ESTROGEN RECEPTOR GENE POLYMORPHISM IN VECHUR AND CROSSBRED CATTLE OF KERALA

KIRANA SASIDHARAN

Thesis submitted in partial fulfilment of the
requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Veterinary and Animal Sciences University
Pookot,Wayanad

2011

Department of Animal Breeding, Genetics & Biostatistics
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

ACKNOWLEDGEMENTS

Words are hard to find when the heart is too full to speak. I am at a loss of words to express my deep sense of gratitude to ***Dr. K.A.Bindu****, Associate Professor, Department of Animal Breeding, Genetics and Biostatistics, and Chairman of the Advisory Committee for the able guidance and constant encouragement that helped bring out the best in me.*

I am thankful to ***Dr. K. V. Raghunandanan****, Retd. Director, Centre for Advanced Studies in Animal Genetics and Breeding for his constant support, timely help and memorable concern.*

I wish to express my sincere gratitude to ***Dr.K.C.Raghavan****, , Centre for Advanced Studies in Animal Genetics and Breeding, for her shrewd suggestions, indispensable guidance, and timely help in pursuit of this study.*

I am indebted to ***Dr.T.V.Aravinakshan****, Professor, Centre for Advanced Studies in Animal Genetics and Breeding, for his valuable guidance and critical suggestions and incessant encouragement. His ability to trouble shoot problems is amazing. . A scientist in every sense of the word, he shall always remain my inspiration.*

It is my good fortune that ***Dr.Siju Joseph****, Associate Professor and Head, Department of Microbiology is a member of the Advisory Committee. His thought provoking ideas, his valuable guidance, perceptiveness helped shape my research programme.*

Dr. Thirupathy Venkatachalapathy*,* ***Dr. Radhika, Dr.Naicy Thomas****, Assistant Professors, Department of Animal Breeding, Genetics and Biostatistics have always been a source of great help and encouragement.*

I am extremely privileged to be associated with ***Dr. Stephen Mathew****, Professor, Department of Animal Genetics and Breeding who helped me a lot with his excellent and professional technical support for my research and also with his warm heart.*

Co operation rendered by ***Dr.Joseph Mathew****, Professor and Head, University Livestock Farm, Mannuthy in according access to the farms for the collection of samples and extending all possible help is gratefully acknowledged.*

I owe my sincere thanks to ***Dr. Rani Alex,*** *Research assistant, Centre for Advanced Studies in Animal Genetics and Breeding for her wholehearted help, genuine support and suggestions offered for statistical analysis part of this work.*

The special concern shown by ***Dr.Rajendra Ashok Bhosle, Dr.Suraj Sivaraman and Dr. Pramod*** *for designing the primers is greatly acknowledged.*

The help rendered by ***Dr. K. A. Mercey,*** *Professor and Head,* ***Dr. Gleeja*** *and* ***Dr. Unnikrishnan****, Assistant Professor, Department of Statistics, is gratefully acknowledged.*

I wish to record my heartily appreciation to ***Nitya Ramakrishnan*** *whose friendship, mental support and sharing experiences in research which helped me in overcoming the hardships faced during the period of study and research work.*

Narrow border of language could never express my gratitude to ***Mr. Arun Ravindran*** *for the help extended for the lab work of my research.*

The help, friendship, mental support and timely advice and co operation of my batch-mates ***Dr. Deepa.C.N, Dr. Archana.S*** *and* ***Dr.Fajre****.P were instrumental in the completion of my research work.*

I really enjoyed the company of my junior colleagues ***Dr.Karthikeyan E.B, Dr.Mittu .T. Kurian, Dr. Raghavendran.***

I feel fortunate to have pleasant company of my seniors like **Dr. Sudina K, Dr. Aparna, Dr. Remya John, Dr. Muhammed E. M.** *and* **Dr. Arpitha** *for their support during difficult times in the period of study.*

I place on record my sincere thanks to **Dr.Varuna Panikar** *and* **Dr. Lijo John***, Department of Veterinary Biochemistry for their help and support.*

I fondly remember the timely help rendered by **Dr. Meenu Basheer, Dr. Abhinay, Dr. Ambily, Dr. Vamshi** *and* **Dr. Surya P.S.** *,Department of Veterinary Microbiology.*

I express my heartfelt gratitude to my friends **Drs. Sreelekha, Divya, Dia, Nithina, Shiny, Soumya, Rosemary, Chinnu, Irene** *and many more for their support & back up.*

It seems very difficult to express in words what I owe to my juniors **Aneesha, Shefeena, Subi, Anjali, Nisha, Neelima, Viji** *and especially to my roommate* **Aparna Madhavan** *for their help and moral support during the hostel days.*

I would like to acknowledge **Raghu Ram***, the* **staff** *and* **laborers** *of* **Vechur Conservation Unit, University Livestock Farm, Mannuthy** *for their wholehearted cooperation during the study.*

I am grateful to the **Dean***, Faculty of Veterinary and Animal Sciences, for providing the facilities for the study.*

The love and affection showered on me by **my parents** *and* **sister** *have indeed been the elixir of life for me. I am at loss for words to thank them for their prayers and support.*

Last but not the least, my sincere solicitude to the **Almighty God***, for all he has given…*

Kirana Sasidharan

CONTENTS

LIST OF TABLES

LIST OF PLATES

DECLARATION

I hereby declare that this thesis, entitled **“ESTROGEN RECEPTOR GENE POLYMORPHISM IN VECHUR AND CROSSBRED CATTLE OF KERALA”** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy

KIRANA SASIDHARAN

1. INTRODUCTION

Milk occupies a pride of place as the most coveted food in the Indian diet. Dairy products are a culinary delight as well as major source of cheap and nutritious food to millions of people in India and the only acceptable source of animal protein for large vegetarian segment of Indian population. Dairy industry is of crucial importance to India as one of the activities is aimed at alleviating the poverty and unemployment. Milk production in India has come a long way over the years from a low volume of 17 million tons in 1951 to 115 million tons in 2010. Today, India is the world leader in milk production. The Indian dairy industry presently contributes about 15 % to the total milk production of the world.

