTACAGGGGTTAGATGAACCTCA</td>
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<td>AACTGTATTCTCTAATAGCAC</td>
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<td>(AC)$_5$/(CA)$<em>9$/(AC)$</em>{14}$</td>
<td>Ciampolini et al.</td>
<td>56</td>
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<td></td>
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<td>GCAAGACATATCTCCATTCTCTT</td>
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<td>Ciampolini et al.</td>
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<td></td>
<td></td>
<td>AAAACCACAGAAATGTTGGAAG</td>
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<td>CCCAAGGACAGAAAAGACT</td>
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<td>Mommens et al.</td>
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<td></td>
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<td>CTCAAGATAAGACCACACC</td>
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<td>19. MM12</td>
<td>9</td>
<td>CAAGACAGGTGTTTCAATCT</td>
<td>FAO</td>
<td>GT***</td>
<td>Mommens et al.</td>
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<td></td>
<td></td>
<td>ATCGACTCTGGGGATGATGT</td>
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<td>20. TGLA122</td>
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<td>CCCTCCTCCAGGTAATCAGC</td>
<td>Bishop et al.</td>
<td>(AC)$_{12}$</td>
<td>Barensen et al.</td>
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<td>AATCACATGGCAAATAAGTACATA</td>
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<td>ARKFSQ00002790</td>
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</tbody>
</table>

*Ciampolini et al. 1995 citing Vaiman et al. 1994

** Repeat motif not available

*** Repeat motif number not available
Figure 3.1 Polyacrylamide gel image of microsatellites BM1824 amplified in Brakmas cattle


Figure 3.2 Polyacrylamide gel image of microsatellites ILSTS030 amplified in Brakmas cattle

Lane M: 25 bp molecular marker; Lane 1: 146/154, Lane 2: 146/154, Lane 3: 146/154, Lane 4: 146/154, Lane 5: 140/146, Lane 6: 140/146, Lane 7: 140/146, Lane 8: 140/146, Lane 9: 146/154, Lane 10: 146/154, Lane 11: 144/154, Lane 12: 140/146, Lane 13: 146/154, Lane 14: 140/146, Lane 15: 140/146, Lane 16: 140/146, Lane 17: 140/146, Lane 18: 140/146, Lane 19: 140/146 and Lane 20: 140/146. Alleles present in this photograph are four (140, 144, 146, 154).
3.2.8 Microsatellite analysis

All data of allele size were tabulated in Excel. For the microsatellites, the data was imported for analysis using Powermarker V3.0 program (Liu, 2004). The program can be obtained at http://statgen.ncsu.edu/powermarker/. The analysis carried out was number of alleles per locus, allele frequencies, observed heterozygosity ($H_o$), expected heterozygosity ($H_e$), gene diversity within population ($F_{IS}$), Polymorphism Information Content (PIC), and $f$ value. UPGMA (unweighed pair-group method using arithmetic average) was used to reconstruct the phylogeny.

3.2.8.1 Allele number

The average number of alleles observed at each locus for each breed is considered to be a good indicator of genetic variability due to allelic diversity (Grzybowski and Prusak, 2004b).

3.2.8.2 Allele frequency

Allele frequency is the percentage or proportion of an allele at a genetic locus within a population. It is used as a measure for genetic diversity. Allele frequencies influence migration, mutation and genetic drift (Grzybowski and Prusak, 2004a). The effects of selection could influence results and subsequently the genetic structure of a population may be wrongly estimated. Comparison in variation of allele frequencies is a powerful method for detecting effects of selection (Tomajian, 2004). To estimate genetic diversity, the frequency of the most common allele should not exceed 0.95 in order to consider a locus as polymorphic (Mandak et al. 2005).

3.2.8.3 Heterozygosity

Heterozygosity refers to the state of being a heterozygote and refers to the fraction of individuals in a population that are heterozygous for that locus.
3.2.8.4 Observed heterozygosity (Ho)

Observed heterozygosity (Ho) is the proportion of heterozygotes observed in a population sample. The accuracy of an estimate for average heterozygosity is dependent on sample size, the number of loci typed and presence of null allele.

3.2.8.5 Expected heterozygosity

Expected heterozygosity or gene diversity (Nei, 1973) is calculated as one minus the sum of all squared allele frequencies at a locus. This statistic is equivalent to observed heterozygosity only when a population is in Hardy-Weinberg Equilibrium. The statistical accuracy is more influenced by the number of loci than sample size. The general concordance between the observed and expected heterozygosities within the populations would suggest that the populations are in Hardy-Weinberg Equilibrium.

Nei and Roychoudhury (1974) have previously demonstrated that the standard error of gene diversity is minimised when the number of loci is maximised and therefore, sample size is less critical than number of loci.

3.2.8.6 Deviations from Hardy-Weinberg Equilibrium

A diploid population is considered to be in Hardy-Weinberg Equilibrium at a polymorphic genetic locus if the genotype proportions observed in the population can be completely specified by the allele frequencies at the locus in question. In other words, alleles at the locus are randomly distributed throughout the population and there is no association between the pair of alleles that an individual receives from its parents.

3.2.8.7 Polymorphic Information Content

Polymorphism Information Content (PIC) is a better index of weighing polymorphism of the fragment. Botstein et al. (1980) have proposed weighing much indexes of Polymorphism Information Content of the degree level of genetic variation at first. As PIC>0.5, it is highly polymorphic loci, PIC 0.25<PIC<0.5, it is middle polymorphic loci and PIC<0.25, it is low polymorphic loci.
3.2.8.8 F statistics

F-statistics are a measure of correlation of alleles within individuals and related to inbreeding coefficients. F statistics describe the amount of inbreeding effects within subpopulation (\(F_{\text{ST}}\) or \(\theta\)), among subpopulation (\(F_{\text{IS}}\) or \(f\)) and within the entire population (\(F_{\text{IT}}\) or \(F\)). \(F_{\text{IS}}\) is inbreeding coefficient within subpopulation.

3.2.8.9 Phylogenetic analysis

Phylogenetic analysis attempts to describe the evolutionary relatedness of a group of animals. Phylogenetic trees or cladograms group species into a diagram that represents their relative evolutionary divergence. Branchings of the tree that occur furthest from the root separate individual species; branchings that occur close to the root group species into kingdoms, phyla, classes, families, genera, and so on. In this study, allele frequencies were used to compute a phylogenetic tree. Bootstrap resampling (\(n = 1000\)) was performed to test the robustness of the dendogram topology.

3.3 Results

3.3.1 Numbers of alleles

A total of 154 alleles were detected across the 20 microsatellites loci analysed in all 37 of the Brakmas cattle. All the loci were polymorphic and the number of alleles varied between 3 (INRA63) and 18 (ILST011) as shown in Table 3.2. It is also observed that this Brakmas herd has a higher number of alleles. The mean number of alleles detected at each locus was therefore 7.7. Five microsatellites were detected to have a number of alleles more than 10. These microsatellites were CSSM66, HEL9, ILSTS006, TGLA122 and ILSTS011 with allele number of 11, 11, 11, 12 and 18, respectively.

It is observed that generally Brakmas displayed lower allele size in most of the microsatellites observed compared to those published by ISAG. Out of 20 microsatellites studied, 16 microsatellites shows the allele size ranges lower than reference except for microsatellites HEL9, ILSTS005, INRA063 and TGLA122.
3.3.2 Allele frequency

Allele frequencies of each microsatellite in Brakmas were as shown in Table 3.4. The frequency of allele varies between microsatellites with no common trend observed.

In some microsatellites such as BM1824, ETH3, ETH10 and MM12, allele frequency was dominated by certain alleles present at high frequency. In ETH10, allele size 200 has frequency of 61.11%. In microsatellites ETH3, BM1824 and MM8, dominant allele present at frequency of 55.00%, 60.29% and 70.83%, respectively.

On the other hand, in some microsatellites such as CSRM60, CSSM66 and HEL9, a few alleles contributed at about similar frequency. For example, in CSSM66, allele size 184, allele size 166 and allele size 176 present at frequency of 19.12%, 17.65% and 14.71%, respectively. It was also observed that certain alleles in microsatellites studied present at very low frequency. For example, in microsatellites HEL9, allele size 140, 142 and 144 all present at 1.39%. The lowest allele frequency present in Brakmas population was at 1.35% which was observed within several microsatellites including CSSM66, ILSTS011 and the highest frequency was at 70.83% which was present in microsatellites MM12.

3.3.3 Observed heterozygosity

The observed heterozygosities in this population ranged from 0.00 to 0.9730. This results in a mean value of 0.4628 for the Brakmas cattle. The highest observed heterozygosity was 0.9706 and was observed in microsatellites ILSTS030, followed by observed heterozygosity at 0.9706 at microsatellites CSSM66 and INRA63. No heterozygosity was observed in microsatellites BP28, ETH152 and ILSTS006.

Several microsatellites showed heterozygosity. These microsatellites were CSSM66, ILSTS030 and INRA023 which were present at 0.9706, 0.9730 and 0.9706 compared to expected heterozygosity at 0.8733, 0.7527 and 0.7066, respectively. All other microsatellites showed that the heterozygosity is lower than the expected heterozygosity with three microsatellites observed absent of heterozygosity (0.0000)
Table 3.2 Allelic ranges data and allele number of the Brakmas cattle compared to reference data

<table>
<thead>
<tr>
<th>Microsatellites</th>
<th>Allele size range</th>
<th>No of allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brakmas REF 1 REF 2</td>
<td>Brakmas REF</td>
</tr>
<tr>
<td>1. BP28</td>
<td>190-216 202-208 Bishop et al. 1994</td>
<td>8 4 Bishop et al. 1994</td>
</tr>
<tr>
<td>2. BM1824</td>
<td>166-186 180-192 Bishop et al. 1994</td>
<td>5 6 Bishop et al. 1994</td>
</tr>
<tr>
<td>5. CSRM60</td>
<td>83-97 93-111* Niemczewski et al. 2002 93-111 CadBase#</td>
<td>6 6 CadBase#</td>
</tr>
<tr>
<td>6. CSSM66</td>
<td>163-185 179-199* Niemczewski et al. 2002 179-199 CadBase#</td>
<td>11 10 CadBase#</td>
</tr>
<tr>
<td>7. ETH3</td>
<td>93-121 105-125 Bishop et al. 1994</td>
<td>4 8 Bishop et al. 1994</td>
</tr>
<tr>
<td>8. ETH10</td>
<td>176-220 210-226 FAO 207-219 CadBase#</td>
<td>5 7 CadBase#</td>
</tr>
<tr>
<td>9. ETH152</td>
<td>160-190 192-204 Bishop et al. 1994</td>
<td>6 6 Bishop et al. 1994</td>
</tr>
<tr>
<td>10. HEL9</td>
<td>141-167 143-165 Bishop et al. 1994</td>
<td>11 12 Bishop et al. 1994</td>
</tr>
</tbody>
</table>

* Data from deer

Table 3.2 (continued) Allelic ranges data and allele number of the Brakmas cattle compared to reference data

<table>
<thead>
<tr>
<th>Microsatellites</th>
<th>Allele size range</th>
<th>No of allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>REF 1</td>
</tr>
<tr>
<td>14. ILSTS030</td>
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<td>15. INRA023</td>
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<td>220**</td>
</tr>
<tr>
<td>16. INRA032</td>
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<td>190**</td>
</tr>
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<td>17. INRA063</td>
<td>181-191</td>
<td>180**</td>
</tr>
<tr>
<td>18. MM8</td>
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</tr>
<tr>
<td>19. MM12</td>
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</tr>
<tr>
<td>Total</td>
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</tr>
<tr>
<td>Mean</td>
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<td></td>
</tr>
</tbody>
</table>

* Data from deer
** No allele ranges available

### Table 3.3 Allele number of the Brakmas cattle compared to reference data

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<tbody>
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<td>ETH3</td>
<td>BK 4 PR 8 H 3 HF 5</td>
<td>PD 7 H 8 HF 7</td>
<td>HF 7 BRP 6 EF 5 BB 8</td>
</tr>
<tr>
<td>ETH10</td>
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<td>PD 6 H 5 HF 6</td>
<td>HF 8 BRP 8 EF 5 BB 7</td>
</tr>
<tr>
<td>BM1824</td>
<td>BK 5 PR 5 H 6 HF 10</td>
<td>PD 4 H 5 HF 5</td>
<td>HF 5 BRP 5 EF 8 BB 4</td>
</tr>
<tr>
<td>TGLA122</td>
<td>BK 12 PR 9 H 5 HF 6</td>
<td>PD 13 H 11 HF 14</td>
<td>HF 14 BRP 14 EF 14 BB</td>
</tr>
</tbody>
</table>

BK Brakmas; PR Polish Red; H Hereford; HF Holstein Friesian; Piedmontese; BRP Belgian Red Pied; EF East Flemish; BB Belgian Blue
Table 3.4 Allele frequency of microsatellites in Brakmas

<table>
<thead>
<tr>
<th>Mc</th>
<th>Allele</th>
<th>Allele frequency</th>
<th>Mc</th>
<th>Allele</th>
<th>Allele frequency</th>
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<td>1. INRA063</td>
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<td>9. ILSTS006</td>
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<td>190</td>
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<td>215</td>
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<td>192</td>
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<td>0.1081</td>
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<td>17. HEL9</td>
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<td>0.0139</td>
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<td></td>
<td>144</td>
<td>0.0139</td>
<td></td>
<td>270</td>
<td>0.0811</td>
<td></td>
<td>140</td>
<td>0.0147</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>0.0588</td>
<td></td>
<td>146</td>
<td>0.2361</td>
<td></td>
<td>272</td>
<td>0.0811</td>
<td></td>
<td>142</td>
<td>0.1471</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>0.1029</td>
<td></td>
<td>148</td>
<td>0.0694</td>
<td></td>
<td>274</td>
<td>0.0270</td>
<td></td>
<td>144</td>
<td>0.0294</td>
</tr>
<tr>
<td></td>
<td>172</td>
<td>0.0294</td>
<td></td>
<td>150</td>
<td>0.0972</td>
<td></td>
<td>278</td>
<td>0.1351</td>
<td></td>
<td>146</td>
<td>0.0294</td>
</tr>
<tr>
<td></td>
<td>174</td>
<td>0.0725</td>
<td></td>
<td>152</td>
<td>0.0833</td>
<td></td>
<td>280</td>
<td>0.3514</td>
<td></td>
<td>148</td>
<td>0.2059</td>
</tr>
<tr>
<td></td>
<td>176</td>
<td>0.1471</td>
<td></td>
<td>154</td>
<td>0.1944</td>
<td></td>
<td>282</td>
<td>0.0541</td>
<td></td>
<td>150</td>
<td>0.2794</td>
</tr>
<tr>
<td></td>
<td>178</td>
<td>0.0147</td>
<td></td>
<td>156</td>
<td>0.1250</td>
<td></td>
<td>284</td>
<td>0.1351</td>
<td></td>
<td>152</td>
<td>0.0294</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>0.1029</td>
<td></td>
<td>164</td>
<td>0.0139</td>
<td></td>
<td>286</td>
<td>0.0270</td>
<td></td>
<td>154</td>
<td>0.0441</td>
</tr>
<tr>
<td></td>
<td>184</td>
<td>0.1912</td>
<td></td>
<td>166</td>
<td>0.1389</td>
<td></td>
<td>290</td>
<td>0.0270</td>
<td></td>
<td>162</td>
<td>0.0588</td>
</tr>
<tr>
<td>20. ILSTS011</td>
<td>230</td>
<td>0.0135</td>
<td></td>
<td>234</td>
<td>0.0270</td>
<td></td>
<td>236</td>
<td>0.0588</td>
<td></td>
<td>238</td>
<td>0.0588</td>
</tr>
</tbody>
</table>
3.3.4 Expected heterozygosity or gene diversity
Expected heterozygosity in Brakmas ranges between 0.4769 and 0.9072 with a mean value of 0.7206. All expected heterozygosity were more than 0.5000 except MM12 with a value of 0.4769. The highest heterozygosity is 0.9072 and was observed in ILSTS011. This was followed by 0.8733 in CSSM66 and 0.8495 in HEL9. TGLA122 and ILSTS011 also were observed to have very high expected heterozygosity which are at 0.8486 and 0.8181, respectively. As mentioned earlier in paragraph 3.3.3, all expected heterozygosity observed in Brakmas were higher than observed heterozygosity except microsatellites CSSM66, ILSTS030 and INRA023.

3.3.5 Deviations from Hardy-Weinberg Equilibrium
Departures from Hardy-Weinberg Equilibrium involving eleven loci were detected. The deviation from Hardy-Weinberg equilibrium (p<0.05) was observed in microsatellites BP28, BM1824, BM4005, ETH3, ETH152, ILSTS005, ILSTS006, ILSTS030, INRA032, INRA063 and MM12.