According to National Bureau of Animal Genetic Resources (NBAGR) there are 30 recognized cattle breeds in India. Among these myriad species of cattle in India, Vechur is the insignia of bovine wealth in Kerala. Vechur cows derived their name from their origin, a village near the temple town of Vaikom in Kottayam district in the state. This legendary short statured cow has carved out a niche for itself in the increasingly popular zero-input farming. It is a boon to landless and peri-urban livestock keepers, especially women. In general, the Vechur cattle are the ideal choice of a farmer who cannot afford the sophisticated dairy management practices but wants milk just enough for home consumption. Efforts are being taken to increase the milk production per animal, conserving the native germplam of Vechur cattle.

Due to the increase in demand for milk, the prime need of the hour is to increase the milk production per animal. Therefore, crossbreeding and upgrading was employed. Henceforth, Kerala witnessed a remarkable increase in the milk production. Selection of cattle for crossbreeding was initially performed based on the individual and pedigree records. But the rate of genetic progress made was very slow as their generation interval was long. To overcome this, scientists set foot to the world of molecular technology.

Current technologies enable scientists to improve the accuracy and efficiency of traditional selection by applying genetic markers. This can be done through marker assisted selection (MAS), which combines information on molecular genetics polymorphisms (marker loci) with data on phenotypic variation among individuals (and their relatives). DNA markers can be used to decrease generation interval by allowing selection at earlier stage in life (Kinghorn *et al*., 1991)[48] . Methods like Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP), Single Strand Conformation Polymorphism (SSCP), Amplified Fragment Length Polymorphism (AFLP) and microsatellite marker analysis reveal the polymorphic patterns of various genes. Niemann-Sorenson and Robertson (1961)[73] were the first to discuss the possible use of molecular marker information directly in a breeding program.

Milk production is an economically important trait of cattle, which is under the control of multiple genes. Screening favorable alleles for selection at the DNA level provides an ideal tool for marker-assisted selection. Some of the candidate genes associated with milk production are growth hormone, prolactin, κ-casein, α and β lactoglobulin, pitutary- specific transcription factor 1 gene (Pit-1) and estrogen receptor gene (ESR). Pitutary- specific transcription factor 1 gene (Pit-1) and estrogen receptor gene (ESR), of vechur and crossbred cattle were analyzed in this study. The present study is aimed at screening the Vechur and crossbred population of Kerala for these candidate genes.

Keeping this in view, the following objectives were undertaken:

(1) To characterize the Estrogen receptor gene and Pitutary- specific transcription gene by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP).

(2) Compare the allelic frequency of ER gene and Pit-1 gene in Vechur and crossbred cattle.

(3) Analyze the correlation, if any, between various allelic combinations with lactation yield.

2. REVIEW OF LITERATURE

2.1 ESTROGEN RECEPTOR GENE

Estrogen receptor is a member of the nuclear receptor superfamily that regulates target genes in response to estrogen and with input from other transduced signals. It does so by binding as a homodimer to a conserved estrogen receptor element in the regulatory region of target genes and via two discrete transactivating domains. Estrogen receptors regulate various vital processes such as reproduction, cell growth and differentiation, mammary gland development, lactogenesis, homeostasis and oncogenesis. Due to these functions, estrogen receptors and their genes are considered as candidate markers for production and functional traits in farm animals, including cattle (Szreder and Zwierzchowski, 2004)[109].

2.1.1 Structure of the gene

Metivier *et al*. (2000) [67] identified that the consensus for estrogen response element sequence was 5'-GGTCANNNTGACC-3'.

According to Kos *et al.* (2001) [57], Estrogen receptor (*ERα*) protein was coded by 8 exons in humans and the 5' region of the estrogen receptor genes contained additional exons that do not code for proteins, but code for transcripts of different length with different 5'-UTR (untranslated region).

In the sequenced 2853-bp 5' region of the bovine *ERα* gene, (GenBank Accession No. AY340597), Szreder *et al*. (2004) [109] localised the non-coding exons A, B, C and their putative promoters.

Munoz *et al*. (2004) [70] amplified four overlapping cDNA fragments covering exons one to eight with primers designed from the pig *ESR2* cDNA sequence (AF164957).

2.1.2 Function of estrogen receptor gene

Smith *et al*. (1971)[98] reported that injection of estrogen and progesterone caused formation of normal colostrums.

Smith and Schanbacher (1973)[99] showed that the treatment with estrogen and progesterone initiated lactation in nonpregnant and nonlactating cows.

Rothschild *et al*. (1996)[89] proposed *ER* gene as a candidate marker for prolificacy in pigs.

Rothschild *et al*. (1996)[89] constructed a linkage map near the estrogen receptor gene and found the ESR gene polymorphism to be responsible for the phenotypic variation in beneficial traits.

Enmark *et al*. (1997)[26] reported that the major expression sites of the estrogen receptor and its transcripts in the reproductive organs in females are uterus, vagina and ovary, and in case of males in the testes, epididymis, and prostate.

Estrogen receptor alpha (*ERα*) and estrogen receptor beta (ERβ) colocalized in certain tissues such as the ovary, uterus (Byers *et al*, 1997[10]) and testes (Rosenfeld *et al*, 1998)[87] but differential expression of the two receptors depended on tissue cell type. The uterus had greater expression of *ERα* than ERβ, whereas, ERβ had greater concentrations than *ERα* within the ovary (Byers *et al*, 1997) [10].

Rollerova and Urbancikowa. (2000)[88] suggested that the alternative splicing regulates the expression of ER genes in different tissues.

Pfaffl *et al*. (2001)[76] suggested the high expression of estrogen receptors in other organs and tissues such as liver, lungs, mammary gland, kidneys, pituitary, gut, brain, and stomach.

Tkaczyk and Kalita, (2001)[115] reported that estrogen influenced maturation of milk gland during embryogenesis as well as graafian follicle during menstruation cycle.

2.1.3 Polymorphism of estrogen receptor genes.

Lehrer *et al*. (1990)[58] suggested specific mutations within the estrogen receptor gene as a cause for brain and endometrium cancers.