3.3.6 Polymorphic Information Content
In Brakmas, PIC ranges from 0.4551 in MM12 to 0.9 in ILSTS011 with a mean value of 0.6850. All microsatellites loci were highly polymorphic loci except PIC of locus BM1824, INRA63 and MM12 were considered middle polymorphic (0.4861, 0.4908 and 0.4551 respectively).

3.3.7 F statistics
All microsatellites in this study showed that there were excess homozygote in this population except for microsatellites CSSM66, ILSTS030 and INRA023. The f value ranges from -0.2794 to 1.00 with a mean value of 0.3701. Three microsatellites, BP28, HEL9 and ILSTS011 were observed to have f value of 1.0000. The f value for excess of heterozygote in microsatellites in this population were -0.0967, -0.2553 and -0.2794 for microsatellites CSSM66, INRA023 and ILSTS030, respectively.

3.3.8 Phylogenetic analysis
Based on phylogeny tree, it is observed that Brakmas display four main clusters. Each individual was observed distant from each other.
Table 3.5. Heterozygosity (Het), P-value for Hardy-Weinberg (HW), Polymorphic information content (PIC) and $f$ value over 20 microsatellites in Brakmas cattle

<table>
<thead>
<tr>
<th>Microsatellites</th>
<th>Het-obs</th>
<th>Het-exp</th>
<th>P-value</th>
<th>PIC</th>
<th>PIC REF</th>
<th>REF</th>
<th>$f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. BM1824</td>
<td>0.4412</td>
<td>0.5502</td>
<td>0.0091</td>
<td>0.4861</td>
<td>*</td>
<td>Radko et al. 2005</td>
<td>0.2124</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Peelman et al. 1998</td>
<td></td>
</tr>
<tr>
<td>2. BM4005</td>
<td>0.2941</td>
<td>0.7279</td>
<td>0.0002</td>
<td>0.6936</td>
<td></td>
<td></td>
<td>0.6055</td>
</tr>
<tr>
<td>3. BMC4216</td>
<td>0.6111</td>
<td>0.7944</td>
<td>0.6159</td>
<td>0.7708</td>
<td></td>
<td></td>
<td>0.2440</td>
</tr>
<tr>
<td>4. CSRM60</td>
<td>0.6216</td>
<td>0.7348</td>
<td>0.0640</td>
<td>0.6890</td>
<td></td>
<td></td>
<td>0.1674</td>
</tr>
<tr>
<td>5. CSSM66</td>
<td>0.9706</td>
<td>0.8733</td>
<td>0.1681</td>
<td>0.8603</td>
<td>-0.0967</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. ETH3</td>
<td>0.1500</td>
<td>0.6213</td>
<td>0.0000</td>
<td>0.5722</td>
<td>*</td>
<td>Radko et al. 2005</td>
<td>0.7692</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Peelman et al. 1998</td>
<td></td>
</tr>
<tr>
<td>7. ETH10</td>
<td>0.4444</td>
<td>0.5849</td>
<td>0.0731</td>
<td>0.5526</td>
<td>*</td>
<td>Radko et al. 2005</td>
<td>0.2533</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Peelman et al. 1998</td>
<td></td>
</tr>
<tr>
<td>8. HEL9</td>
<td>0.4722</td>
<td>0.8495</td>
<td>0.0655</td>
<td>0.8322</td>
<td></td>
<td></td>
<td>0.4554</td>
</tr>
<tr>
<td>9. ILSTS005</td>
<td>0.5000</td>
<td>0.7850</td>
<td>0.0070</td>
<td>0.7534</td>
<td>0.59</td>
<td>Kemp et al. 1995</td>
<td>0.3760</td>
</tr>
<tr>
<td>10. ILSTS011</td>
<td>0.6216</td>
<td>0.9072</td>
<td>0.8127</td>
<td>0.9000</td>
<td>0.67</td>
<td>Kemp et al. 1995</td>
<td>0.3271</td>
</tr>
</tbody>
</table>

* Comparison between breed as in Table 3.6
Table 3.5 (continued). Heterozygosity (Het), P-value for Hardy-Weinberg (HW), Polymorphic information content (PIC) and $f$ value over 20 microsatellites in Brakmas cattle

<table>
<thead>
<tr>
<th>Microsatellites</th>
<th>Het-obs</th>
<th>Het-exp</th>
<th>P-value</th>
<th>PIC</th>
<th>PIC REF</th>
<th>REF</th>
<th>$f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>11. ILSTS030</td>
<td>0.9730</td>
<td>0.7527</td>
<td>0.0005</td>
<td>0.7179</td>
<td>0.65</td>
<td>Kemp et al. 1995</td>
<td>-0.2794</td>
</tr>
<tr>
<td>12. INRA023</td>
<td>0.9706</td>
<td>0.7976</td>
<td>0.0716</td>
<td>0.7688</td>
<td>0.77</td>
<td>Vaiman et al. 1994</td>
<td>-0.2553</td>
</tr>
<tr>
<td>13. INRA032</td>
<td>0.6364</td>
<td>0.7066</td>
<td>0.0473</td>
<td>0.6510</td>
<td>0.54</td>
<td>Vaiman et al. 1994</td>
<td>0.1146</td>
</tr>
<tr>
<td>14. INRA063</td>
<td>0.0541</td>
<td>0.5727</td>
<td>0.0000</td>
<td>0.4908</td>
<td>0.46</td>
<td>Vaiman et al. 1994</td>
<td>0.9080</td>
</tr>
<tr>
<td>15. MM8</td>
<td>0.3438</td>
<td>0.6235</td>
<td>0.0134</td>
<td>0.5621</td>
<td></td>
<td></td>
<td>0.4613</td>
</tr>
<tr>
<td>16. MM12</td>
<td>0.4167</td>
<td>0.4769</td>
<td>0.9598</td>
<td>0.4551</td>
<td></td>
<td></td>
<td>0.1400</td>
</tr>
<tr>
<td>17. TGLA122</td>
<td>0.7353</td>
<td>0.8486</td>
<td>0.9090</td>
<td>0.8239</td>
<td>*</td>
<td>Radko et al. 2005</td>
<td>0.1402</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Peelman et al. 1998</td>
<td></td>
</tr>
<tr>
<td>18. BP28</td>
<td>0.0000</td>
<td>0.7553</td>
<td>0.0000</td>
<td>0.7189</td>
<td></td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>19. ETH152</td>
<td>0.0000</td>
<td>0.6311</td>
<td>0.0000</td>
<td>0.5996</td>
<td></td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>20. ILSTS006</td>
<td>0.0000</td>
<td>0.8181</td>
<td>0.0000</td>
<td>0.8010</td>
<td>0.72</td>
<td>Kemp et al. 1995</td>
<td>1.000</td>
</tr>
<tr>
<td>Mean</td>
<td>0.4628</td>
<td>0.7206</td>
<td>0.6850</td>
<td></td>
<td></td>
<td></td>
<td>0.3701</td>
</tr>
</tbody>
</table>

* Comparison between breed as in Table 3.6
Table 3.6  Polymorphic Information Content (PIC) of the Brakmas cattle compared to reference data

<table>
<thead>
<tr>
<th>Microsatellites</th>
<th>Radko et al. 2005</th>
<th>Peelman et al. 1998</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BK</td>
<td>PR</td>
</tr>
<tr>
<td>ETH3</td>
<td>0.5722</td>
<td>0.732</td>
</tr>
<tr>
<td>ETH10</td>
<td>0.5526</td>
<td>0.700</td>
</tr>
<tr>
<td>BM1824</td>
<td>0.8239</td>
<td>0.559</td>
</tr>
<tr>
<td>TGLA122</td>
<td>0.4861</td>
<td>0.627</td>
</tr>
<tr>
<td>INRA023</td>
<td>0.7688</td>
<td>0.825</td>
</tr>
</tbody>
</table>

BK Brakmas; PR Polish Red; H Hereford; HF Holstein Friesian; BPR Belgian Red Pied; EF East Flemish; BB Belgian Blue
Figure 3.3 Phylogenetic tree of Brakmas showing relationship within the population. The tree is constructed by the neighbour-joining. This is an unrooted tree. Number 1-37 refer to Brakmas cattle and code number as in Table 2.1. No 38-43 is animal from reference database http://projects.roslin.ac.uk/cdiv/refdna.html. No 38: Holstein; No 39: Ayrshire; No 40: Limousin; No 41: Charolais; No 42: Aberdeen Angus; No 43: Piemontese.
3.4 Discussion

3.4.1 Numbers of alleles

The average number of alleles observed at each locus for each breed is a good indicator of genetic variability due to allelic diversity when the population is at equilibrium between mutation and genetic migration and the sizes of populations compared are similar (Grzybowski and Prusak, 2004b). It can also provide a good indicator for inbreeding or outbreeding, population bottlenecks and selection intensity. Ewens (1972) has previously demonstrated that the sample size dependency of allelic diversity only becomes problematic with high values of effective population size and mutation rate. MacHugh et al. (1997) stated that that allelic diversity patterns they found are likely to reflect distinctive population histories rather than a sampling artefact using population sizes similar to those studied here.

A total of 154 alleles were detected across the 20 microsatellites loci analysed in the population of 37 individuals of the Brakmas cattle. All the loci were polymorphic and the number of alleles varied between 3 (as in INRA63) and 18 (ILST011) as shown in Table 3.2. The mean number of alleles detected at each locus was therefore 7.7. As a comparison, based on breed reported by Moazami-Goudarzi et al. (1997) Jersey, Maine-Anjou, Limousin, Holstein and Charolais, is 6.0, 6.29, 6.35, 6.76 and 7.7, respectively. Bishop et al. (1994) reported a mean number of 6.8 alleles per locus but MacHugh et al. (1997) reported mean allele number as 8.8 in various breeds included African Zebu. However, compared to results by another study on nine different breeds (Grzybowski and Prusak, 2004b), Brakmas displayed a lower number of alleles in most of the microsatellites. Although it is also observed that this Brakmas herd has higher number of alleles than the ISAG populations which are from highly selected herds, compared to results of Grzybowski and Prusak (2004b) which included rare livestock breeds, local breeds as well as highly selected breeds, there was a lower number of alleles in most of the microsatellites.

Five microsatellites were detected to have a number of alleles more than 10. These microsatellites were CSSM66, HEL9, ILSTS006, TGLA122 and ILSTS011 with allele number of 11, 11, 11, 12 and 18, respectively. The high number of alleles for microsatellites CSSM66 and HEL9 are similar to those reported by Bishop et al. (1994). However, number of alleles in microsatellites ILSTS011 observed in the
Brakmas population is much higher than reported by Bishop et al. (1994) which is only five instead of eighteen in Brakmas. INRA063 was less informative, detecting only three alleles but this is consistent with previous researchers who detected five alleles (Russell et al. 2000).

Microsatellites TGLA122 and ILSTS006 in Brakmas were also observed to have higher number of alleles, 12 and 11 instead of 8 and 7, respectively as reported by Bishop et al. (1994). Higher allele number also had been reported for microsatellites TGLA122 in various breeds (Mioili et al. 2004; Peelman et al. 1998) as shown in Table 3.3. Comparison of microsatellites ETH3, ETH10 and BM1824 in Brakmas with other breeds also shows that the number of alleles in Brakmas were similar to other breeds. In the case of ETH10, the number of alleles is the same as reported in breed Hereford (Mioili et al. 2004) and East Flemish (Peelman et al. 1998), although it was lower than other breeds such as Polish Red and Holstein-Friesian, as well as Hereford, as reported by Radko et al. (2005); Piemontese and Holstein-Friesian as reported by Mioili et al. (2004); and Holstein-Friesian, Belgian Red Pied and Belgian Blue as reported by Peelman et al. (1998).

This shows that in some microsatellites, number of alleles in Brakmas at lower range but in some others were at higher range. Although direct comparison should not be applied, as a different microsatellites panel had been used in each of the experiments, the numbers can give a general idea about the diversity levels present in different populations and breeds. Overall, the number of microsatellite alleles in Brakmas, when compared to other population studies, is at the higher end of the range. This indicates that Brakmas is not strongly inbred even in relatively small herd. This results also indicates that Brakmas is diverse compared to European breed and could offer another source of diversity to the world of cattle breeds.

### 3.4.2 Size of allele

It was observed that generally Brakmas displayed lower allele size in most of the microsatellites observed compared to those published by ISAG largely for Bos taurus accessions. This result is in agreement with results reported by MacHugh et al. (1997) that allelic size distribution of *Bos indicus* is frequently lower than taurine (their figure 1 shows four microsatellite markers and they note that there are characteristic zebu-associated alleles, all four of which are shorter than the taurine alleles); Brakmas
is categorised as the *Bos indicus* subspecies. However, McHugh does not note any reason why the *Bos indicus* loci may be shorter, and no obvious reason can be offered.

### 3.4.3 Allele frequency

Allele frequency is important and can be used to detect effect of selection. Several studies show that certain alleles may be specific to certain breeds (Kim *et al.* 2002; Metta *et al.* 2004). Allele frequencies of each microsatellites in Brakmas were as shown (Table 3.4). The frequency of alleles varies between microsatellites with no common trend observed.

In some microsatellites such as BM1824, ETH3, ETH10 and MM12, allele frequency was dominated by a certain allele and present at high frequency. In ETH10, allele size 200 has frequency of 61.11%. In microsatellites ETH3, BM1824 and MM8, dominant alleles were present at frequencies of 55.00%, 60.29% and 70.83%, respectively. On the other hand, in some microsatellites such as CSRM60, CSSM66 and HEL9, several alleles contributed at about similar frequency. For example, in CSSM66, allele size 184, allele size 166 and allele size 176 are present at frequencies of 19.12%, 17.65% and 14.71%, respectively. It was also observed that certain allele in microsatellites studied present at very low frequency. For example, in microsatellites HEL9, allele sizes 140, 142 and 144 are all present at 1.39%. The lowest allele frequency present in Brakmas population was at 1.35% which was observed within several microsatellites including CSSM66, ILSTS011 and the highest frequency was at 70.83% which present in microsatellites MM12. It was observed that the frequency allele ranges as low as 1.35% to as high as 70.83%. This trend is similar as compared to other studies although it was done on different breed and different microsatellites (Ciampolini *et al.* 1995). The same author shows that allele frequency ranges from very low to very high frequencies depending on breeds and type of marker (data summarised in figure 1 of Ciampolini *et al.* 1995)

Sometimes, several alleles are found uniquely in certain breeds that can be used as a marker for that particular breed. Most of the alleles found in Brakmas breed are unlikely to be useful as breed markers because of their low frequencies. However a few alleles that were found at higher frequency as mentioned above and may not be abundant in other breeds might be used for Brakmas characterisation. Apart from their higher frequency characteristics, a high proportion of allelic variants seem to be
unique alleles for this breed. However, further research is required with higher sample size population to confirm this, as well as for searching for unique allele in Brakmas.

3.4.4 Observed heterozygosity

Observed heterozygosity is the proportion of heterozygotes observed in a population which reflected by allele number. It can be used to detect inbreeding when animals from different herds are used as well as be able to detect linkage disequilibrium and selection for hybrids/heterozygosity.

The observed heterozygosities in this population ranged from 0.15 to 0.9730. This results in a mean value of 0.4628 for the Brakmas cattle. The highest heterozygosity was 0.9706 and was observed in microsatellites ILSTS030, CSSM66 and INRA63. No heterozygosity was observed in microsatellites BP28, ETH152 and ILSTS006. The microsatellites used have been selected by the ISAG to identify within and between breed polymorphisms; the *Bos indicus* genetic background may not be polymorphic at some loci. Several microsatellites were observed with higher than expected heterozygosity. These microsatellites were CSSM66, ILSTS030 and INRA023 which present at 0.9706, 0.9730 and 0.9706 compared to expected heterozygosity at 0.8733, 0.7527 and 0.7066, respectively. All other microsatellites showed heterozygosity that was lower than the expected heterozygosity.

The lower levels of observed heterozygosity observed in this population presumably reflects consequences of inbreeding in the relatively small overall population. However, looking at number of allele present in Brakmas, it is unlikely due to inbreeding.