Lehrer *et al*. (1990)[58] reported that amino acid substitution at position 86 of *ERα* protein—Ala/Val resulted in mutations in the transactivating domain which was a putative cause of spontaneous abortions in women.

Korach (1994)[56] found a point mutation in domain B of *ERα*, related to mammary cancer in mouse. This resulted from amino acid substitution creating additional recognition site for endogenous proteases.

Rothschild *et al*. (1996)[89] identified nucleotide sequence polymorphisms in coding and non-coding regions of the porcine *ERα* gene by RFLP *–PvuII* and established an association of one of these mutations with the mean number of piglets born per litter.

Drogemuller *et al*. (1997)[24] performed RFLP-*Ava1* and RFLP-*MspAI* in swine and revealed two mutations in exon VIII of *ERα* gene (transitions—T/C at position +1665 and A/G at position +1754, respectively).

Short *et al.* (1997)[95] studied the estrogen receptor gene polymorphism in four Large White based commercial lines and found that the B allele had an additive effect for the total number of pigs born (TNB), number of pigs born alive (NBA), back fat and average daily feed consumed.

Rohrer *et al.* (1999)[85] failed to detect an effect of the *ESR/Pvu*II SNP on ovulation rate and uterine capacity in a multigeneration Meishan ×White composite experimental population.

Speirs et al. (2000)[104] observed that deletion of exon V in the estrogen receptor beta gene deteriorated ligand-binding capacity of the receptor.

Chen et al. (2000)[14] studied the effect of the ERα gene in different breeds, especially in Chinese pig populations and observed that sows of beneficial homozygote BB had in total 1.40–3.37 piglets per litter and 0.63–3.58 live born piglets more than AA homozygotes.

van Rens et al. (2000) [116] observed no difference between AA and AB ESR genotypes for pre-ovulatory hormones viz luteinizing hormone, estradiol and progesterone, number of embryos at day 35/36 of gestation, weight of embryos and viable embryos in Meishan/Landrace animals.

No significant B allele additive effect was observed on ovulation rate in a Landrace/Large White population studied by Linville et al. (2001) [62].

Isler et al. (2002)[41] genotyped a population of Large White Yorkshire and crossbred animals and found that the AA genotype had higher values for total weight of animals born (kg) and total weight of animals born alive (kg) than the AB or BB genotypes.

Koh et al. (2002)[54] suggested an association of restriction fragment length polymorphism (RFLP- PvuII and RFLP- XbaI) in the coding region of the human ERα gene with early osteoporosis in men.

In the study by Kmiec´ et al (2002)[52], the ERα allele B was significantly associated with the litter size with an average of 1.15 piglets more per litter in synthetic line with Meishan breed and 0.42 piglets more in Polish Landrace breed.

Gibson et al. (2002) [28] studied the polymorphism at the PvuII restriction site in the ERα gene and observed no statistically significant association with reproduction traits in a Meishan · Large White F2 population

Zhang *et al*. (2002)[129] analyzed the linkage between *ERα* gene polymorphism in pigs and size of reproductive organs and litter size. The results showed that sows with bigger reproductive organs and more litter size carried genotype BB of *ERα* and AA of ERβ gene.

Xu *et al*. (2003)[125] studied allele distribution of *ERα*, FSHRβ, and prolactin receptor genes in three lines of Jinhua pigs and showed significant associations for the *ERα* locus with the number of piglets born alive in later parities and the total number of piglets born of all parities.

Kamin´ ski *et al*. (2004)[45] evaluated the possible relations between the ESR/*AvaI* polymorphism (T/C transitions at position +1665 in exon 8) and carcass performance traits in Polish Large White boars. No significant differences were found for daily gain and for selection index.

Goliasova and Wolf (2004) [30] analyzed a Large White population for the ESR polymorphism and found that AA genotype had better performance over all parities for number of piglets born alive, total number of piglets born per litter, number of piglets weaned per litter and litter weight at weaning.

Munoz *et al.* (2004)[70] uncovered a polymorphism at *Hsp92*II recognition site in the estrogen receptor beta gene in two Iberian pig populations. No statistically significant association between this estrogen receptor beta SNP and litter size was found.

2.1.4 PCR-RFLP in Estrogen Receptor Gene

Santana *et al.*(2006)[93] assessed the allele frequency of ESR/*Pvu II* polymorphism and evaluated the effect of genotypes on the expected progeny differences (EPD) for litter size (LS), average daily weight gain (DWG) and back fat thickness (BT) in Brazilian Large White, Landrace and Pietrain herds.

Terman *et al.* (2006)[111] estimated the frequencies of *ESR/Ava*I and *ESR/Pvu*II gene mutations and found AA allele with highest mean ejaculate volume (220.5 cm^3), while the lowest in *BB* genotype – 207.9 cm^3 (*ERα*) boars and the recorded differences were statistically significant at $P \leq 0.01$.

Humpoliček *et al.* (2007) [38] evaluated the effect of three candidate genes viz estrogen receptor gene, follicle stimulating hormone gene and myogenin genes in one herd of Large White breed by PCR-RFLP and found that AB genotypes of *ER* gene had longer service period and insemination interval than AA genotypes.

Munoz *et al.* (2004)[70] studied the polymorphism in porcine *ERα* and *ERβ* genes located in the coding region of these two genes, and evaluated the effects on litter size traits in a composite Chinese-European pig line. They detected five silent SNPs within exons in ESR 1.

2.2 PITUITARY SPECIFIC TRANSCRIPTION FACTOR 1 GENE

Pituitary-specific transcription factor 1 is responsible for pituitary development and hormone expression in mammals and is a member of the POU family of transcription factors that regulate mammalian development. The POU family is so named because the first 3 members identified were *PIT-1* and octamer transcription factor 1 proteins of mammals, and neural Unc-86 of *Caenorhabditis elegans.*

2.2.1 Pituitary specific transcription factor 1

The POU domain was first identified as a novel DNA-binding motif from the cloning of mammalian transcriptional regulators *PIT-1*, Oct-1, Oct-2, and the Caenorhabditis elegans developmental modulator Unc-86 (Bodner *et al*., 1988[8]; Clerc *et al*., 1988[16]; Fnney *et al*.,1988; [27] Herr *et al*., 1988[35]; Ingraham *et al*., 1988[39]; Ko *et al*., 1988[53]).

Cohen *et al*., (1997)[18] stated that *PIT-1*, also termed Growth hormone factor 1(GHF1), was responsible for pituitary development and hormone expression in mammals

PIT-1, a member of the POU domain containing proteins belonging to a group of transcriptional regulators had critical role in differentiation and proliferation of cells (Mangalam *et al*., 1989[63]).

The human *PIT-1* gene was 14kb in size and located on chromosome 3p11 (Ohta et al., 1992[74]), whereas the mouse PIT-1 gene on chromosome 16 (Li et al., 1990[59]), and the bovine PIT-1 gene on chromosome 1 (Moody et al., 1995[68]).

More than 20 other members of the POU family have been identified in metazoan organisms, ranging from Drosophila to humans (Jacobson et al., 1997[43]) and are grouped into six or seven classes based on the amino acid sequence of their POU domains and conservation of the variable linker region (Wegner et al., 1993[122]; Ryan and Rosenfeld, 1997[90]).

Theill et al. (1989)[112] and Ingraham et al. (1990) [40] opined that conserved domain at the N terminus, which was rich in serine and threonine residues (serine/threonine activation domain, STA), mediated transcriptional activation.

.

Anderson and Rosenfeld. (1994)[1] stated that the POU-homeodomain is a 60-amino acid region near the C terminus that had considerable homology to the homeobox, which was a conserved sequence motif identified in genes that regulated developmental processes.

Anderson and Rosenfeld. (1994)[1] explained the POU-specific domain as a 75-amino acid region that was 5' to the POU-homeodomain and separated by a nonconserved linker.

Theill et al. (1989)[112] and Ingraham et al. (1990) [40] opined that POU-specific domain was necessary for high affinity binding and accurate recognition of PIT-1 response elements.

Theill et al. (1989) [112] and Anderson and Rosenfeld. (1994) [1]; found that POU-specific domain contributed to DNA-dependent *PIT-1-PIT-1* interaction.

Ingraham et al., (1990)[40] analyzed alpha helical domains and conserved structures in *PIT-1* and suggested that POU domain proteins interacted with their DNA recognition sites with both the POU-HD and POU-S domain contacting DNA.

By crystallographic studies, Klemm et al., (1994)[51] suggested that POU proteins contain four alpha helices in the POU-S and three alpha helices in the POU-homeodomain.

According to Ingraham et al., (1990)[40], the amino alpha helical regions of the POU specific domain was necessary for DNA binding and were responsible for mediating protein-protein interactions, whereas the carboxyl alpha-helical regions were involved in site specificity.

Ryan and Rosenfeld. (1997)[90] studied the linker located between the two subdomains and explained that it increased the local concentrations of POU-specific and POU homeodomains by tethering these two subdomains, and helped high affinity, site-specific DNA binding by the two POU subdomains. The length of the linker region, which ranged in size from 15 to 56 amino acids, played a role in enabling the POU specific domain to have different orientations relative to the POU homeodomain and in determining flexibility with which the POU domain recognized DNA-binding site.

Ingraham et al. (1990)[40] proved that *PIT-1* is monomeric in solution but associates as a dimer on most DNA response elements.

Cohen et al., (1997)[18] opined that some *PIT-1* response elements bind only to homodimers (such as the distal site in the GH promoter).

A high resolution x-ray analysis of *PIT-1* POU domain bound to DNA as a homodimer showed that the POU specific domain and the homeodomain attached to the perpendicular face of the DNA and the dimerization interface turned out to be the site of a mutational hot spot in pituitary hormone deficiency (CPHD) in human (Jacobson et al., 1997[43]).

According to Voss et al., (1991a) [120], the heterodimers of *PIT-1* and Oct-1 were found to bind preferentially to the prolactin Prl-1P site.

Ingraham et al. (1990)[40], Rosenfeld (1991) [88], Holloway et al. (1995)[36] and Jacobson et al. (1997)[43] stated that *PIT-1* preferentially binds as a monomer and with high affinity on certain sites, such as prolactin Prl-1d.

Jacobson et al., (1997)[43] reported that *PIT-1* binds to a complex pattern of sites within the promoters and enhancers of the genes it regulates. The sequences of these sites varied widely around a weak consensus sequence (A/T)/(A/T)TATNCAT. This suggested that *PIT-1* configured differently on the different sites.

According to Nelson et al., (1988)[72], the expression of the GH gene was controlled by a pituitary specific promoter that contained two binding sites for *PIT-1*, GH-I (-96 to –70) and GH-II (-134 to –106).

Nelson et al., (1988)[72] explained that transcription of the prolactin gene depended on a distal enhancer segment (-1830 to -1530) and a proximal promoter region (-422 to -36).

Smith et al., (1995) [100] opined that the transcriptional regulation was sensitive to the placement of the most proximal *PIT-1* binding site and also the spacing between the TATA box and the proximal binding site on the prolactin gene.

According to Mason et al., (1993)[64], the 5’ flanking region of the TSH-b gene (-128 to -92) had a *PIT-1* binding site. Three more upstream regions within the 5’ flanking region of the TSH- promoter at –274 to –258 (TSH A), at –336 to –326 (TSH B), and at –402 to –385 (TSH C) contained sequences similar to the *PIT-1* consensus binding sites.

Chen et al., (1990)[15]; McCormick et al., (1990)[65] and Cohen et al., (1997) [18] reported that the *PIT-1* promoter was autoregulated as a consequence of *PIT-1* binding to two *PIT-1* binding elements, PitB1 and PitB2, with sequence similarity to the consensus sequence of (T/A)/(T/A)TATNCAT.

Rhodes et al., (1993)[83] and Cohen et al., (1997) [18] propounded that the *PIT-1* enhancer contained five binding sites for *PIT-1*, four of which conformed to the TATNCAT/A consensus. Three of the binding sites contributed to enhancer activity.

Rosenfeld (1991) [87] and Cohen et al., (1997) [18] proved that phosphorylation of *PIT-1* affected its binding. Results suggested that phosphorylation changes the conformation of *PIT-1* on DNA, which resulted in different interaction between *PIT-1* and its binding site at the target gene.