3.4.5 Deviations from Hardy-Weinberg equilibrium

Deviation from Hardy-Weinberg equilibrium reflects substructure and nature of allele segregation within population. Lotfus *et al.* (1999) reported that significant deviation was observed across all loci in one breed due to heterozygote deficiency. Such deviation from HWE may result from the population substructure of null alleles. Furthermore, low copy number of template could have led to the selective amplification of one allele (Lotfus *et al.* 1999). In Brakmas, departures from Hardy-Weinberg equilibrium involving eleven loci were detected. Null alleles are likely to be segregating for these loci as samples from a number of animals failed to amplify for
up to 10 loci: this would not be surprising in a breed which is rather distant geographically and morphologically from the breeds where the markers were developed. Hence it is possible that non-amplifying alleles are segregating in some of the animals (Pemberton et al. 1995)

Of the 37 samples, 17 samples failed to amplify for ETH3. In total it was observed that 13 loci have null alleles ranges from one (HEL9 and ETH10) to 17 (ETH3) with a mean of 3.6 null allele for that. Given that value, it is not feasible to specify any samples as homozygous for a non-amplifying allele. Due to the presence of a null allele, there is a possibility to overestimate the value for mean \( f \) estimates. This could be due to the presence of null alleles in these loci or be the consequences of several years of intensive selection on these breeds (Usha et al. 1995). Mis-scoring of alleles would also suggest deviations from HWE; although the gel resolution was enough to distinguish alleles, stutter in bands may have meant some bands were incorrectly scored as homozygotes or the wrong sizes.

The deviation, either positive or negative, indicated that there are locus-specific effects that suggest selection affecting some of the loci (Barker et al. 1997).

### 3.4.6 Polymorphic Information Content

The PIC is an indication of degree of informativeness of markers and can be used as index to evaluate polymorphism within population. In Brakmas, PIC ranges from 0.4551 in MM12 to 0.9 in ILSTS011 (Table 3.5). All microsatellites loci were highly polymorphic loci except the PIC of loci BM1824, INRA63 and MM12 were considered middle polymorphic (0.4861, 0.4908 and 0.4551 respectively). The mean value of observed PIC was 0.6850, a value similar to the 0.6 reported by Vaiman et al. (1994).

Comparing the PIC value in Brakmas to other breeds shows that in microsatellites, there is no common trend observed (Table 3.6). For example, PIC value of ETH10 is similar with Holstein-Friesian (Radko et al. 2005) but lower than other breeds such as Polish Red and Herefords (Radko et al. 2005) and Holstein-Friesian, Belgian Red Pied, East Flemish and Belgian Blue (Peelman et al. 1998). For microsatellites BM1824, it was observed that the PIC value in Brakmas is lower than all the breeds reported by Radko et al. (2005) and Peelman et al. (1998). However, the PIC value for TGLA 122 was observed higher than the breeds in both studies.
It can be concluded that Brakmas microsatellites presented a high polymorphism in Brakmas. It is also reported that the di-nucleotide microsatellites were found to be more polymorphic than tri-nucleotide (Metta et al. 2004). All the microsatellites in this study were dinucelotide repeat microsatellites except ILSTS005 and INRA032.

3.4.7 F statistics

F-statistics are a measure of correlation of alleles within individuals and can be use to describe the amount of inbreeding effects within subpopulation. The F_{IS} value in Brakmas was 0.37. This suggests that the sample represented individuals of inbred populations. However, this result is similar to another study in different breed. Metta et al. (2004) observed F_{IS} value of 0.36 in the Ongole breed, Indian cattle breed. This could also due to the small sample size. There is also possibility that individuals considered unrelated during collection may share common parentage in the history beyond known pedigree. This is exactly the advantage of using molecular marker in revealing the actual genetic relationship (Metta et al. 2004). Another possibility of this F_{IS} value in Brakmas is due to lower observed heterozygosity that might be as a contribution of null allele.

3.4.8 Phylogenetic analysis

To obtain the best topology using microsatellites as a genetic markers, genetic distance was employed (Takezaki and Nei, 1996). The genetic distance was used to construct a neighbour joining tree (Saitou and Nei, 1987). Allele frequency was used to generate genetic distance for each pair of individual in Brakmas cattle. A neighbour joining tree based on genetic distances is shown in figure 3.3. Clearly, Brakmas in this study display four main clusters. This result also indicates that there is a very low probability of finding animals with identical genotypes at all loci. This result also indicates that the animals are unique and quite distant from each other. This result is in agreement with Gryzbowski and Prusak (2004b) in Polish Red cattle. It was also reported that under uncontrolled breeding, a breed will be at risk of becoming genetically uniform in the future (Ibeagha-Awemu and Erhardt, 2005). From this study, the results indicate there is no evidence that Brakmas has become a genetically
uniform breed, although the study of one set of data cannot indicate any temporal trends.

3.4.9 Conclusion

In total, Brakmas display a high microsatellite polymorphism but within the range of other studies of less intensively bred cattle and particularly the *Bos indicus* types. This is proved by the considerable number of alleles identified, high mean number of alleles at each locus and high PIC value. The result is consistent with their relatively high morphological variation and low level of uniformity.

Brakmas do not display evidence for a genetic bottleneck, a decrease in genetic diversity or loss of alleles. As the genetic variability can be characterised by number of alleles, loss of alleles due to genetic drift or inbreeding consequently decreased the genetic variability (Rochambeau, 2000). The high degree of genetic variability in Brakmas indicates that the herd is rich reservoir of genetic diversity. This is a strong justification for preservation of the breed apart from others, because of the productivity as well capability of adaptation in local tropical climates.

Further information on microsatellites is still necessary for the improvement of the breed. Such relationships could have both positive (spread of useful genes, increase in within breed diversity) and negative (genetic uniformity, reduction in between breed diversity, reduced chances of improvement, spread of undesirable genes) consequences. The results presented here provide a baseline for future studies to track whether there is drift in allele composition and frequency, or whether selection pressures are leading to loss of diversity within the breed. It also indicates which markers may be useful in selection of parents for breeding programmes. The markers may also be applicable should a herdbook be established for the breed, and if phenotypic recording becomes more extensive.
Chapter 4: Structural analysis of genes associated with growth

4.1 Introduction and aims

Growth is an economically important trait in livestock. Selection for a higher growth rate combined with efficient feed utilization is required for the most cost efficient production system. Advances in molecular techniques enable the improvement of cattle through selection at the genetic level. DNA polymorphisms can be linked and associated to growth traits and subsequently the identified alleles can be used as a marker for selection of desirable traits and selection against loci correlated with poor performance (Zhou et al. 2005).

Growth is controlled by multiple genes (Zhou et al. 2005). Among these genes, four important genes that have been identified are the Growth Hormone (GH) genes and Growth Hormone Receptor Gene (GHR), Insulin-like Growth Factor 1 gene (IGF1) and Pituitary-specific Transcription factor 1 gene (PIT1).

The objectives of current study were to analyse the DNA sequence of various genes that had been associated to growth for detection of the alleles in Brakmas cattle, to measure the amount of polymorphisms, and to analyze these polymorphisms to see if current knowledge of the alleles allows prediction of growth and associated carcass traits in Brakmas cattle. For identification of polymorphisms within genes, I aimed to apply molecular markers such as SNP and PCR-RFLP to screen and select desirable alleles in Brakmas.

Specifically, the objectives were:

1. to identify polymorphisms within growth hormone gene, growth hormone receptor gene, insulin-like growth factor 1 gene and pituitary-specific transcription factor 1 gene using SNPs detected from sequence analysis
2. to determine presence of SNP with a G to C change that alters an amino acid residue from Val to Leu in the growth hormone gene
3. to determine if there is presence of shorter or longer TG-repeats in the growth hormone receptor gene allele
4. to determine the presence of a point mutation T-to-C (resulting in the A allele for T nucleotide and B allele for C nucleotide) for BB genotype in insulin-like growth factor 1 gene

5. To determine HinfI restriction sites and polymorphisms in the pituitary-specific transcription factor 1 gene

The results generated will be used to predict the growth performance that might be contributed by the polymorphisms within these genes, introns or immediately flanking regions.

### 4.2 Materials and Methods

#### 4.2.1 Primer design and PCR amplification

As in chapter 2

#### 4.2.2 Cloning, sequencing and sequence analysis

See chapter 2.

#### 4.2.3 PCR-RFLP

As in chapter 2.

### 4.3 Results

#### 4.3.1 Growth hormone gene

Primers were designed for the study to amplify fragments of between 400 – 700 bp and four primer pairs were generated to amplify products named GH1, GH2, GH3 and GH4 (Table 4.1). As shown in the Figure 4.1, each primer pair spans an intron which is likely to maximize SNP discovery, and there is some overlap of the products within exons.

![Figure 4.1. Schematic diagramme of growth hormone gene. Arrows indicate the fragment amplified. E refers to exon and GH refer to the product from each primer pair (Not drawn to scale).](image)

89
Table 4.1 Names of genes, chromosome location (Chr), primers used, region amplified and sequence of primer pairs, annealing temperature (Anne Temp), primers reference and Genebank accession number used for growth related genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Primer name</th>
<th>Region amplify</th>
<th>Primer sequence 5'- 3'</th>
<th>Anne Temp</th>
<th>Primer Reference</th>
<th>Genbank Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>19</td>
<td>GH1</td>
<td>Partial E1, I1 and</td>
<td>CAGGACCCAGTTCACCAGAC AACAGGCGGGCAAAAGGAC</td>
<td>60°C</td>
<td></td>
<td>M57764</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>partial E2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>19</td>
<td>GH2</td>
<td>Partial I1, E2, I2</td>
<td>GGCCCTCTCTGGTCTCTCC TGCCACTCACTGATTTCTGC</td>
<td>63°C</td>
<td></td>
<td>M57764</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>and partial E3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>19</td>
<td>GH3</td>
<td>Partial E3, I3 and</td>
<td>CTACATCCCAGGG?ACAG TCCAGGTCCTCTAGCTCTTC</td>
<td>60°C</td>
<td></td>
<td>M57764</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>partial E4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>19</td>
<td>GH4</td>
<td>Partial E4, I4 and</td>
<td>GCTTCGATCTCACTGCTC GATGGCTGGCAACTAGAAAAGG</td>
<td>60°C</td>
<td></td>
<td>M57764</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>partial E5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHR</td>
<td>20</td>
<td>GHR</td>
<td>5’ end</td>
<td>GTGCTCTAATCTTTTCTGGTACCAGG CCTCCCAATCAATTACATTTTCTC</td>
<td>65°C</td>
<td>Lucy et al. 1998</td>
<td>U15731.2</td>
</tr>
<tr>
<td>IGF1</td>
<td>5</td>
<td>IGF1</td>
<td>5’ end and E1</td>
<td>ATTACAAACCTGCTGCCCC TTAATAATTGGGAGAGTTGAGACTGC</td>
<td>60°C</td>
<td>Ge et al. 2001</td>
<td>AF017143</td>
</tr>
<tr>
<td>PIT1</td>
<td>1</td>
<td>PIT1</td>
<td>Partial I5, E6 and</td>
<td>AAACCATCATTCCCTCTT AATGTACAATTGCTCTGAGG</td>
<td>57°C</td>
<td>Woolard et al. 1994</td>
<td>X12657</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>partial I6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E Exon; I Intron; Anne Temp Annealing temperature
4.3.1.1 PCR amplification of primers and cloning of GH gene

GH1 primer pair was used to amplify regions of exon 1 to exon 2 in growth hormone gene (figure 4.1). This primer pair gave a product size of 409 bp shown in Figure 4.2(A). Primers pair GH2 was used to amplify region between exon 2 and exon 3 and it gave a product size of 537 bp as shown in Figure 4.2(B). Primer pair GH3 was used to amplify between exon 3 and exon 4 gave product size of 475 bp (Figure 4.2(C)) whereas GH4 were used to amplify region between exon 4 and exon 5 and gave a product size of 641 bp (Figure 4.2(D)). Upon amplification, the bands from each lane were cut from the gel and DNA was extracted, cloned and sequenced.

4.3.1.2 Sequence analysis

4.3.1.2.1 Fragment GH1

Four animals were selected for cloning and one clone for each animal was sent for sequencing. The sequences of GH1 fragment were aligned with ClustalW using default settings (Figure 4.3). Forward and reverse primers were included and the sequences were aligned with Genbank accession M57764 as a reference. The polymorphisms are given in Table 4.2.
Figure 4.3a. DNA sequence alignment of primer GH1 of growth hormone gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 4.3b. DNA sequence alignment of primer GH1 of growth hormone gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
The results from the sequences show that there are four SNPs detected within this fragment. All the SNPs detected were within intron 1. SNPs at position 73 and 141 involves transversion of G→T and G→C, respectively. SNP at position 126 involves transition of C→T whereas at position 246, there was insertion of G nucleotide. This G insertion occurs in all the animals (frequency 1.00) compared to the other SNPs (0.5) within the fragment GH1.

Table 4.2. Details of SNPs identified in fragment GH1 of growth hormone gene in Brakmas. Position of polymorphism is based from start of fragment amplified. Sequence changes were reported according to variants compared to GenBank accession M57764. Frequency is based on changes observed within the four sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change</th>
<th>Frequency</th>
<th>Amino acid change (blank indicates intron)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73</td>
<td>Intron 1</td>
<td>Transversion</td>
<td>G→T</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>126</td>
<td>Intron 1</td>
<td>Transition</td>
<td>C→T</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>141</td>
<td>Intron 1</td>
<td>Transversion</td>
<td>G→C</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>246</td>
<td>Intron 1</td>
<td>Insertion</td>
<td>G</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

4.3.1.2.2 Fragment GH2

Four animals were selected for cloning and one clone for each animal was sent for sequencing. The sequences of GH2 fragment were aligned with ClustalW using default settings (Figure 4.4). Forward and reverse primers were included and the sequences were aligned with Genbank accession M57764 as a reference. The polymorphisms are given in Table 4.3.

Two SNPs were detected within fragment GH2 in Brakmas cattle. Both SNPs were detected within exon 2. SNPs detected within this fragment were at frequency of 0.25. At position 111, transition of C→T resulted in changes of codon TCC→TCT. However this change is a silent mutation of amino acid
Figure 4.4a. DNA sequence alignment of primer GH2 of growth hormone gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 4.4b. DNA sequence alignment of primer GH2 of growth hormone gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
serine. On contrary, at position 122, transition of $C \rightarrow T$ resulted in changes of codon $GCC \rightarrow GTC$ which results in change of amino acid alanine to valine.

Table 4.3. Details of SNPs identified in fragment GH2 of growth hormone gene in Brakmas. Position of polymorphism is based from start of fragment amplified. Sequence changes$^a$ were reported according to variants compared to GenBank accession M57764. Frequency$^b$ is based on changes observed within the four sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change$^a$</th>
<th>Frequency$^b$</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>111</td>
<td>Exon 2</td>
<td>Transition</td>
<td>$C \rightarrow T$</td>
<td>0.25</td>
<td>Silent mutation</td>
</tr>
<tr>
<td>2</td>
<td>122</td>
<td>Exon 2</td>
<td>Transition</td>
<td>$C \rightarrow T$</td>
<td>0.25</td>
<td>Ala$\rightarrow$Val</td>
</tr>
</tbody>
</table>

**4.3.1.2.3 Fragment GH3**

Three animals were selected for cloning and one clone for each animal was sent for sequencing. The sequences of GH3 fragment were aligned with ClustalW using default settings (Figure 4.5). Forward and reverse primers were included and the sequences were aligned with Genbank accession M57764 as a reference. The polymorphisms are given in Table 4.4.

Ten SNPs were detected within fragment GH3 in Brakmas cattle. Nine out of ten SNPs detected occurred within intron 3. All SNPs were in the form of transition of nucleotides except at position 175 and 185 which involves insertion of nucleotides T and G, respectively, both deletions being present in all three sequences (frequency 1.00). Other SNPs, where high frequencies involved transition of nucleotides, were at position 182, 302, 303 and 328.

At position 452, transition of $C \rightarrow T$ resulted in changes of codon $GTC \rightarrow GTT$. However this change is a silent mutation in the codon for the amino acid valine and occurred at lower frequency rate (0.33).
Table 4.4. Details of SNPs identified in fragment GH3 of growth hormone gene in Brakmas. Position of polymorphism is based from start of fragment amplified. Sequence changes$^a$ were reported according to variants compared to GenBank accession M57764. Frequency$^b$ is based on changes observed within the three sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change$^a$</th>
<th>Frequency$^b$</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>116</td>
<td>Intron 3</td>
<td>Transition</td>
<td>G$\rightarrow$A</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>117</td>
<td>Intron 3</td>
<td>Transition</td>
<td>G$\rightarrow$A</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>175</td>
<td>Intron 3</td>
<td>Insertion</td>
<td>T</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>182</td>
<td>Intron 3</td>
<td>Transition</td>
<td>C$\rightarrow$T</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>185</td>
<td>Intron 3</td>
<td>Insertion</td>
<td>G</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>302</td>
<td>Intron 3</td>
<td>Transition</td>
<td>G$\rightarrow$A</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>303</td>
<td>Intron 3</td>
<td>Transition</td>
<td>A$\rightarrow$G</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>318</td>
<td>Intron 3</td>
<td>Transition</td>
<td>C$\rightarrow$T</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>328</td>
<td>Intron 3</td>
<td>Transition</td>
<td>C$\rightarrow$T</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>452</td>
<td>Exon 4</td>
<td>Transition</td>
<td>C$\rightarrow$T</td>
<td>0.33</td>
<td>Silent mutation</td>
</tr>
</tbody>
</table>

4.3.1.2.4 Fragment GH4

Four animals were selected for cloning and one clone for each animal was sent for sequencing. The sequences of GH4 fragment were aligned with ClustalW using default settings (Figure 4.6). Forward and reverse primers were included and the sequences were aligned with Genbank accession M57764 as a reference. The polymorphisms are given in Table 4.5.