According to Cohen et al., (1997)[18] *PIT-1* phosphorylated at two major sites, serine 115 and threonine 220, and one minor site, threonine 219, through the protein kinase A (PKA) and protein kinase C (PKC) pathways.

2.2.2 Structure of *PIT 1* gene

Sturm et al. (1988)[107], Ingraham et al. (1990)[40] and Verrijzer et al. (1990) [117] described the POU domain as a bipartite DNA-binding.

Ingraham et al. (1990) [40] and Anderson and Rosenfeld. (1994)[1] reported that POU-S was responsible for high affinity DNA binding with site specificity and POU-HD was responsible for low affinity binding with relaxed specificity.

Ryan and Rosenfeld, (1997)[90] and McEvilly and Rosenfeld, (1999) [66] reported that many of the POU proteins regulated the key developmental processes, which are associated with the nervous system in in vivo conditions.

Theill et al. (1992) [113] cloned *PIT-1* cDNA in several mammalian species including rat, human, bovine, and swine and reported that it had six exons and that most of the distinct functional domains of PIT-1 are encoded by separate exons.

Theill et al. (1992)[113] and Konzak and Moore. (1992)[55] revealed that *PIT-1* had three different splicing variants in mammals, the major type, *PIT 1a*, and two other splicing variants, *PIT-1b* and *PIT-1T* . All of the splicing variants are biologically active. These *PIT-1* variants acted differentially on the promoters of target genes. *PIT-1a* strongly activated *PIT-1a* and *PIT-1*b promoters (Voss et al., 1991b; Tanaka et al., 1999)[110].

Mangalam et al., (1989)[63] and Delhase et al., (1995)[19] described a 26-amino acid insert in the transactivation domain of *PIT-1b* because of alternative splicing of the *PIT-1* gene transcript at the end of intron one. Consequently it lost the ability to activate the PRL and *PIT-1* promoters and activated the GH gene promoter (Konzak and Moore, 1992).

Haugen et al., (1993, 1994)[34] reported that *PIT-1T* contained a 14-amino acid insert in the transactivation domain because of an alternate 3’ splice acceptor.

Klemm and Pabo, (1996)[50] and Ryan and Rosenfeld, (1997) [90]stated that *PIT 1* consisted of two highly conserved regions joined by a variable linker. The two protein domains, POU-specific domain (POU-S), which was unique to these factors, and POU-homeodomain (POU-HD), which was related to that found in the homeobox proteins, are both necessary for DNA binding (Ingraham et al., 1990[40]; Anderson and Rosenfeld, 1994)[1].

In chicken, two pituitary expressed PIT-1 mRNAs encoding cPIT-1a and cPIT-1g, composed of 335 and 327 amino acid residues, respectively, were found. They differed at the N-terminal regions and cPIT-1a had higher expression than cPIT-1g (Tanaka et al., 1999[110]).

2.2.3 Regulation of PIT-1 gene

Bodner et al. (1988) [8], Ingraham et al. (1988)[39], Nelson et al. (1988) [72] and Mangalam et al. (1989) [63] cloned *PIT-1* based on its affinity for these A/T-rich sites and was shown to control transcription of growth hormone and prolactin.

Simmons et al. (1990)[96], Steinfelder et al. (1991)[106], Gordon et al. (1993)[31] and Lin et al. (1994) [61] reported that *PIT-1* regulated the transcription of thyroid-stimulation hormone b-subunit (TSHb) genes.

Lin et al. (1992)[60] opined that *PIT-1* regulated the transcription of growth hormone releasing hormone receptor (GHRH-R) genes.

Rhodes et al. (1993)[83] stated that *PIT-1* regulated the transcription of *PIT-1* gene itself.

By immunohistological analysis, Dollé et al. (1990)[23] and Simmons et al. (1990)[96] revealed high expression of *PIT-1* in three cell types in the anterior pituitary, somatotropes, lactotropes, and thyrotropes, which secreted the pituitary hormones GH, PRL, and TSHb, respectively.

Li et al. (1990)[59] demonstrated the role of *PIT-1* in cell development and hormone secretion by a single autosomal recessive mutation on the *PIT-1* gene in Snell dwarf mouse (dw) which possessed the first genetically transmitted dwarfism.

Sinha et al. (1975) [97] reported that the single autosomal recessive mutation on the *PIT-1* gene in Snell dwarf mouse prevented the development of the pituitary cells that secreted growth hormone, thyrotrophin, and prolactin. It led to the absence of growth hormone, prolactin, and TSH-b gene expression and resulted in a failure of somatotrope, lactotrope, and thyrotrope proliferation.

Li et al. (1990)[59] studied another dwarf mouse, the Jackson mouse (dwJ) that had a gross structural alteration of the *PIT-1* gene and revealed that it possessed hypoplastic anterior pituitary gland and combined pituitary hormone deficiency of GH, PRL, and TSH-b.

Cohen et al. (1995) [17] opined that naturally occurring mutations in the *PIT-1* gene caused combined pituitary hormone deficiency (CPHD) in humans.

Association studies showed that *PIT-1* was associated with growth rate, carcass traits, and milk production in domestic animals. *PIT-1* was found to be related to birth weight (Yu et al., 1996)[127], weaning weight, average daily gain and backfat thickness (Yu et al., 1995) [126], as well as lean to fat ratio (Stancekova et al., 1990[105]), in pigs.

Research involving the naturally occurring dwarf mouse strains, Snell and Jackson, showed that the lack of functional PIT-1 had no effect on the initial activation of the *PIT-1* gene, but did impair sustained expression after ~e18 (embryo day 18). These results indicated that, the initial activation of *PIT-1* is not regulated by *PIT-1* itself, but *PIT-1* carried out a function of autoregulating its sustained expression (Rhodes et al., 1993[83]).

DiMattia et al. (1997)[22] found that a pituitary-specific paired-like homeodomain protein, Prophet of *PIT-1* (Prop-1), was capable of binding to the enhancer and regulating expression of the *PIT-1* gene.

Sornson et al. (1996)[103] revealed a mutation in the Prop-1 gene of dwarf Ames mouse which had similar phenotypes to Snell and Jackson dwarf mice and this indicated that Prop-1 is necessary for *PIT-1* expression.

Simmons et al., (1990)[96], Dolle et al., (1990) [23], Rhodes et al., (1993)[83] studied the mouse pituitary and opined that activation of *PIT-1* gene expression occurred on or before embryonic Day 13 as it was initially detectable at embryonic Day 14. Once the amount of *PIT-1* protein has reached a critical level, *PIT-1* transcription is maintained by autoregulation and additional transcriptional regulation.

According to Chen et al. (1990)[15] and McCormick et al. (1990)[65], *PIT-1* autoregulation was carried out via two *PIT-1* response elements. One binding site, PitB1, located at position -60 to -45 had a positive effect on transcription. Another *PIT-1* recognition element, PitB2, located downstream of the start site of transcription, had an inhibitory effect. These two elements worked together to precisely control the basal level of *PIT-1* expression.

Chen et al. (1990) [15] studied the regulation of *PIT-1* by cAMP and revealed two binding sites for cAMP regulatory element binding protein (CREB in the *PIT-1* promoter region around positions –200 and –155.

McCormick et al. (1990)[65] reported that environmental cues that affected the intracellular level of cAMP regulated *PIT-1* gene transcription.

In cattle, *PIT-1* was found to be associated with body weight, and milk, protein and fat yields (Renaville et al. 1997[81]).

Xu et al. (1998)[124] explained that the activity of *PIT-1* was determined by a regulated balance between a co-repressor complex that contained N15 CoR/SMRT, mSin3A/B, and histone deacetylases, and a co-activator complex that included the CREB-binding protein (CBP) and p/CAF.

2.2.4 Polymorphism of pit 1 gene

Woollard *et al.* (1994)[123] identified two alleles for *PIT-1* gene by PCR-RFLP using the restriction enzyme HinfI and confirmed the autosomal Mendelian inheritance of *PIT-1* gene.

Moody *et al.* (1995)[68] amplified a 1.3kb fragment of *PIT-1* gene and studied the restriction pattern using HinfI in *Bos taurus* cattle.

Sabour *et al.* (1996) [91] revealed the allelic frequencies at PIT/*HinfI* locus as 0.21 and 0.79 for A and B allele, respectively in Canadian Holstein bulls.

Renaville *et al.* (1997)[81a] studied the PCR-RFLP of PIT/*HinfI* in Belgian Blue cattle and reported a frequency of 0.53 and 0.47 for A and B allele, respectively.

Renaville *et al.* (1997b)[82] investigated HinfI polymorphism and examined the effect of *PIT-1* genotypes on milk yield and body conformation inItalian Holstein Friesian bulls. They found that A allele showed a significant superiority over B allele for milk yield and body conformation traits.

Klauzinska *et al.* (2000) [49] proposed that polymorphisms of *PIT-1* gene with enzyme HinfI were distributed according to Hardy Weinberg equilibrium. They observed a frequency of 0.26 and 0.74 for A and B allele in Polish Black and White cattle.

Di Stasio *et al.* (2002)[21] genotyped Piedmontese cattle for HinfI polymorphism and found no association of *PIT-1* gene variants with meat production traits.

Carrijo et al. (2003)[11] reported highest frequencies of A allele (0.86) in Canchim cattle.

Hori-Oshima and Barreras-Serrano. (2002) [37] studied the relationship between *DGAT*-1 and *PIT-1* genes polymorphism and milk yield in Holstein cattle. They found that interaction between *DGAT*-1 and *PIT-1* genes had significant effect on milk yield.

Oprzadek *et al*. (2003)[95] studied the association of *PIT-1* polymorphism with growth, feed conversion and carcass quality in Black and White bulls and reported that AA homozygotes had higher chest girth and higher circumference of round, while BB homozygotes had higher length and width of round.

Zhao *et al* .(2004) [130] studied the polymorphism at four loci of *PIT-1* gene which included three introns and one exon and reported no significant association of polymorphism in *PIT 1* gene with growth and carcass traits in Angus beef cattle.

Mukesh *et al.* (2008) [69] analyzed the bovine PIT/HinfI gene polymorphism in 16 Indian zebuine cattle and found AA homozygote in one individual of Dangi cattle.

Zakizadeh *et al.* (2007) [128] studied the polymorphism of *PIT 1* gene in three Iranian native breeds and Holstein Cattle of Iran and found no significant association of genotype with milk production of three Iranian native breeds.

2.3 VECHUR CATTLE

Girija (1994)[29] reported the diploid chromosome number of dwarf cattle of Kerala as 60 with 29 pairs of acrocentric autosomes, submetacentric X chromosomes and acrocentric Y chromosome. She stated that dwarf cattle of Kerala have unique characteristics and reiterated the necessity of their conservation.

The milk composition of Vechur cattle was described by Venkatachalapathy (1996)[118] and observed that the mean fat globule size in Vechur milk (3.12 μ) was lower than that of crossbred cattle (4.87 μ) and Murrah buffalo (5.85 μ), but higher than goat (2.6 μ). He suggested that increased phospholipid content in small fat globules aided in the nervous tissue development and fat digestion.

Venkatachalapathy and Iype (1997)[119] reported that Vechur cattle had unique characteristics of its own with separate identity from other breeds of cattle not only by its small size but also due to its milk protein variants, composition of milk, size of fat globules and level of saturated fatty acids.

Iype and Venkatachalapathy (2001)[42] prepared breed descriptor for Vechur cattle and explained that Vechur cattle was characterized by small size with an average height of 89.43±6.55 cm in bull and 87.75±0.77 cm in cow and average weight of about 170 kg for bulls and 130 kg for cows. These animals needed less feed as compared to crossbreds with a daily milk yield of 2-3 kg and they were adapted to hot and humid environment.

Anilkumar and Raghunandanan (2003) [2] prepared breed descriptor for four genetic groups of cattle in Kerala, namely High range dwarf, Vechur, Vatakara and Kasargode.