Ten SNPs were detected within GH4 fragment. Eight SNPs were detected within intron 4 and two within exon 5. Five out of ten polymorphisms detected were transition of nucleotides, three were transversions and two were insertions.

The insertion of C nucleotide was detected at position 182 with high frequency (1.00) and insertion of three nucleotides ACG were detected at position 229-231 with lower frequency (0.25). Figure 4.7 shows the insertion of ACG. Both insertions were within intronic regions. Transversions at three
Figure 4.6a. DNA sequence alignment of primer GH4 of growth hormone gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 4.6b. DNA sequence alignment of primer GH4 of growth hormone gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
different positions also had been observed with different frequency, 0.25, 0.5 and 1.00 for transversion of T→A at position 546, transversion of C→G at position 202 and transversion of T→G at position 238, respectively.

At position 521, transition of C→T resulted in changes of codon CTC→CTT. However this change resulted in silent mutation of amino acid leucine with frequency of 0.75. But transversion at position 546 resulted in changes of amino acid phenylalanine (TTC) to isoleucine (ATC) with frequency of 0.25. All other transitions (position 218, 271, 316 and 347) were at frequency rate of 0.5

Figure 4.7. Sequence traces of two clones showing differences in sequence of Brakmas. The lower trace show the insertion of the ACG tri-nucleotide after the first GC
Table 4.5. Details of SNPs identified in fragment GH4 of growth hormone gene in Brakmas. Position of polymorphism is based from start of fragment amplified. Sequence changes<sup>a</sup> were reported according to variants compared to GenBank accession M57764. Frequency<sup>b</sup> is based on changes observed within the four sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Frequency&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>182</td>
<td>Intron 4</td>
<td>Insertion</td>
<td>C</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>202</td>
<td>Intron 4</td>
<td>Transversion</td>
<td>C→G</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>218</td>
<td>Intron 4</td>
<td>Transition</td>
<td>G→A</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>229-231</td>
<td>Intron 4</td>
<td>Insertion</td>
<td>ACG</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>238</td>
<td>Intron 4</td>
<td>Transversion</td>
<td>T→G</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>271</td>
<td>Intron 4</td>
<td>Transition</td>
<td>T→C</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>316</td>
<td>Intron 4</td>
<td>Transition</td>
<td>A→G</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>347</td>
<td>Intron 4</td>
<td>Transition</td>
<td>G→A</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>521</td>
<td>Exon 5</td>
<td>Transition</td>
<td>C→T</td>
<td>0.75</td>
<td>Silent mutation</td>
</tr>
<tr>
<td>10</td>
<td>546</td>
<td>Exon 5</td>
<td>Transversion</td>
<td>T→A</td>
<td>0.25</td>
<td>Phe→Ile</td>
</tr>
</tbody>
</table>

4.3.2 Growth hormone promoter gene

4.3.2.1 PCR amplification of primers and cloning of GHR gene

GHR primer pairs were used to amplify the 5’ flanking region of the growth hormone receptor gene. This primer pair gave a product size of 94 bp shown in Figure 4.8.
Figure 4.8. Agarose gel separation of PCR amplification products of a growth hormone receptor gene flanking region. Fragment sizes are indicated above. Lane M: Yorkbio Q-Step 2 marker; Lane 1: B5222; Lane 2: B7159; Lane 3: B8040; Lane 4: B9058

4.3.2.2 Sequence analysis

Four animals were selected for cloning and one clone for each animal was sent for sequencing. The sequences of GHR fragment were aligned with ClustalW using default settings (Figure 4.9). Forward and reverse primers were included and the sequences were aligned with Genbank accession U15731.2 as a reference. The polymorphism is given in Table 4.6.

Table 4.6. Details of polymorphisms identified in fragment GHR of growth hormone receptor gene in Brakmas. Position of polymorphism is based from start of fragment amplified. Sequence changes\(^a\) were reported according to variants compared to GenBank accession U15731.2. Frequency\(^b\) is based on changes observed within the four sequences analysed from Brakmas

<table>
<thead>
<tr>
<th>No.#</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change(^a)</th>
<th>Frequency(^b)</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>5' end</td>
<td>Deletion</td>
<td>(GT)_7</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

Deletion of (GT)_7 at position 50 results in presence of (GT)\(_{11}\) microsatellites in Brakmas sequences compared to U15731.2 (GT)\(_{10}\). This resulted in 16 bp shorter amplification products
Figure 4.9. DNA sequence alignment of primer GHR of growth hormone gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
4.3.3 Insulin-like Growth Factor 1 gene

4.3.3.1 PCR amplification of primers and cloning of \textit{IGF1} gene

\textit{IGF1} primer pair was used to amplify region of 5' end and exon 1 of insulin-like growth factor 1 gene. This primer pair gave products size of 846 bp shown in Figure 4.10.

![Agarose gel separation of PCR amplification products of IGF1](image)

Figure 4.10. Agarose gel separation of PCR amplification products of \textit{IGF1}; fragment sizes are indicated above. Lane M: Yorkbio Q-Step 2 marker; Lane 1: B5222; Lane 2: B7159; Lane 3: B8040; Lane 4: B9058

4.3.3.2 Sequence analysis

Six clones were selected from four animals and sent for sequencing. The sequences of \textit{IGF1} fragment were aligned with ClustalW using default settings (Figure 4.11). Forward and reverse primers were included and the sequences were aligned with Genbank accession AF017143 as a reference. The polymorphisms are given in Table 4.7a.

Nineteen polymorphisms were detected within this fragment and the six clones were the same in all locations except at position 682 and 687 (0.167). All polymorphisms detected were within 5' upstream region of the gene. The polymorphisms detected were nine involving transition, seven involving transversion, one insertion, one deletion of one nucleotide and one deletion of 2 bp.
Figure 4.11a. DNA sequence alignment of primer IGF of insulin-like growth factor 1 gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 4.11b. DNA sequence alignment of primer IGF of insulin-like growth factor 1 gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 4.11c. DNA sequence alignment of primer IGF of insulin-like growth factor 1 gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Table 4.7a. Details of SNPs identified in fragment *IGF1* of insulin-like growth factor 1 gene in Brakmas. Position of polymorphism is based from start of fragment amplified. Sequence changes were reported according to variants compared to GenBank accession AF017143. Frequency is based on changes observed within the six sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change</th>
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<td>1.000</td>
<td></td>
</tr>
<tr>
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<td>351</td>
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<td>Transition</td>
<td>G→A</td>
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<td></td>
</tr>
<tr>
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<td>353</td>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>Transversion</td>
<td>C→G</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>392</td>
<td>5' end</td>
<td>Transition</td>
<td>C→T</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>416</td>
<td>5' end</td>
<td>Transition</td>
<td>G→A</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>417</td>
<td>5' end</td>
<td>Transversion</td>
<td>A→C</td>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>427</td>
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<td>G→T</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>428</td>
<td>5' end</td>
<td>Transversion</td>
<td>G→T</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>430</td>
<td>5' end</td>
<td>Deletion</td>
<td>G</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>546-547</td>
<td>5' end</td>
<td>2 bp deletion</td>
<td>GA</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>618</td>
<td>5' end</td>
<td>Transversion</td>
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<td></td>
</tr>
<tr>
<td>15</td>
<td>682</td>
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<td></td>
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<tr>
<td>16</td>
<td>687</td>
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<td>17</td>
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<tr>
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<td>1.000</td>
<td></td>
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<tr>
<td>19</td>
<td>850</td>
<td>5' end</td>
<td>Transition</td>
<td>G→A</td>
<td>1.000</td>
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</table>

Comparison with Genbank accession AF404761 also had been done and the polymorphisms are shown in Table 4.7b. However the 2bp deletion (GA) at position 546-547, transition of A→G and C→T at position 682 and 687, are the
polymorphisms that also had been detected as compared with AF017143. The additional polymorphism detected within Brakmas compared to this reference are the transition of T→C and A→G at position 298 and 534, respectively, insertion of nucleotide A at position 505 and deletion of 42 bp nucleotides. All these polymorphisms were analysed in the 5' flanking region of the gene (“5’ end”).

Table 4.7b. Details of SNPs identified in fragment IGF1 of insulin-like growth factor 1 gene in Brakmas. Position of polymorphism is based from start of fragment amplified. Sequence changes were reported according to variants compared to GenBank accession AF404761. Frequency is based on changes observed within the six sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
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<td></td>
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<tr>
<td>2</td>
<td>505</td>
<td>5’ end</td>
<td>Insertion</td>
<td>A</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>534</td>
<td>5’ end</td>
<td>Transition</td>
<td>A→G</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>546-547</td>
<td>5’ end</td>
<td>Deletion</td>
<td>2 bp</td>
<td>0.167</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>548-588</td>
<td>5’ end</td>
<td>Deletion</td>
<td>41 bp</td>
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<td></td>
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<tr>
<td>6</td>
<td>682</td>
<td>5’ end</td>
<td>Transition</td>
<td>A→G</td>
<td>0.167</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>687</td>
<td>5’ end</td>
<td>Transition</td>
<td>C→T</td>
<td>0.167</td>
<td></td>
</tr>
</tbody>
</table>

4.3.3.3 In situ hybridization analysis of IGF1-1 clone
Fluorescence in situ hybridization analysis of IGF1-1 clone on Kedah-Kelantan and Charolais-Kedah Kelantan cattle was performed to investigate their chromosomal distribution. In situ hybridization was carried out under high stringency to ensure identification of site specific to the probe. Chromosomes were stained with DAPI (blue fluorescence) and clone of IGF1-1 probe was labelled with biotin-dUTP detected by Alexa 594 (red fluorescence).

Figure 4.12 shows Kedah-Kelantan cattle chromosomes stained with DAPI and hybridized with IGF1-1 clone in red. Several signals were detected within these chromosomes. It was present in various sizes as single dots, double dots or more at different signal strength. It seems that this probe has homology to
Figure 4.12. Metaphase spread of Brakmas cattle. (A) DAPI stained in Brakmas chromosomes. (B) *In situ* hybridization of IGF1-1 probe labelled with biotin and detected by Alexa 594 in Brakmas cattle. (C) Overlay of A and B image produce magenta coloured signal indicates co-localization of red signals on Brakmas DAPI stained chromosome. In C the IGF1-1 probe is shown to hybridize to some chromosome. More strongly in some, dispersed with single and double dots along the chromosome. The localization of two IGF1-1 signals to two larger chromosome is marked with arrows. Bars represent 10 μm. (D) Enlargement of chromosome number 5.
various chromosomal sites. Additional weak signals were present suggesting the sequences homologous to $IGF1$-1 clone probe were dispersed throughout the genome of Kedah-Kelantan. However these trends are not consistent to all the metaphases analysed except for the double dots. Furthermore, the double dots signals were much stronger. However, identification of the chromosome was not carried out. Presence of background signal was also detected but it seems weaker and it may be caused by the noise. This result suggests that $IGF1$-1 clone present as a single copy in Kedah-Kelantan.

### 4.3.4 Pituitary-specific Transcription factor gene

#### 4.3.4.1 PCR amplification of primers and cloning of $PIT1$ gene

$PIT1$ primer pairs were used to amplify a region including part of intron 5, exon 6 and partial of intron 6 of pituitary-specific transcription factor 1 gene. This primer pair gave a product size of 454 bp shown in Figure 4.13.

![Figure 4.13: Agarose gel separation of PCR amplification products of a $PIT1$ fragment; sizes are indicated above. Lane M: Yorkbio Q-Step 2 marker; Lane 1: B5222; Lane 2: B7159; Lane 3: B8040; Lane 4: B9058](image)

#### 4.3.4.2 Sequence analysis

Four animals were selected for cloning and one clone for each animal was sent for sequencing. The sequences of the $PIT1$ fragment were aligned with ClustalW using default settings (Figure 4.14). Forward and reverse primers were included and the sequences were aligned with Genbank accession NM174579 as a reference. The polymorphisms are given in Table 4.8.
Six SNPs were detected within *PIT1* fragment. Two out of six polymorphism detected were within exon 6. At position 65, transversion of G→C resulted in changes of codon GCG→GCC. At position 209, transition of A→G resulted in changes of codon CTG→CTA. However both changes are a silent mutation (alanine and leucine). At position 281, there was insertion of 4 bp (GTGT). SNPs at position 312 and 352 involved insertion of nucleotides C and A, respectively. At position 387 the SNPs was a transition of T→C. The SNPs were present in all four animals except at position 209 and 387.

Table 4.8. Details of SNPs identified in fragment *PIT1* of growth hormone gene in Brakmas. Position of polymorphism is based from start of fragment amplified. Sequence changes^a^ were reported according to variants compared to GenBank accession NM17457. Frequency^b^ is based on changes observed within the four sequences analysed from Brakmas

<table>
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<th>Position</th>
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<th>Sequence change^a^</th>
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<td>G→C</td>
<td>1.00</td>
<td>Silent mutation</td>
</tr>
<tr>
<td>2</td>
<td>209</td>
<td>Exon 6</td>
<td>Transition</td>
<td>A→G</td>
<td>0.5</td>
<td>Silent mutation</td>
</tr>
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<td>281-285</td>
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<td>GTGT</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>312</td>
<td>Intron 6</td>
<td>Insertion</td>
<td>C</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>352</td>
<td>Intron 6</td>
<td>Insertion</td>
<td>A</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>387</td>
<td>Intron 6</td>
<td>Transition</td>
<td>T→C</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.14a. DNA sequence alignment of primer PIT1 of pituitary transcription factor 1 gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion and gaps at the beginning or at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 4.14b. DNA sequence alignment of primer PIT1 of pituitary transcription factor 1 gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within sequences indicate deletion and gaps at the beginning or at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
4.3.4.3 Identification of RFLP site and PCR-RFLP

Hinfl polymorphisms were reported within the PIT1 gene (Wollard et al. 1994; Renaville et al. 1997a; 1997b; Zhoa et al. 2004). Therefore screening of Hinfl (G\(^A\)ANTC) polymorphism on Brakmas cattle were carried out. Figure 4.15 shows some of the animals screened.

Homozygotes exhibit two distinct bands of 207 and 244 which indicates the BB genotype whereas heterozygotes showed three DNA bands of 207, 244 and 454 bp indicating the AB genotype. Screenings of the population revealed that 15 out of 35 animals were AB genotype and 20 out of 35 animals were BB genotypes. No AA homozygotes were detected within this Brakmas population.

![Figure 4.15 Gel images of agarose gel separation showing PIT1 fragment amplified by PCR digested with Hinfl.](image)

1 2 3 4 5 6 M

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>YorkBio Q-Step 2 marker</td>
</tr>
<tr>
<td>1</td>
<td>B8040</td>
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<td>B8107</td>
</tr>
<tr>
<td>3</td>
<td>B9002</td>
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<td>B9037</td>
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<tr>
<td>7</td>
<td>B9071</td>
</tr>
<tr>
<td>8</td>
<td>B9178</td>
</tr>
</tbody>
</table>

The A allele homozygote would produce a single fragment of 454 bp (not seen in this sample of Brakmas cattle), B allele homozygote produces 207 and 247 bp fragments (lanes 4 and 5), while the AB heterozygote has fragments of 454, 207 and 247 bp (lanes 1, 2, 3 and 6).

4.4 Discussion

The ultimate applied goals of identification of polymorphism in this work is to identify possible contributing factors that affect traits of economic value in the phenotype, develop markers for aiding selection and to look for evidence of linkage disequilibrium or allele/heterozygosity fixation. Fundamental research
goals include the identification of new polymorphisms and the measurement of
diversity levels in the Malaysian composite cattle breed Brakmas, a baseline
study that will provide background data for future monitoring of changes. In this
chapter, identification of polymorphisms in Brakmas within four genes related to
growth were carried out. Polymorphisms were detected using molecular
technique such as single nucleotide polymorphisms (SNPs) in sequence analysis.
In some instances, SNPs detected were at a very low frequency (one out of four
DNA sequenced analysed) and could also be caused by PCR errors. These SNPs
are still discussed as they present potential candidates, but need to be verified by
future analyses. It was shown that PCR-RFLP are successfully used for
identification and detection of polymorphisms in a wider range of animals.