2.3.1 Studies conducted on Vechur

Aravindakshan *et al*. (2003) [6] analyzed the genetic polymorphism at the third exon of prolactin (PRL) gene in Vechur cattle by PCR-RFLP. They observed that PRL genotypes did not vary significantly for milk fat percentage, peak yield, first lactation yield and lactation length.

PCR-RFLP analysis of β -lactoglobulin locus in Vechur cattle revealed two alleles, A and B and the frequency of desirable B allele was found to be very high (Aravindakshan *et al*., 2004[5]).

Suprabha *et al*. (2004)[108] analyzed genetic variation among dwarf cattle of Kerala and found that Vechur, Vatakara and combination of the two were more uniform.

The Vechur animals were screened for the presence of the bovine leukocyte adhesion deficiency (BLAD), a genetic defect using PCR-RFLP and DNA sequencing methods and found that none of the 129 Vechur animals studied carried the BLAD allele (Aravindakshan *et al.*, 2006).

PCR-RFLP analysis at the third intron of growth hormone gene using restriction enzyme, *Msp*1 revealed two alleles namely (+) allele (612, 93 and 63 bp fragments) and (-) allele (705 and 63 bp fragments). The frequencies of *Msp*1(+)/*Msp*1(-) alleles were 0.35/0.65 and 0.48/0.52 for Vechur and Kasargode cattle, respectively. Association studies revealed that the genotypes were not significantly different for birth weight and body measurements (Aravindakshan *et al*., 2007).

Rajeev (2007) [80] cloned and sequenced alpha-lactalbumin (α-LA) gene of Vechur cattle and reported 99% homology with *Bos taurus*, 98% with yak and 95% with sheep. He suggested α-LA as a genetic marker to increase milk production in Vechur cattle.

2.8 MILK PRODUCTION TRAITS IN VECHUR CATTLE

Girija (1994)[29] reported the mean total lactation yield of 471 kg in Vechur cattle while the average daily milk yield reported was 2.17 kg. The average lactation length and peak yield recorded by her was 217 days and 3.71 kg, respectively.

Venkatachalapathy (1996)[118] studied the milk composition of Vechur cattle and reported the average milk fat and total solids as 6.18% and 15.16%, respectively. The average milk fat percentages at 7th and 13th week of lactation were recorded as 5.56±0.13% and 5.77±0.13%, respectively.

According to Venkatachalapathy and Iype (1998)[114], the fat and total solids percentages of Vechur milk showed an increasing trend as the lactation advanced. The least squares means of milk fat, total solids and solids-not-fat in percentages were 6.13±0.12, 15.02±0.14 and 8.89±0.13, respectively for one to forty four weeks of lactation.

Iype and Venkatachalapathy (2001) recorded average daily milk yield (2.2 kg), peak yield (3.6 kg), lactation length (232 days), fat percentage (4.7%) and solids not fat (8.87 %) in Vechur cattle.

The fat percentage of Vechur milk was reported by Raghunandanan (2006) [79] as 4.5 to 5.0%. He observed the average fat globule size in Vechur milk as 3.2 micron and recommended for infant feeding due to easy digestibility. The average lactation milk yield and daily milk yield in Vechur cow were reported as 561.1±13.8 kg (242±9.4 days of lactation) and 2.5-3.5 kg, respectively.

2.9 MILK PRODUCTION TRAITS IN CROSSBRED CATTLE

To improve the milk production, local cattle of Kerala were crossed with exotic breeds Jersey, Brown Swiss and Holstein Friesian. Now Kerala has a mosaic population of exotic and local cattle.

Chacko and Jose (1988) [12] reported the average first lactation milk yield (2300 to 2700 kg), first lactation length (274.3 days) and milk fat percentage (4%) in crossbred cattle of Kerala.

Radhika (1997)[78] evaluated the production performance of crossbred cattle population maintained in Kerala Agricultural University Farms and reported the average 305 day milk yield as 1829.68±34.128 kg. The milk fat percentages of crossbred cattle in Kerala during early, mid and late lactation were reported as 3.46±0.04, 3.65±0.04 and 3.97±0.04%, respectively.

The average daily milk yield of pooled population consisting of purebreds and crosses of Sahiwal, Jersey and Friesian breeds was reported as 8.64 kg by Kant and Prasad (1997).

Kannan *et al*. (2000) [46] studied the production performance of crossbred dairy cattle and reported a mean 305 day lactation yield of 2113±54.98 kg.

Sathian (2001)[94], studying the quality of milk in crossbred cows, observed an average value of 4.64% for milk fat percentage.

According to Thirumurugan and Saseendran (2006)[114], the average daily milk yield of crossbred cattle maintained in various housing systems in Kerala Agricultural University livestock farms was ranged from 7.78±0.12 to 8.86±0.13kg.

Chandran (2007)[13] reported the mean 305 day milk yield of cattle from different zones of Kerala as 2406±16.6 kg.

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Blood samples collected from 30 Vechur cattle belonging to Vechur conservation unit of College of Veterinary and Animal Sciences, Mannuthy and 100 crossbred cattle, from University Livestock Farm, Mannuthy formed the materials for the present study on estrogen receptor gene (*ESR*) and pituitary specific transcription factor-1 (*PIT* 1) gene polymorphism by PCR-RFLP.

3.2 COLLECTION OF SAMPLES

From each animal, 5 ml of blood was collected from the jugular vein into sterile 15 ml polypropylene centrifuge tube containing Ethylene Diamine Tetra Acetic acid (EDTA) as anticoagulant (1 mg/ml of blood). The samples were brought to the laboratory at 4ºC, temperature being maintained with the aid of ice packs and stored at -20ºC until needed for DNA extraction.

3.3. ISOLATION OF DNA FROM WHOLE BLOOD

DNA was extracted from whole blood using the standard phenol chloroform extraction procedure (Sambrook and Russell, 2001)[92]. The procedure followed was

1. To 5 ml blood, double the volume of ice cold RBC lysis buffer (150 m*M* ammonium chloride, 10 m*M* potassium chloride and 0.1 m*M* EDTA) was added and kept in ice cold condition with occasional mixing for 10 min for complete lysis of red blood cells.
2. The leukocytes were pelleted by centrifuging at 4000 rpm for 10 min, and the supernatant containing lysed RBCs was discarded.
3. The pellet was resuspended in ice-cold RBC lysis buffer and the above two steps were repeated till a clear pellet was obtained.