4.4.1 Polymorphisms in growth hormone gene

Analysis of the bovine growth hormone in Brakmas cattle revealed a total of 25
SNPs over 2068 bp of sequence that were assayed; four in intron 1, two in exon
2, nine in intron 3, eight in intron 4 and two in exon 5. Out of twenty five SNPs
detected, only four SNPs occurred within the exonic regions. SNPs at position
111 of GH2 and 521 of GH4 are silent mutations. SNPs at position 122 of GH2
and position 546 of GH4 induce amino acid substitution Ala/Val and Phe/Ile,
respectively (Table 4.5).

Several polymorphisms had been reported within the bovine growth hormone gene but the substitution of C—»G in exon 5 which induce amino acid Leu/Val has been associated to growth (Reis et al. 2001; De Stasio et al. 2003). Sequence analysis carried out in Brakmas shows that Brakmas carries the Leu variant in all the sequences analysed here. The Leu variant has been associated with higher weight gain, meat deposition and carcass weight compared to Val variant. There is possibility that Brakmas might have higher growth rate traits. However, further verification has to be carried out to confirm the association of Leu/Val polymorphisms in growth hormone gene in Brakmas. As shown by Zwierchowski et al. (2001) there is an effect of breed. Furthermore, in another study it was observed that there is no association between Val/Leu polymorphism with meat production traits in Piedmontese cattle (Di Stasio et al. 2003).
The Leu homozygous genotype also had been associated with higher milk production (Lucy et al. 1993). With the fact that Brakmas has Leu variants, there is possibility to look at Brakmas as multi-purpose cattle: Brakmas had never been considered for dairy cattle use due to low milk production. Although a large number of genes contribute to milk production, the presence of variation at this locus means that selection at some loci might improve dairy production: there is a possibility that milk production traits in Brakmas have not been noted as superior characteristics due to the tropical hot and humid environmental factors, not due to lack of superior genes. However, further verification is required for this assumption.

Raised milk fat is a major trait of economic value, with milk prices heavily dependent on butterfat content; raised protein levels, measured and paid for as milk solids, are also desirable traits. In Danish Holstein and Polish Black and White cattle, the Leu variant of the GH gene has been associated to higher milk fat content but less protein content (Zwierchowski et al. 2002). However, in Danish Jersey, the Val variant had been associated with higher fat and protein content as well as milk production (Zwierchowski et al. 2002). In another study also done on Black and White cattle (Holstein-Friesian type), Leu had been associated with higher milk production and fat and protein content (Dybus 2002). Therefore, there is the possibility to use Leu/Val polymorphism in marker assisted selection. Looking at the scenario, marker assisted selection used in different breeds might be different as well.

4.4.2 Polymorphisms in growth hormone receptor gene

Analysis of the bovine growth hormone receptor gene in Brakmas showed that there was presence of a TG11 repeat (Fig 4.8/Table 4.6). This short allele resulted in a 94 bp PCR product, whereas the long allele repeat such as TG16 has a product size of 104 bp. The result in Brakmas is in agreement with results reported (Lucy et al. 1998) earlier. Lucy et al. (1998) reported that the Bos indicus subspecies carries the shorter TG-repeat compared to Bos taurus which usually carries a longer TG-repeat, TG16- TG20. Angus cattle (Bos taurus) were shown to be polymorphic, with the TG16- TG20 allele most frequent but 9% of individuals being heterozygous containing TG11/ TG16-20 allele (Hale et al. 2000).
It was shown that Brahman and Nellore (*Bos indicus*) cattle were homozygotes for a 94 bp allele. Hale *et al.* (2000) reported that TG\textsubscript{11} in growth hormone receptor gene alleles have decreased growth rate at weaning and slaughter.

Based on short TG repeat polymorphism within growth hormone receptor gene, it is predicted that Brakmas will have lower weight gain. Selection and screening of TG polymorphism is important. There is possibility that this polymorphism will reduce 16 kg and 12 kg of weaning weight and carcass weight as the results shown by Hale *et al.* (2000). However, the same authors proved that carcass fat deposition and longissimus muscle areas are not affected. Effect on short TG repeat in Brakmas cattle in association to growth rate is unknown. However, if short TG repeat was proven to reduce the growth rate in Brakmas, this polymorphism can be use in marker assisted selection in order to avoid this allele.

### 4.4.3 Polymorphisms in insulin-like growth factor 1 gene

Analysis of sequence of bovine insulin-like growth factor 1 gene over 846 bp in Brakmas cattle revealed a total of 23 SNPs (19 SNPs as compared to AF017143 and seven as compared to AF404761 but sharing three SNPs). All SNPs detected within this fragment were intronic. Polymorphisms in promoter region have more effect and association to production traits compared to polymorphism within introns (Ge *et al.* 2001).

The transition of T→C within insulin-like growth factor 1 gene at position 512 in the gene indicates presence of BB genotype (Ge *et al.* 2001). Position 512 in the study reported by Ge *et al.* (2001) corresponds to position 298 in this study on Brakmas cattle. The BB genotype has been associated with higher weight gain during the first 20 days after weaning. Sequence analysis carried out in Brakmas showed that Brakmas carried a C nucleotide which indicates BB genotype associated with higher weight gain. Ge *et al.* (2003) consider the potential of polymorphisms in insulin-like growth factor 1 as a genetic marker in Angus cattle. The same authors also stated that there are differences in production traits in different populations associated with the polymorphisms detected within insulin-like growth factor 1 and further verification is required to established the association. Although verification of
association of BB genotype to higher growth rate is required, there is a possibility to use this polymorphism as a marker in a marker assisted selection program in Brakmas cattle.

Calves with BB genotypes weight gain is 4.69 kg more in than the AA genotype whereas the heterozygous AB genotype calves gain 4.78 kg higher than AA genotype (Ge et al. 2001). BB genotype can be used as marker for identification of calves with higher weight gain during first 20 days after weaning. Although the importance of weight gain characteristics and the relationship to weaning of calves may be of particular importance in suckler herds such as Brakmas; genotype information can be gathered and potentially is use in understanding the mechanism of growth as well as formulation of management strategies.

4.4.4 Polymorphisms in pituitary-specific transcription factor 1 gene

Investigation of the genetic variability at bovine pituitary-specific transcription factor 1 gene over 451 bp in Brakmas cattle has been carried out and analysis of sequence of bovine pituitary-specific transcription factor 1 gene (PIT1) revealed that total of six SNPs; two SNPs occurred within exon 6 and four SNPs occurred within intron 6. The SNPs at exon however are silent mutations.

Allele B was associated with higher body weight gain at 7 months of age in Belgian Blue (Renaville et al. 1997a). Allele B which corresponds to Hinfl polymorphism (Wollard et al. 1994) in exon 6 was reported to be associated with early-age body weight gain in beef cattle. However, no association to meat production was reported in Piedmontese (Di Stasio et al. 2002) as well as no relationship reported to be associated to growth and carcass in Angus (Zhoa et al. 2004). Sequence analysis of pituitary-specific transcription factor 1 gene showed that Brakmas carries AB or BB genotype and given the observed frequency of the two alleles, there is no significant deficiency of the AA homozygotes. PCR-RFLP carried out in Brakmas cattle population revealed the same trends indicating presence of AB or BB genotype.

The PCR-RFLP result displays presence of allele A an B in Brakmas cattle. In beef production, allele BB is preferable as BB allele animals has been
shown to have higher weight gain. In Angus cattle, PIT1-HinflI correlates with growth traits (Zhao et al. 2004). However, in other studies, it was found that there is no association between growth performance and carcass traits in Black and White cattle (Zwierzchowki et al. 2002) and (Dybus et al. 2004).

On the other hand, in dairy cattle, both allele A and allele B have important contributions. The A allele in Italian Holstein-Friesian breed had been shown to be superior for milk and protein yields but allele B superior for fat percentage (Renaville et al. 1997b). In Black and White cattle, Allele A also had been associated to higher milk production (Zwierzchowki et al. 2002) although there is no association between PIT1-Hinfl polymorphism and milk production traits (Dybus et al. 2004) was reported in the same breed.

PIT1 is a strong candidate in dairy cattle for association with growth (Renaville et al. 1997a). As beef cattle, it is expected that presence of allele B indicates a higher weight gain in Brakmas cattle. However, as association of higher weight gain and PIT1-HinflI in Brakmas cattle need to be verified before this polymorphism is confirmed and can be used in marker-assisted selection programs.

4.5 Implications

The results provide a valuable reference for marker assisted selection, especially since the work has identified a number of SNPs within the genes in the Brakmas population which have previously been associated with production-related traits. In this study as well, we were successful in identifying both novel alleles not previously found, and various polymorphisms in different growth-related genes in cattle in the Brakmas population.

The polymorphisms identified in the growth hormone gene, growth hormone receptor gene and pituitary-specific transcription factor 1 offer potential markers for growth rate, whereas polymorphisms in insulin-like growth factor 1 gene could be a potential marker for post-weaning growth. Most of the published SNPs that are associated with key growth QTLs have been identified in temperate Bos taurus type cattle. At present, the best hypothesis is that the same SNPs will have the same effect in Bos indicus types such as Brakmas. Given the
large numbers of polymorphisms between the two subspecies, verification on
effect of associated of production traits with the molecular markers has to be
established in Brakmas as the effect might be different between breeds.
Chapter 5: Structural analysis of genes associated with meat quality

5.1 Introduction and aims

Improvement of meat quality through genetic selection is difficult as quality parameters such as tenderness and marbling of the cattle are most accurately measured after slaughter and except for use of stored semen or embryos, at that time it is too late to use these cattle for breeding. Advances in molecular techniques enable the improvement of meat quality based on selection at the genetic level, using DNA polymorphisms which can be linked to such economic traits in cattle. For trait association, molecular methods can also be combined with non-invasive imaging methods such as ultrasound or nuclear magnetic resonance imaging (MRI). Several studies have indicated a relationship between meat tenderness and calpain gene alleles, and marbling to leptin, thyroglobulin (\(TG\)) and diacylglycerol \(O\)-transferase (\(DGAT1\)); recent work based on the polymorphism within these genes has associated certain alleles with desirable tenderness and marbling traits.

The aim for this chapter is to analyse the DNA sequences of fragments of several genes in Brakmas cattle, and compare these with meat quality in this breed and reference cattle. Analyses of polymorphisms of genes can be used to characterise the roles of these identified genetic polymorphisms on meat quality and important economic traits in cattle. Knowledge of effects of polymorphism in livestock is important for the manipulation of meat production genetics in livestock. Only a few molecular markers have been identified to link with meat quality and thus genetic improvement through marker assisted selection for breeding purposes is limited.

In this chapter, I aimed to isolate genes that are related to meat quality, such as calpain for tenderness of meat and leptin, \(TG\) and \(DGAT1\) for marbling from Brakmas by PCR amplification, cloning and sequencing. From sequence analysis of calpain, leptin, \(TG\) and \(DGAT1\) within Brakmas, I examined the range of types of mutation such as transition, transversion, insertions and deletions that were presents. High levels of similarity were found in these genes as compared to
those in Genbank. SNP and PCR-RFLP was use as a molecular marker approach for detection of polymorphisms. PCR-RFLP screening within Brakmas indicated a range of trends associated to these genes. Various PCR-RFLP in published articles which were reported to be associated to meat quality were also observed within these studies. Given the level of polymorphism included in Brakmas and the similarity of some alleles with those tested in larger experiments in other breeds, the results can be used to chose parents for breeding with the most valuable alleles for meat quality.

Specifically, the objectives were:

1. to identify polymorphisms within calpain gene, leptin gene, thyroglobulin gene and diacylglycerol O-transferase gene using SNPs detected from sequence analysis
2. to determine presence of Iso/Val genotype at position 530 and Gly/Ala genotype at position 316 in calpain gene
3. to determine if there is presence of point mutation A to T which alters an amino acid residue from Thy to Phe in leptin gene
4. To determine presence of point mutation T (allele ‘2’) to C (allele ‘3’) in thyroglobulin gene
5. To determine Lys/Ala genotype in diacylglycerol O-transferase gene

The results generated will be used to predict meat quality in Brakmas cattle.

5.2 Materials and Methods
5.2.1 Primer design and PCR amplification
As in chapter 2

5.2.2 Cloning, sequencing and sequence analysis
Cloning was done as in chapter 2. Clones were sequenced at the PNACL Sequencing Facility of Leicester University for Thyroglobulin gene and John Innes Centre (JIC), Norwich for the other genes.

The sequences of the gene fragment clones were aligned with the ClustalW using default setting. Forward and reverse primers were included within the sequence and were aligned with GenBank reference accessions (Table
5.1). For alignments, the forward primer was used as a start site. The Brakmas sequences were submitted to EMBL-EBI Database with the accession numbers AM263422 to AM263425 and AM266809 to AM266811.

5.2.3 PCR-RFLP

Based on sequences analysis, polymorphisms were recorded and restriction enzyme sites particularly those spanning polymorphisms were identified. These and additional restriction enzymes reported in published articles (regardless of polymorphisms seen in the new sequences) which have been associated to tenderness and marbling traits in these gene fragments were also used to screen the Brakmas cattle population in this study. Upon identification of restriction enzyme site polymorphisms for the fragment amplified, most accessions of the population studies here (see Chapter 2; animal codes given in figure legends) were genotyped by PCR-RFLP.

5.3 Results

5.3.1 Calpain gene

In order to investigate the genetic structure of the calpain gene in Brakmas, three set of primers were used, CAE13I14, CAI7E10 and CAE14I15.

5.3.1.1 Fragment CAE13I14

5.3.1.1.1 PCR amplification of primers and cloning of CAE13I14 fragment of calpain gene

Fragment CAE13I14 (Table 5.1) was amplified by PCR and this primer pair gave products of approximately 568bp (Figure 5.1). The bands from each lane which represent different animals were cut from the gel and the DNA was extracted, cloned and sequenced.
Table 5.1 Names of genes, chromosome location (Chr), primers used, region amplified and sequence of primer pairs, annealing temperature (Anne Temp), primers reference and Genebank accession number used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Primer name</th>
<th>Region amplify</th>
<th>Primer sequence 5’-3’</th>
<th>Anne Temp</th>
<th>Primer Reference</th>
<th>Genbank Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpain</td>
<td>29</td>
<td>CAI7E10</td>
<td>Partial I7, E8, I8, E9, I9, Partial E10</td>
<td>CCCCGTGACCTTCAGCAGCAC GTTGCGGAACCTCTGGCTCTTGAG</td>
<td>68°C</td>
<td>Page et al. 2003</td>
<td>AF252504</td>
</tr>
<tr>
<td>Calpain</td>
<td>29</td>
<td>CAI13I14</td>
<td>Partial E13, I13, E14, partial I14</td>
<td>GAGCCCAACAACGAAGGT AATACAGCCCAATGATGAGG</td>
<td>60°C</td>
<td>Page et al. 2003</td>
<td>AF248054</td>
</tr>
<tr>
<td>Calpain</td>
<td>29</td>
<td>CAE14I15</td>
<td>Partial E14, partial I14</td>
<td>TTCAGGCCAATCTCCCCGACG GATGTGAACTCCACCAGGCCAG</td>
<td>60°C</td>
<td>Juszczuk-Kubiak et al. 2004</td>
<td>AF248054</td>
</tr>
<tr>
<td>Leptin</td>
<td>4</td>
<td>LA</td>
<td>Partial I1, E2, partial I2</td>
<td>GATTCCGCGCCACCTCTC CCTGTGCAAGGCTGCACAGCC</td>
<td>60°C</td>
<td>Lagonigro et al. 2003</td>
<td>AJ512638</td>
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<tr>
<td>TG</td>
<td>14</td>
<td>TG</td>
<td>5’ end</td>
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<td>60°C</td>
<td>Thaller et al. 2003</td>
<td>M35823</td>
</tr>
<tr>
<td>DGAT1</td>
<td>14</td>
<td>DGAT1</td>
<td>Partial I6, E7, I7, E8, I8, E9, partial I9</td>
<td>GCACCATTCCTCTCTCCAAG GGAAGCGCTTTCCGATG</td>
<td>60°C</td>
<td>Thaller et al. 2003</td>
<td>AY065621</td>
</tr>
</tbody>
</table>

E Exon, I Intron
5.3.1.1.2 Sequence analysis

Four animals were selected for cloning and a total of eight clones were sent for sequencing in which two sequences represented each animals. The sequences were aligned with ClustalW using default settings (Figure 5.2). Forward and reverse primers were included. The sequences were aligned with reference to GenBank accession AF248054. The polymorphisms are given in Table 5.2.

In total, 11 SNPs were detected within this fragment. Out of that 11, one SNP was detected within exon 13, one within intron 13, one within exon 14 and eight within intron 14. A mutation at position 351-354 resulted in a 4bp deletion (Figure 5.3) and within the introns, SNPs were detected at position 124, 282, 306, 395, 512 and in Brakmas. The SNP at position 34 which involved a change from T→C resulted in a change of amino acid phenylalanine (TTC) to leucine (CTC). The SNP at position 175 which involving changes of nucleotide C→T resulted in a silent mutation (amino acid aspartic acid – GAC→GAT).
Figure 5.2a. DNA sequence alignment of primer CAE13114 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the beginning of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.2b. DNA sequence alignment of primer CAE13114 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the beginning of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.2c. DNA sequence alignment of primer CAE13I14 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the beginning of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Table 5.2. Details of SNP identified in fragment CAE13I14 Calpain gene in Brakmas. Sequence changes were reported according to variants compared to GenBank accession AF248054. Frequency is based on changes observed within the eight sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change</th>
<th>Frequency</th>
<th>Amino acid change</th>
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</thead>
<tbody>
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<td>Phe→Leu</td>
</tr>
<tr>
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<td>Intron 13</td>
<td>Transition</td>
<td>C→T</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>175</td>
<td>Exon 14</td>
<td>Transition</td>
<td>C→T</td>
<td>0.125</td>
<td>Silent mutation</td>
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<td>4</td>
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<td>G→C</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>306</td>
<td>Intron 14</td>
<td>Transition</td>
<td>C→T</td>
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</tr>
<tr>
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<td>Intron 14</td>
<td>Deletion</td>
<td>C</td>
<td>0.875</td>
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<tr>
<td>7</td>
<td>352</td>
<td>Intron 14</td>
<td>Deletion</td>
<td>G</td>
<td>0.875</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>353</td>
<td>Intron 14</td>
<td>Deletion</td>
<td>A</td>
<td>0.875</td>
<td></td>
</tr>
<tr>
<td>9</td>
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<td>T</td>
<td>0.875</td>
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<td>10</td>
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<td>11</td>
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<td>Intron 14</td>
<td>Transition</td>
<td>T→C</td>
<td>0.125</td>
<td></td>
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</tbody>
</table>

Figure 5.3. Sequence trace of two different positions showing polymorphisms. Upper sequence refers to SNP in exon 13 (C→T, grey shading) and the lower sequences shows the 4 bp deletion of CGAT in intron 14 detected in some Brakmas.
5.3.1.1.3 Identification and analysis of PCR-RFLP site

The nucleotide polymorphisms found in the sequences were examined to see if they lay within restriction enzyme recognition sequences. From the sequence, a restriction site was identified in intron 14, indicating that the enzyme *PvuI* (CGAT\(^{+}\)CG) could be used to detect a 4 bp deletion in Brakmas. Cleavage of this products using *PvuI* enzyme yielded two fragments of 358 bp and 210 bp (Figure 5.4). All Brakmas in the population studied were genotyped by PCR-RFLP. It was observed that 27 out of 37 animals screened showed present of this 4 bp deletion.

5.3.1.2 Fragment CA17E10

5.3.1.2.1 PCR amplification of primers and cloning of CA17E10 fragment of calpain gene

Fragment CA17E10 was amplified as shown in Table 5.1 by PCR. This primer pair gave products of approximately 998 bp (Figure 5.5). The bands from each lane which represent different animals were cut from the gel and the DNA was extracted, cloned and sequenced.

![Figure 5.5. Agarose gel separation of PCR amplification products of a fragment of the calpain gene amplified by primer pair CA17E10. Lane M: Bioline Hyperladder II marker; Lane 1: B5222; Lane 2: B7159; Lane 3B8040; Lane 4: no amplification](image)

5.3.1.2.2 Sequence analysis

Three animals were selected for analysis and a total of six clones were sent for sequencing in which two sequences represented each animals. The sequences were of the fragment CA17E10 were aligned with ClustalW using default settings (Figure 5.6). Forward and reverse primers were included within the sequence and were aligned with GenBank accession AF252504 as a reference.
Figure 5.4. Agarose gel separation of PCR amplification products digested with *Pvu*I. The images are inverted to show faint bands clearer. Fragments sizes are indicated above. Cattle without 4bp deletion will shows two fragments of 210 and 358 bp where cattle with 4bp deletion will not be cut. Lane M: York Bio Q-Step II marker; Lane 1: B129; Lane 2: B135; Lane 3: B140; Lane 4: B145; Lane 5: B151; Lane 6: B184; Lane 7: B307; Lane 8: B1857; Lane 9: B2241; Lane 10: B3102; Lane 11: B40280; Lane 12: B4064; Lane 13: B5222; Lane 14: B5230; Lane 15: B5288; Lane 16: B7159; Lane 17: B8032; Lane 18: B8040; Lane 19: B8107; Lane 20: B9002; Lane 21: B9004; Lane 22: B9014; Lane 23: B9030; Lane 24: B9037; Lane 25: B9058; Lane 26: B9062; Lane 27: B9071; Lane 28: B9130; Lane 29: B9132; Lane 30: B9178; Lane 31: B9181; Lane 32: B9183; Lane 33: B9187; Lane 34: B20149; Lane 35: B20154; Lane 36: B20197; Lane 37: B62127
Figure 5.6a. DNA sequence alignment of primer CA17E10 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the beginning and at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.6b. DNA sequence alignment of primer CA17E10 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the beginning and at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.6c. DNA sequence alignment of primer CA17E10 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the beginning and at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.6d. DNA sequence alignment of primer CA17E10 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the beginning and the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
The polymorphisms are given in Table 5.3. The results from the sequences showed that there are 14 SNPs present within this fragment. Six SNPs were detected within intron 7, two within intron 8, one within exon 9, four within intron 9 and one within exon 10.

Based on sequences analysis, nine of the thirteen mutations involve transitions except at position 69 and 726 which involve transversions, compared to an expectation of 25%, and at position 905 which involves deletion of one nucleotide. All Brakmas sequences showed a deletion at this position compared to Bos taurus and Bos taurus X Bos indicus reference sequences (Figure 5.7). The SNPs occurring in exon 9 (from C→G at position 726) resulted in amino acid change from alanine (GCC) to glycine (GGC). The change of C→A at position 940 in exon 10, from codon TCC to TCA is a silent mutation of the amino acid serine.

Page et al. (2002) reported a variant at position 10876 which corresponded to position 721 in this experiment that resulted in an amino acid changes from alanine. Alanine (GCC) was associated to tender meat whereas glycine (GGC) was associated to less tender meat. However, this SNP was not observed within the three Brakmas sequences obtained here, all carrying the G variant. Screening the whole population was not done as no restriction enzyme site is present at this SNP.

Figure 5.7. Sequence trace of two clones showing presence of five C nucleotide at position 905 in intron 5 (table 5.3). Reference sequences detected six C which indicates a deletion in Brakmas.
Table 5.3. Details of SNPs identified in fragment CA17E10 of the calpain gene in Brakmas. Sequence changes\textsuperscript{a} were reported according to variants compared to GenBank accession AF252504. Frequency\textsuperscript{a} is based on changes observed within the six sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change\textsuperscript{a}</th>
<th>Frequency</th>
<th>Amino acid change</th>
</tr>
</thead>
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</tr>
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<td>4</td>
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<td>T→C</td>
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<tr>
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<td>Transition</td>
<td>C→T</td>
<td>0.167</td>
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<td>C→A</td>
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<td>Silent mutation</td>
</tr>
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</table>

5.3.1.2.3 Identification and analysis of PCR-RFLP site
The nucleotide polymorphisms found in the sequences were examined to see if they lay within restriction enzyme recognition sequences. However, no enzyme identified.

5.3.1.3 Fragment CAE14I15
5.3.1.3.1 PCR amplification of primers and cloning of CAE14I15 fragment of calpain gene
Primers CAE14I15 were used to amplify CAE14I15 fragment (Table 5.1) This primer pair gave products of approximately 685 bp (Figure 5.8). The bands from
each lane which represent different animals were cut from the gel and the DNA was extracted, cloned and sequenced.

Figure 5.8. Agarose gel separation of PCR amplification products of a fragment of the calpain gene amplified by primer pair CAE14115. Lane M: Bioline Hyperladder II marker; Lane 1: B5222; Lane 2: B7159; Lane 3: B8040; Lane 4: B9058

5.3.1.3.2 Sequence analysis

Four animals were selected for cloning and a total of eight clones were sent for sequencing in which two sequences represented each animals. However, two sequences from animal B5222 and B9058 were not used due to poor reading. The sequences of the fragment CAE14115 clones were aligned in ClustalW using default setting (Figure 5.9). Forward and reverse primers were included. The sequences were aligned with reference of GenBank accession AF248054. The polymorphism are given in Table 5.4.

Eight SNPs were detected within this fragment and all of them were in intron 14. The first 389 bp present in this sequence is overlapping with fragment CAE13114 discussed previously and therefore the first six SNPs present in this sequence are the same as in fragment CAE13114. Extension of flanking region within intron and exon 15, only results in two further two SNPs and both are in intron 14. SNP at position 400 and 402 resulted in nucleotide changes from T—>C. SNP at position 127 result in transition of C—>T creating a FokI restriction enzyme site.
Figure 5.9a. DNA sequence alignment of primer CAE14115 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.

Figure 5.9b. DNA sequence alignment of primer CAE14115 of Calpain gene in Brakmas using ClustalW. Each nucleotide is.
Figure 5.9c. DNA sequence alignment of primer CAE14115 of Calpain gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps within Brakmas sequences indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Table 5.4. Details of SNPs and a deletion identified in fragment CAE14I15 of the Calpain gene in Brakmas. Sequence changes\textsuperscript{a} were reported according to variants compared to GenBank accession AF248054. Frequency\textsuperscript{a} is based on changes observed within the six sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change\textsuperscript{a}</th>
<th>Frequency</th>
<th>Amino acid change</th>
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<td>0.167</td>
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Figure 5.10. Sequence traces of two clones at position 127 (C or T, shaded grey) showing polymorphism within fragment CAE14I15

5.3.1.3.3 Identification of RFLP site and PCR-RFLP

Upon identification of enzyme restriction sites at position 127 fragment CAE14I15 in Brakmas, the PCR-RFLP method was used for the detection of nucleotide sequence polymorphism in intron 14 of the bovine \textit{CAPN1} gene. \textit{FokI} (GGATG) cuts the T allele producing a single fragment of two 685 bp whereas C allele produce 530 and 155bp fragments. Seventeen out of 37 Brakmas cattle produce C allele.
Figure 5.11. Gel images of agarose gel separation showing CAE14I15 fragments amplified by PCR and digested with FokI. The images are inverted to show faint bands clearer. Lane M: York Bio Q-Step II marker; Lane 1: B129; Lane 2: B135; Lane 3: B140; Lane 4: B145; Lane 5: B151; Lane 6: B184; Lane 7: B307 and Lane 8: B1857. C allele produce 530 and 155 bp (not seen as below bottom of gel) fragment and T allele produce single fragment of 685 bp.

5.3.2 Leptin gene

5.3.2.1 PCR amplification of primers and cloning of exon 2 leptin gene
The primer used amplified a 467 bp fragment (Figure 5.12). The band from each lane which represent different animals were cut from the gel and the DNA was extracted, cloned and sequenced.

Figure 5.12. Agarose gel separation of PCR amplification products of the leptin gene. Lane M: Bioline Hyperladder II marker; Lane 1: B5222; Lane 2: B7159; Lane 3: B8040; Lane 4: B9058

5.3.2.2 Sequence analysis
Four animals were selected for cloning and a total of eight clones were sent for sequencing in which two sequences represented each animal. The sequences were aligned with ClustalW using default setting (Figure 5.13). The sequences
Figure 5.13a. DNA sequence alignment of Leptin gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.13b. DNA sequence alignment of Leptin gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps indicate deletion and gaps at the end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
were aligned with reference of GenBank accession AY138588 and AJ512638. The details of polymorphism were simplified as in Table 5.5.

From the sequence, four SNPs were detected, two in intron 1, and one each in exon 2 and intron 2. In intron 1, SNP involve insertion of one bp of G nucleotide at position 26 and a transition of A→G at position 98 whereas in intron 2, the SNP is transition of C→T at position 389. Transition of G→A in exon 2 at position 298 result in changes of coding of amino acid from GTG → GTA but it was a silent mutation valine.

Table 5.5. Details of SNPs identified in a fragment of the leptin gene in Brakmas. Sequence changes were reported according to variants compared to GenBank accession AJ512638. Frequency is based on changes observed within the eight sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change</th>
<th>Frequency</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>Intron 1</td>
<td>Insertion</td>
<td>G</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>98</td>
<td>Intron 1</td>
<td>Transition</td>
<td>A→G</td>
<td>0.167</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>298</td>
<td>Exon 2</td>
<td>Transition</td>
<td>G→A</td>
<td>0.167</td>
<td>Silent mutation</td>
</tr>
<tr>
<td>4</td>
<td>389</td>
<td>Intron 2</td>
<td>Transition</td>
<td>C→T</td>
<td>0.167</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.14. Sequence traces of two clones showing presence of four G nucleotide (Reference sequence have three G nucleotide)

5.3.2.3 Identification and analysis of PCR-RFLP site

All of the SNPs observed in these sequences were not within restriction enzyme site. However, Clal was shown to generate a polymorphism by Lagonigro et al.
Therefore, the Brakmas population were also genotyped for polymorphism present in an A/T variant with Clal (AT^CGAT) in attempts to associate Brakmas bovine leptin gene with feed intake as reported by Lagonigro et al. (2003). In the Clal digestion, the T allele will give a single fragment of 468bp or two fragments of 253 and 215 bp for allele A. A/T allele had been associated with up to 19% greater mean feed intake compared to A/A genotype (Lagonigro, et al. 2003). The results of genotyping with Clal in few Brakmas cattle were as in Figure 5.15. The Brakmas cattle in this experiment showed no T variant allele present either by restriction digestion or in the sequences.

![Figure 5.15. Agarose gel separation showing Leptin fragments amplified by PCR and digested with Clal. Lane M: York Bio Q-Step II marker; Lane 1: B129; Lane 2: B135; Lane 3: B140; Lane 4: B145; Lane 5: B151; Lane 6: B184; Lane 7: B307 and Lane 8: B1857. The A variant allele produces 253 and 215 bp fragments and the T allele (not seen above) would produce a single fragment of 468 bp.]

5.3.3 Thyroglobulin gene

5.3.3.1 PCR amplification of primers and cloning of thyroglobulin gene
The primer used amplified a 467 bp fragment (Figure 5.16) at 5' end of thyroglobulin gene (TG). Prominent bands at the expected size from each lane which represent different animals were cut from the gel and the DNA was extracted, cloned and sequenced.
5.3.3.2 Sequence analysis

Four animals were selected for cloning and a total of eight clones were sent for sequencing in which two sequences represented each animals. The sequences of the fragment TG clones were aligned in ClustalW using default setting (Figure 5.17). The sequences were aligned with reference of GenBank accession M35823. There were 15 SNPs detected within 5' end of thyroglobulin in Brakmas cattle. Transition occurs in 12 positions and four position involve in transversion of nucleotides. The polymorphisms are given in Table 5.6.

5.3.3.3 Identification of RFLP site and PCR-RFLP

The first restriction enzyme detected within the thyroglobulin gene is HpyCH4V (TG^CA). The A nucleotide allele at position 334 will result in formation of three fragments of 100, 114 and 334 bp whereas the G allele will result in a single fragment of 548 bp. Present of nucleotide A allele at position 29 results in fragment of 29, 100, 114 and 305 bp. Screening of SNPs at position 29 shows that 12 out of 37 animals detected this polymorphism.

The second restriction enzyme is BstYI (R^GATCY) where R represents purine and Y represent pyrimidine. T allele generate two fragments of 75 and 473 bp whereas C allele generate three fragments of 75, 178 and 473 bp. Genotyping of Brakmas cattle shows that all animals carry the C allele.
Figure 5.17a. DNA sequence alignment of Throglobulin gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.17b. DNA sequence alignment of Thyroglobulin gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Table 5.6. Details of SNPs identified in fragment TG gene fragments in Brakmas. Sequence changes\(^a\) were reported according to variants compared to GenBank accession M35823. Frequency\(^a\) is based on changes observed within the eight sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change(^a)</th>
<th>Frequency</th>
<th>Amino acid change</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>5' end</td>
<td>Transition</td>
<td>T→C</td>
<td>0.500</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>5' end</td>
<td>Transition</td>
<td>G→A</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>184</td>
<td>5' end</td>
<td>Transition</td>
<td>A→G</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>189</td>
<td>5' end</td>
<td>Transversion</td>
<td>G→T</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>196</td>
<td>5' end</td>
<td>Transition</td>
<td>T→C</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>203</td>
<td>5' end</td>
<td>Transition</td>
<td>T→C</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>206</td>
<td>5' end</td>
<td>Transition</td>
<td>C→T</td>
<td>0.750</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>223</td>
<td>5' end</td>
<td>Transversion</td>
<td>C→A</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>284</td>
<td>5' end</td>
<td>Transition</td>
<td>A→G</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>300</td>
<td>5' end</td>
<td>Transversion</td>
<td>T→A</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>335</td>
<td>5' end</td>
<td>Transition</td>
<td>C→T</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>336</td>
<td>5' end</td>
<td>Transition</td>
<td>G→A</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>486</td>
<td>5' end</td>
<td>Transition</td>
<td>C→T</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>501</td>
<td>5' end</td>
<td>Transition</td>
<td>T→C</td>
<td>0.625</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>521</td>
<td>5' end</td>
<td>Transversion</td>
<td>C→A</td>
<td>0.125</td>
<td></td>
</tr>
</tbody>
</table>
5.3.4 Diacylglycerol O-transferase

5.3.4.1 PCR amplification of primers and cloning of $DGAT1$ gene

$DGAT1$ amplification produces an approximately 413 fragment (Figure 5.19). Prominent bands at the expected size from each lane which represents different animals were cut from the gel and the DNA was extracted, cloned and sequenced.

Figure 5.19. Agarose gel separation of PCR amplification products of the $DGAT1$ gene. Lane M: Bioline Hyperladder II marker; Lane 1: B5222; Lane 2: B7159; Lane 3: B8040; Lane 4: B9058
5.3.4.2 Sequence analysis

Four animals were selected for cloning and in total of four clones were sent for sequencing in which one clone represented each animal. The sequences of the fragment *DGAT1* clones were aligned in ClustalW using default settings (Figure 5.20). Forward and reverse primers were included. The sequenced were aligned with reference to GenBank accessions AY065621 and AJ318490. The details of polymorphism were simplified as in Table 5.7.

The results for sequences shows that there were five polymorphism detected within *DGAT1* in Brakmas with two SNPs detected on exon 8, two on Introns 8 and one on exon 9. All the mutations in this fragment involved transition except at position 290 which involved insertion of G. Sequence traces of this polymorphism are in Figure 5.22.

Changes at position 223 from C→T result in amino acid changes from glutamin (CAG) to stop codon (TAG). Changes A→G at position 229 result in changes of amino acid threonine (ACC) to alanine (GCC) whereas transition from G→T at position 368 resulted in amino acid changes from cysteine (TGC) to phenylalanine (TTC).

Table 5.7. Details of SNP identified in fragment the *DGAT1* gene fragment in Brakmas. Sequence changes were reported according to variants compared to GenBank accession AY065621. Frequency is based on changes observed within the four sequences analysed from Brakmas.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>Region</th>
<th>Nature</th>
<th>Sequence change</th>
<th>Frequency</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>223</td>
<td>Exon 8</td>
<td>Transition</td>
<td>C→T</td>
<td>0.125</td>
<td>Glu→Stop</td>
</tr>
<tr>
<td>2</td>
<td>229</td>
<td>Exon 8</td>
<td>Transition</td>
<td>A→G</td>
<td>0.125</td>
<td>Thr→Ala</td>
</tr>
<tr>
<td>3</td>
<td>290</td>
<td>Intron 8</td>
<td>Insertion</td>
<td>G</td>
<td>0.500</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>305</td>
<td>Intron 8</td>
<td>Transition</td>
<td>T→C</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>368</td>
<td>Exon 9</td>
<td>Transversion</td>
<td>G→T</td>
<td>0.125</td>
<td>Cys→Phe</td>
</tr>
</tbody>
</table>
Figure 5.20a. DNA sequence alignment of Diacylglycerol-\(O\)-acyltransferase gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps indicate deletion and gaps at the beginning and end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.20b. DNA sequence alignment of Diacylglycerol-\(O\)-acyltransferase gene in Brakmas using ClustalW. Each nucleotide is coloured in a different colour. Gaps indicate deletion and gaps at the beginning and end of reference sequences indicate no information available. Clones from Brakmas are shaded. Forward and reverse primers used to amplify the fragments are indicated in red and blue arrows at above and below the nucleotides, respectively.
Figure 5.21. Sequence trace of two clones showing presence of 7 or 6 G nucleotides. Reference sequence detected 6 C nucleotide which indicate an insertion in Brakmas.

5.3.4.3 Identification of RFLP site and PCR-RFLP

No restriction enzyme was present at a polymorphism we detected in this fragment but Thaller et al. (2003) identified that polymorphism within this fragment resulting in a change from lysine (AAG) to alanine (GCG) that had some effect on marbling. For this SNP restriction enzyme EaeI (Y^GGCCCR) were used. Therefore Brakmas were screened for this polymorphism. PCR-RFLP was carried out on Brakmas and Figure 5.22 showed that no polymorphism was detected at this site.

![Figure 5.22. Gel images of agarose gel separation showing DGAT1 fragment amplified by PCR digested with EaeI. Lane M: Hyperladder II marker; Lane 1: B8040; Lane 2: B8107; Lane 3: B9002; Lane 4: B9014; Lane 5: B9030; Lane 6: B9037; Lane 7: B9071 and Lane 8: B9178. C allele produces 203 and 208 bp fragment (not seen in Brakmas cattle) and T allele produce single fragment of 413 bp as seen here.](image-url)
5.4 Discussion

Analyses of Brakmas sequences from genomic DNA sequences of fragments of four genes that have associations with meat quality have demonstrated that the sequences are polymorphic both within Brakmas and between the Malaysian breed and European breeds used as a reference. Various types of SNPs were detected within Brakmas in both exons and introns of the genes, with transitional changes being most frequent, followed by transversions, deletions and insertions of nucleotides. Some SNPs were present at low frequency and still need verification to exclude PCR errors. Various restriction enzyme sites were detected within polymorphic regions of the sequences of these various genes. Some were not previously reported and were found from the polymorphisms in the new sequence. Other polymorphic sites of this restriction enzyme reported in the literature, and known to have effects on traits were used to screen the Brakmas population and relate its alleles to reported meat quality traits.

5.4.1 Polymorphisms in calpain gene

Analysis of sequences of bovine calpain genes in Brakmas cattle revealed a total of 33 SNPs; fourteen SNPs were observed within intron 7 and exon 10, eleven SNPs within exon 13 and intron 14 and eight within intron 14 and exon 15.

Several polymorphisms in the gene have been described to have effects on carcass traits. Detection of QTL at BTA29 has associated to isoleucine/valine and glycine/alanine allele in \textit{CAPN1} with tenderness. As noted in the introduction, Page \textit{et al.} (2002) found that glycine rather than alanine at position 316 and isoleucine rather than valine at position 530 were associated with decreased meat tenderness. All of the Brakmas sequences have only the glycine codon at position 316 (corresponding to position 721 of fragment CAI7E10), and the valine codon at position 530 (corresponding to position 179 of fragment CAE13I14). Thus the Brakmas breed has one amino acid correlated with increased meat tenderness (valine) and one with decreased tenderness (glycine). In a study done Page \textit{et al.} (2002), isoleucine and valine alleles were detected in Brahman and Angus breed with no breed appearing to be fixed to isoleucine allele. The frequency of isoleucine was 30\% and considered by the author to be sufficiently high to suggest that selection has impact on tenderness. The
frequency of glycine at position 316 in Brahman, Simmental, Gelbvieh, Salers, Maine-Anjou and Chianina was 86%. The same authors suggest that the mutation on the amino acid causes functional change in the μ-calpain protease and functionally different protein lead to variation in myofibrillar proteolysis and subsequently varies in meat tenderness.

Another polymorphism within bovine calpain gene was associated with leanness (Juszczuk-Kubiak et al. 2004). The polymorphisms were found within the third fragment (CA114E15) of calpain gene. Sequence analysis and PCR-RFLP analysis using FokI restriction enzyme has revealed of presence of genotype CC and genotype CT at position 174 within intron 14 in Brakmas cattle. Presence of CT and TT genotype in Brakmas similar to results reported by Juszczuk-Kubiak et al. (2004) that found these genotypte frequently in other breed. However, CT and TT genotypes were found at low frequency in Polish Red cattle.

CC genotype is present at 0.46 in Brakmas cattle. Frequency of CC genotype was observed higher than Brakmas in Polish Red (0.78) and Black and White cattle (0.54) but lower than Brakmas in Red Angus and Hereford (0.33); Simmental (0.3) and Limousin (0.2). Homozygous CC was not presence in Charolais cattle and similar trends exist in Brakmas, where based on PCR-RPLP analysis shows absent of TT genotype.

The same author showed that the TT genotype animals have a leaner carcass compared to CC genotype. The presence of the polymorphism in Brakmas cattle shows that Brakmas have variability in fatness contributed by the calpain gene. However, effect of this polymorphisms with carcass traits within Brakmas cattle need further evaluation.

5.4.2 Polymorphisms in leptin gene

Several polymorphisms within the leptin gene in Brakmas cattle were detected. One involved a nucleotide G insertion in intron 2, where all eight of the Brakmas sequences differed from the reference sequence. The other sites, only one out of four of which was located in an exon, showed polymorphism within the Brakmas and the SNP in exon 2 (position 298), a G→A transition was a silent mutation.
Buchanan et al. (2002) reported that SNPs at first base position of the 25th codon of exon 2 have effect on fat deposition. All the Brakmas sequences showed the C allele (corresponding to position 306 of the fragment sequenced) which is associated with a leaner carcass. This finding is in contrast with result of polymorphism at position 174 of third fragment (CAI14E15) within intron 14 of calpain gene. Based on that result, Brakmas has acquired fatness traits but analysis of leptin gene sequence in Brakmas indicated that Brakmas has leaner traits as well. C allele encoded amino acid cystein and T encoded amino acid thymine. Buchanan et al. (2002) suggest that T allele which contributes to cystein to the protein cause partial loss of biological function and associated to fatter carcass and hence contribute to marbling of meat.

A second polymorphism was at position 252 within exon 2, has been reported to have an effect on feed intake (Lagonigro et al. 2003). All of the Brakmas sequences had A allele rather than T allele at position 253 correlating with the allele for lower mean feed intake. PCR-RFLP analysis using Clal restriction enzyme also confirmed that all Brakmas cattle studied here has A allele. Lagonigro et al. (2003) shows that animals with AT genotype were associated to 19% greater mean feed intake compared to AA genotype. This finding suggests that probably, one of the contribution factor of low body weight and body weight gain in tropical environmental cattle is due to genetically controlled of lower feed intake as a results of this polymorphism or mutation.

5.4.3 Polymorphisms in TG gene
Analysis of the 5' end of TG gene in Brakmas cattle revealed that sixteen SNPs were present within this fragment and 25% of them involved transversional changes. However all these SNPs occurred at low frequencies level except for T→C transition at position 30, C→T transition at position 206 and T→C transition at position 501 which were at frequencies 0.5, 0.75 and 0.625, respectively.

Polymorphisms within this 5' end of bovine TG has been reported by Thaller et al. (2003a). Presence of C allele ('2') and T allele ('3') was identified by using PsuI (R^GATCY) restriction enzyme. Genotyping the Brakmas population with an isoschizomer (BstY1 - R^GATCY) revealed that all animals
had the C allele associated with lower fat content. Thus both TG and leptin genes show that Brakmas cattle have genetic lean alleles.

The results of BstYI restriction enzyme shows that has frequency of C of 1.00 in Brakmas cattle. This result is similar to the results by Casas et al. (2005) who study association of SNPs with carcass traits in Bos indicus cattle. This author found that Brahman cattle have frequency of 0.946 of C allele. In another study done by Thaller et al. (2003b) in Casas et al. (2005), German Holstein and Charolais have shown to have a frequency of 0.22 to 0.25 of allele C. Thaller et al. (2003a) found that there is association of C allele to higher marbling score in longissimus muscle. However, the effect of polymorphisms of carcass traits and marbling score in Bos indicus is inconclusive (Casas et al. 2005). Therefore application of this polymorphisms as a molecular marker in Brakmas require further investigation.

5.4.4 Polymorphisms in DGAT1 gene

Analysis of DGAT1 sequences in Brakmas revealed that five SNPs were detected. It was interesting that three out of these five SNPs detected were within exons. There are no reports correlating these SNPs with meat quality phenotypes, although it would be interesting to see whether the transition of C→T at position 223 which resulted in an amino acid change from glutamic acid to a stop codon actually changes the gene expression or occurs in a pseudogene.

Thaller et al (2003a) reported using enzyme CfrI for identification of a lysine/alanine polymorphism. Screening of Brakmas cattle sequence shows that presence of one the alanine variant. Alanine variant has been associated with low marbling on semitendinosus muscle whereas lysine variant has been associated to high marbling score. Analysis of Brakmas cattle by PCR-RFLP using EaeI (similar to CfrI restriction site) shows that all Brakmas cattle have lysine variant. Frequency of lysine in Brakmas cattle is at 1.00 (base on PCR-RFLP analysis) and this results similar to results reported by Casas et al. (2005). The same author reported high frequency of lysine in Brahman cattle, which is at 0.814. These results indicate that there is possibility that Brakmas have high marbling score within semitendinosus muscle. However, verification on association of this
polymorphisms needs further investigation before application of alanine/lysine variant can be established as molecular marker in MAS.

5.4.5 Implication

Based on sequence analysis as well as screening with PCR-RFLP, Brakmas show a range of meat quality traits, both for tenderness and marbling. A number of SNPs had been identified within the genes in the Brakmas which has been associated to different meat quality. Novel alleles also have been found within different meat quality genes in Brakmas.

The polymorphisms identified in the calpain gene indicate that there is potential to use this polymorphism as a marker for meat tenderness. The polymorphisms detected within leptin gene, thyroglobulin gene and diacylglycerol O-transferase, on the other hand could be potential markers for marbling of meat. The SNPs identified had been associated with meat tenderness and marbling in Bos taurus type cattle but the effect on Brakmas is unknown. Verification on association of the SNPs on meat quality traits has to be carried out before can be used as a molecular markers for Brakmas cattle.
Chapter 6: General discussion: DNA markers and their application in Malaysian Brakmas Cattle

6.1 Diversity of Brakmas cattle

6.1.1 Importance to measure and conserve genetic diversity

Maintaining genetic diversity is important for the improvement of livestock production. Variation within cattle breeds enables selection for animals with superior performance. Capability to select and improve the production performance is important in order to remain competitive in the industry. Furthermore, maintaining genetic variation allows continuous improvement and a programme that can respond to various challenges in cattle industry (Blott et al. 1998a) and adapt with changing conditions such as changes in climate and disease.

Numerous studies have been carried out for the sake of genetic conservation within breeds (Blott et al. 1998a) and between breeds (Blott et al. 1998b; Canon et al. 2001). Studies on genetic diversity allow identification of genetic variation and genetic relationships within and between populations. Subsequently, identification of breeding strategies can be done to prevent genetic loss within the breed (Blott et al. 1998a).

6.1.2 Diversity of microsatellites

Analysis of microsatellite variation in Brakmas cattle reveals phylogenetic information and genetic relationships within the population. In total, Brakmas display microsatellite polymorphisms and this is proven by the considerable number of alleles identified, allele frequency and high degree of PIC value (see Chapter 3.3.1, 3.3.2 and 3.3.6). Brakmas was observed to have a higher number of allele numbers than the ISAG population numbers but lower than reported by Grzybowski and Prusak (2004b) in less highly selected breeds. The number of alleles is proven to be successful in estimating the degree of diversity within cattle. The high degree of genetic variability indicates that Brakmas is rich
reservoir of genetic diversity and therefore it is important for preservation. Furthermore, three prominent groups were observed and these display genetic variation (see Chapter 3.3.8). There was no indication of genetic uniformity.

The results presented here provide a baseline for future studies to track whether there is drift in allele composition and frequency, or whether selection pressures are leading to loss of diversity within the breed. It also indicates which markers may be useful in selection of parents for breeding programmes where lack of relatedness and high levels of polymorphism are defined as criteria. The markers may also be applicable should a herdbook be established for the breed, and if phenotypic recording becomes more extensive.

These results have important implications for the investigation of economic traits in Brakmas cattle as well as for quantitative trait loci. Furthermore microsatellites are useful in any breed conservation programme. Conservation of genetic material in livestock is important to increase sustainable in food production.

6.1.3 Diversity in and around coding regions

Analyses of Brakmas sequences from genomic DNA sequences of fragments of eight genes were carried out. The genes were chosen for their associations with growth and meat quality traits. The results have demonstrated that the sequences are polymorphic both within Brakmas and between the Malaysian breed and European breeds used as a reference. Various types of SNPs were detected within Brakmas in both exons and introns of the genes, with transitional change being most frequent, followed by transversions, deletions and insertions of nucleotides (Chapter 4 and 5). Various restriction enzyme sites were detected within polymorphic regions of the sequences of these various genes. Some were not previously reported and were found from the polymorphisms in the new sequence.

In total, 8038 bp of genomic DNA sequence were analysed in detail and 91 SNPs were detected giving an average of one SNP per 88 bp. This is slightly higher compared to the results reported in other cattle breeds: Konfortov et al. (1999) observed that one SNP on the amyloid precursor protein gene was every 104 bp. Heaton et al. (2001) observed one SNP per 443 bp in cytokine gene and
Konfortov et al. (1999) found one SNP every 434 bp in leptin gene. Vignal et al. (2002) suggests that high densities of SNPs are present in defined regions. The same authors also believe, higher values are found in cattle compared to human due to consequences of pre-selection of fragment that are known to contain SNPs. This is true in Brakmas cattle as the genes studied were selected based on knowledge of previous study of those genes as published in literature. Furthermore, there is possibility that nature and function of gene will have effect on occurrence of SNPs.

Analysis of sequence revealed that Brakmas posses high genetic diversity within and around coding regions. Diversity might reflect adaptability to tropical environments and other environmental factors. Several polymorphisms appeared to be at high frequencies and there is possibility to use this specific marker in Brakmas. However to confirm, the diversity unique to breed needs further investigation. Research on effect of polymorphism and association to production traits will confirm the importance of these specific markers.

The results also demonstrated that there is lack of evidence of a genetic bottleneck in Brakmas cattle. Further investigation looking at the ancestors would probably confirm any evidence in relation to bottleneck. Genetic variation and genetic relationships in Brakmas can be done by looking at Brahman and Kedah Kelantan cattle, the ancestor of Brakmas cattle.

The baseline study done here has generated information that will be valuable for the cattle genetic improvement and conservation purposes.

### 6.1.4 Molecular assessment of diversity in Brakmas

The levels of diversity found in Brakmas cattle using these complementary methods are consistent with the relatively high morphological polymorphism and low level of uniformity with a hybrid origin of the breed and no intensive selection. For example, progeny testing and AI programmes with a small number of elite bulls as is practiced in many dairy breeds with lower levels of polymorphism.

This study is the first report using DNA-based molecular markers to understand genetic diversity of Brakmas. Using Brakmas to understand the genetic backgrounds, the present study contributed to the knowledge of genetic
diversity within Brakmas cattle. Further investigations including other Malaysian and South-East Asian cattle are required and currently there is substantial data accumulating within Indian breeds in particular (Metta et al. 2004) and Korean cattle (Kim et al. 2003). The data would be useful to clarify their origin and relationships between these local breeds.

6.2 Functional Diversity within genes in Brakmas

Polymorphism within coding and promoter area had been associated to various production traits and function. Identification and association of SNPs to production traits and other economic traits can be done through quantitative trait loci. QTL studies can be used to identify quantitative loci that can be used in marker assisted selection programmes. Therefore the SNPs data produced can be exploited for the searching of association of particular allele to production traits.

6.2.1 Previously associated SNPs and QTLS

Based on sequence analysis as well as screening with PCR-RFLP, Brakmas show a range of growth and meat quality genes at least within the sample of diverse individuals analysed here. It shows that there is possibility of Brakmas in possessing different growth and meat quality alleles at different location which contributes differently in both production traits; growth and meat quality. Table 6.1 give the details of polymorphism within genes studied here, in association to production traits. In this table, detail about alleles that had been associated to lower and good quality was given.

This result of Val/Leu genotype in growth hormone is consistent with results from BB genotype in the insulin-like growth factor 1 gene and Hinfl polymorphism in pituitary-specific transcription factor 1 gene which indicate that Brakmas cattle carries the higher growth gene. However the finding of short TG repeat from growth hormone receptor gene contradicts to growth hormone
<table>
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<th>Allele region</th>
<th>Allele position*</th>
<th>High yield/quality allele</th>
<th>Low yield/quality allele</th>
<th>Brakmas genotype</th>
<th>Method used</th>
<th>Predicted traits acquired by Brakmas</th>
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* allele position based on Brakmas sequence. ↑ increased; ↓ reduced
gene, insulin-like growth factor 1 and pituitary-specific transcription factor 1 which indicate Brakmas posses certain lower growth rate alleles. This is not surprising as the fact that growth is controlled by many genes and interaction of those genes are not fully known. The possible explanation is every single allele contributes differently in growth mechanism in the cattle. Therefore quantitative traits loci should be established to determine contribution of various loci in growth.

Brakmas show a range of meat quality traits, both for tenderness and marbling, with some loci being polymorphic while others are fixed, at least within the sample of diverse individuals analysed here. On one side, it shows that Brakmas having meat tenderness allele in certain sequences fragment but not on the other fragment of the same calpain gene. In tenderness traits it shows the possibility of Brakmas in gaining different alleles at different location which contributes differently in meat tenderness traits. This result is consistent with results from leptin and thyroglobulin gene which indicate that Brakmas cattle maintain its leanness. However this result contradicts to calpain fragment CAI14E15 assessment which indicate Brakmas posses certain degree of fatness traits. The possible explanation is every single allele contributes differently in fat deposition mechanism in the cattle.

The same trend occurs within marbling and fat associated traits. Leptin, thyroglobulin gene and diacylglycerol-O-acyltransferase gene revealed that Brakmas is a lean cattle breed but on the other hand fragment CAI14E15 revealed that Brakmas posses its fatness. It was concluded that each single allele variants contribute differently in meat tenderness and fat deposition mechanism. Further studies are required in order to search for the combination effect of each variant for the SNPs and indels identified (Table 6.2).

6.2.2 Novel SNPs – are they anonymous or QTLs?

Currently, the significance of many SNPs and indels detected within the genes studied in Brakmas in relation to the association of production traits is unknown. Knowledge about function of intron is very little (Snustad and Simmons, 1997; Cooper, 2000). As presence of introns within genes was very frequent, it leads to speculation that introns may play an important role but
Table 2a. Novel gene alleles found in Brakmas compared to reported in cattle in Genbank/EMBL database

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1M57764; 2U15731.2; 3AF017143; 4AF404761; 5NM17457; 6AF248054; 7AF252504; 8AJ512638; 9M35823; 10AY065621
Table 2b. Novel gene alleles found in Brakmas compared to reported in cattle in Genbank/EMBL database

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1M57764; 2U15731.2; 3AF017143; 4AF404761; 5NM17457; 6AF248054; 7AF252504; 8AJ512638; 9M35823; 10AY065621
Table 2c. Novel gene alleles found in Brakmas compared to reported in cattle in Genbank/EMBL database

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1M57764; 2U15731.2; 3AF017143; 4AF404761; 5NM17457; 6AF248054; 7AF252504; 8AJ512638; 9M35823; 10AY065621
because it accumulates new mutations more rapidly within exons, it indicates that the intron may not be very important (Snustad and Simmons, 1999). However the function of intron cannot be underestimated. This is based on work done by Juszczuk-Kubiak et al. (2004) on meat production traits on Polish Red and Polish Black and White cattle that had shown that there is association between several production traits and SNP in intron 14. In some cases, polymorphisms detected may themselves be neutral with respect to growth traits, but they may be linked to particular beneficial or detrimental alleles either within the Brakmas breed or more generally. These may be valuable for marker assisted selection but extensive trait testing will be required to link the morphological markers with molecular markers as QTLs.

6.2.3 Microsatellites for genetic/marker assisted selection

Microsatellites are found in large numbers, ubiquitous and evenly spaced throughout the genome (Ihara et al. 2004). Furthermore, analysis in this thesis demonstrates that microsatellites are polymorphic and highly informative for study of gene diversity. However, several factors have to be considered in selection of microsatellites as markers such as mutation rate and occurrence of null alleles (Pemberton et al. 1995). Mutation rates as a result of slipped-strand mispairing is a dynamic process and are not well understood yet (Tautz and Schlotterer, 1994) whereas non-amplification of microsatellite alleles is usually due to sequence variation in the DNA and presence of null allele at high frequency will cause bias in genetic analysis (Ihara et al. 2004). Caution and verification of microsatellites as a marker is important before it is applicable especially in different breed and species.

Microsatellites are exploitable as anonymous and not linked to traits markers. Microsatellites have a potential as a marker for marker assisted selection for the investigation of economic traits in cattle as well as for quantitative trait loci. Microsatellites had been used in bovine genome map and subsequently it will facilitate marker assisted selection.
6.2.4 SNPs in genes: are they useful in Brakmas selection and breeding?

SNPs detected within genes might be important. Several studies had indicated the association of SNPs with production traits. However, as the effect of polymorphism may vary between breed, further investigation on effect of the SNPs on growth or meat quality are required.

6.3 Nature of diversity in Brakmas

6.3.1 Microsatellite diversity

Microsatellites diversity is reflected from the number of allele, allele size as well as allele frequencies. As in all other organisms, microsatellite diversity arises from mutation or slipped strand mispairing in the genome (MacHugh et al. 1997). Primary mechanism which generates variation in length at the loci is due to slipped strand mispairing during replication. Slippage occurs as a result of mispairing of complementary bases on the sequence of microsatellites. Although microsatellites had been associated with high mutation rate, microsatellites had been used widely to measure diversity within and between populations, for pedigree evaluation and fingerprinting due to polymorphism. Microsatellites have been used as a tool for genetic conservation. This is based on the information that can be generated from microsatellites analysis such as evidence of bottlenecks and inbreeding or outbreeding.

6.3.2 SNP diversity

In this study, it was also observed that point mutation involved more of transition changes rather than transversion changes. This is in agreement to reports by Moriyama and Powell (1997). Transition changes involve substitution of purine to purine or pyrimidine to pyrimidine whereas transversion changes involves changes of purine to pyrimidine and vice versa. Transition/transversion bias is important for understanding of the nucleotide substitution mechanism involved (Rosenberg et al. 2003). Transition/transversion rate bias varies among genes (Yang and Yoder, 1999) but transition/transversion mutation rate within
genome and species is still unknown (Yang and Yoder, 1999). The bias affects all codon positions equally (Freeland and Hurst, 1998) but the third base changes usually do not altered the genetic code it represents.

Transition and transversion occur as a result of methylation. Methylation occurs at CpG sites (cytosine-phosphate-guanine site). Methylation of CpG play an important role and have impact on gene activity and expression (Cezar et al. 2003). Several genomic function associated to methylation has been reported including effect on genomic stability, X-chromosome inactivation and tissue-specific gene expression (Cezar et al. 2003). The same author shows the effect of methylation on cloned bovine fetuses and methylation losses may contribute to the development failure.

Comparison of transition and tranversion ratio from SNPs from data base ftp://ftp.hgsc.bcm.tmc.edu/pub/data/Btaurus/snp/Btau20060804/ with transition and transversion ratios in Brakmas was carried out. This database FROM WHAT DATA records 1146 SNPs. From the database, transition and transition was at 78% and 22%, respectively and in Brakmas the percentage is 74% and 26%, respectively. Furthermore, from SNP database at ftp://ftp.hgsc.bcm.tmc.edu/pub/data/Btaurus/snp/Btau20050310/, transition and transversion ratio in different breeds were summarised. Base on 65536 SNPs sample analysed; there are 4353 SNPs in Angus, 4794 SNPs in Brahman, 20123 SNPs in Holstein, 2559 SNPs in Jersey, 638 SNPs in Limousin and 33069 SNPs in Norwegian. The ratio of transition and transversion is 69% and 21%, respectively in Angus and Holstein; 67% and 33% in Jersey and 70% and 30% in Brahman, Limousin and Norwegian. The ratio in Bakmas showed that the ratio of transition and transversion is similar with the database.

### 6.3.3 Chromosomal variation and possible future analyses

Fluorescence *in situ* hybridization analysis on insulin-like growth factor 1 gene on Kedah-Kelantan cattle was performed to investigate their chromosomal distribution. These results suggest that the IGF1-1 clone present as a single copy in Kedah Kelantan breed. Fluorescence *in situ* hybridization can be used for gene mapping.
6.4 Conclusion

Based on evidence presented in this chapter, it is concluded that detection of polymorphisms within Brakmas cattle on several genes related to meat quality is proven successful. Application of molecular technique such as SNPs which was detected in sequence analysis and application of molecular marker for population screening using PCR-RFLP is possible and promising.

PCR-RFLP is cost efficient but it cannot detect all the SNPs and polymorphism present within the genome. It was shown that from eight genes studied which cover a range of 8038 bp and 109 SNPs and indels detected, only seven restriction enzymes were identified to screen the cattle population. Taking the minimum number of animals screened through sequencing, sequence analyses was shown to be the most valuable approach in identifying variation within the genome of the Brakmas breed.

Also, clones of genes from Brakmas cattle had been shown to be used for chromosomal distribution study. It was proven that analysis of fluorescence \textit{in situ} hybridization using probes derived from Brakmas sequence are able to be detected in different breed of Kedah Kelantan. Mean to say this probe can be used directly in screening Brakmas in finding abnormality such as translocation or accumulation of copy number as well as to detect evolutionary diversification, if there is any, in future.

Sequencing work is proven successful. Although the number of sequence for each genes studied was small, it was successful in showing presence of polymorphism in Brakmas cattle; sequencing costs and difficulty are likely to reduce in the future making this approach to diversity analysis even more attractive. Advances in bioinformatics provide useful information in comparing the sequence between different cattle breeds. Brakmas have demonstrated quite a range of differences compared to reference sequence. High levels of similarity were found in these genes as compared to those in Genbank. It is possible in future to further analyse bigger number of animals in order to generate more information and to detect more polymorphism site (which subsequently lead to identifying the contributing allele in growth and meat quality). This indicates that further research is required to fill in the gap of information. Larger number of animals that would be a better representative of this breed and allow one to look
for species breed-specific variants. This will give better overview of the presence of polymorphism in this breed as well as would provide more information and give more general idea about the variation of these breed compared to the other breed. Subsequently, the variation could be linked to the economic traits. There is possibility that more restriction enzyme sites could be identified and detected and be used to screened the population at minimum cost which finally could be used as a marker for future association to meat quality traits and as for marker assisted selection (MAS). In fact, improvement of growth and meat quality carried out through genetic selection can be enhanced by selection for genes which are most accurately measured at finishing age or after slaughter even earlier and save the time and cost incurred. So far, only a few molecular markers have been identified to link with meat quality and thus genetic improvement through marker assisted selection for breeding purposes is limited.

6.4.1 SNPs and possible future analyses

The strategy includes to further sequence a larger number of animals that would be a better representative of this breed and look for breed-specific variants and also to sequence multiple clones (normally about eight clones) from each animal to confirm allelic variants and check directly for heterozygosity. Bigger numbers of animals would provide more information and give a more general idea about the variation within this breed compared to other breeds. Subsequently, the variation could be linked to the economic traits.

6.4.2 Microsatellites and possible future analyses

More microsatellites markers should be tested especially the ones recommended by the ISAG (International Society for Animal Genetics) as in Secondary Guidelines for the Development of National Farm Animal Genetic Resources Management Plan – Measurement of Domestic Animal Diversity (MoDAD) and as in http://www.projects.roslin.ac.uk/cdiv/markers.html. Furthermore, using automated sequencing machine for polymorphism analysis will be helpful for large number animals and microsatellites to be used.
6.4.3 Chromosomal variation and possible future analyses

More in situ hybridization work is also recommended for various different breeds as well as incorporation of other genes. Various chromosome abnormalities had been associated to reproductive failure and problems such as fertility in cattle had been detected by cytogenetics works. Implementation of such work, intensively in Brakmas cattle will enable the detection of any abnormality present and may be used in breeding decision and programme.

6.4.4 Genome project and possible future analyses

Involvement in genome project sequence will give an advantage of having a *Bos indicus* sequence and this incredibly valuable for comparison. Comparison between *Bos taurus* and *Bos indicus* may be worthwhile as the distance between both of them offer lots of differences that can be used to track the responsible polymorphisms related to the cattle performance. Big QTL experiment with lots of genotyping, set up as pedigree design or as an LD/AG type experiment would enable to identify significant variation. However such global-type projects require huge funding and contribution as well as support from government.

In conclusion, molecular markers have a huge potential to be used for the improvement of cattle. Molecular markers can be used as a tool in searching polymorphisms within genomes that have economic impact to the cattle industry and significant impact to human beings.
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