4. The pellet was then washed twice with Tris buffered saline (TBS-140 m*M* sodium chloride, 0.5 m*M* potassium chloride and 0.25 m*M* Tris base) by vigorous vortexing followed by centrifugation at 3000 rpm for 10 minutes.
5. The white blood cell pellet was resuspended in 3 ml saline EDTA buffer (SE-75 m*M* sodium chloride and 35 *mM* EDTA). The cell suspension was incubated at 50ºC in water bath with 0.25 ml of 20 % sodium dodecyl sulphate (SDS) and 25 µl of proteinase-K (20 mg/ml) for a minimum of three hours. Swirled the viscous solution occasionally.
6. The digested samples were cooled to room temperature, 300 µl of 5 M sodium chloride was added and mixed. An equal volume of phenol (pH 7.8) saturated with Tris-hydrochloride was added, mixed by gentle inversion for 10 min and centrifuged at 4500 rpm for 10 minutes.
7. The aqueous phase containing DNA was collected in fresh tubes, to which an equal volume of saturated phenol: chloroform: isoamyl alchohol (25:24:1) was added. The contents were mixed and centrifuged at 4500 rpm for 10 minutes.
8. The aqueous phase was transferred in fresh tubes, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed and centrifuged at 4500 rpm for 10 minutes.
9. The supernatant was transferred to a sterile 50 ml beaker and one tenth volume of 3 *M* sodium acetate (pH 5.5) was added and mixed.
10. To this mixture, equal volume of chilled isopropyl alcohol was added and the precipitated DNA was spooled out using a fresh micropipette tip washed in 70% ethanol and air-dried.
11. Dried DNA was resuspended in 0.5 ml of Tris EDTA buffer (TE-10 m*M* Tris base, 0.1 *mM* EDTA) and stored at -20ºC.

3.4 DETERMINATION OF YIELD, PURITY AND QUALITY OF DNA

Twenty microlitres of the DNA stock solution was diluted to 2 ml with sterile distilled water, giving a dilution of 100 times. Optical densities (OD) were measured at 260 nm and 280 nm using a 2 ml cuvette in a UV spectrophotometer (Jenway, UK). Sterile distilled water was used as blank. Yield and purity of DNA samples were estimated as follows.

3.4.1 Yield Of DNA Samples

An OD of one at 260 nm wavelengths corresponds to approximately 50 µg/ml of double stranded DNA. Concentration of DNA stock solution was calculated as follows.

Concentration of DNA stock solution (µg/ml) = OD_{260} x Dilution factor x 50

3.4.2 Purity of DNA Samples

The purity of DNA samples was assessed by estimating the ratio between the readings at 260 and 280 nm wave lengths. Pure DNA samples have OD_{260}/OD_{280} ratios of 1.7 to 1.9.

3.4.3 Checking quality Of DNA

To determine the quality and molecular weight of DNA samples, 1 µl each of stock solution of DNA was checked electrophoretically using 0.8 percent agarose in 1 x TAE buffer in a horizontal submarine gel electrophoresis unit.

The agarose in 1 x TAE buffer containing 0.5 µg/ml of ethidium bromide was heated until it was a clear solution and was cooled to 50^0C. The comb was kept in proper position in the gel tray on a level surface and the molten agarose was poured carefully into the gel tray avoiding air bubbles. After gelling the comb was removed gently and the gel tray was immersed in the buffer tank with 1 x TAE buffer. From the DNA stock solution 0.5 to 1 µg DNA was mixed with one-sixth volume of 6x gel loading buffer and the samples were loaded into the wells carefully. Electrophoresis was carried out at 80V at room temperature until the bromophenol blue dye migrated more than two-third of the length of the gel. The gel was visualized under a UV transilluminator (Hoefer,USA).

3.5 TEMPLATE DNA PREPARATION FOR PCR

Template DNA for PCR was prepared by diluting the DNA stock solution in sterile triple distilled water to a concentration of 50 ng/ µl and was stored at -20^{0}C.

3.6 PCR-RFLP ANALYSIS OF ESTROGEN RECEPTOR GENE (*ESR* gene)

3.6.1 Primers for PCR

In the present PCR-RFLP analysis, the point mutation, adenine to cytosine at position 503 of estrogen receptor gene in exon-one was investigated using restriction endonuclease *TspRI*. Primers were designed based on the sequence available from GenBank (AY641986) and using the Primer3 software to amplify a 276 bp fragment from exon-one of estrogen receptor gene which is composed of the specified mutation.

Forward primer : 5´AGGTGTACATGGACAGCAGC3´

Reverse primer : 5´AGGTAATAGGGCACCTGTTGG3´

The primers, obtained in the lyophilized form, were reconstituted in sterile distilled water to a concentration of 200 pmol/µl and 10 fold dilutions (20 pmol/µl) were used as working solutions for PCR.

3.6.2 Setting up of PCR

PCR was carried out in a final volume of 20 µl with concentrations of reagents as given in the Table 3.1. A peltier thermal cycler was pre-programmed for temperature and cycling conditions specified in Table 3.3 and used for amplification. The PCR products were cooled down to 4ºC and stored at $^{-}$20ºC till further analysis.

The amplification was checked by agarose gel electrophoresis. Two microlitre of the product was subjected to electrophoresis using 2% agarose gel in 1X Tris Acetic Acid EDTA (TAE) buffer. The product size was confirmed using 100 bp DNA size marker.

3.6.3 Restriction digestion of amplified products

Ten microlitre of the amplified product was digested with 1 µl of restriction enzyme *TspRI* at 65^{O}C for 5 minutes. The composition of reaction mixture in a final volume of 30 µl was as follows

PCR product	-	10.0 µl
10X assay buffer	-	3.0 µl
TspRI enzyme	-	1.0 µl
Distilled water	-	16.0 µl
Total	-	30.0 µl

Digestion was carried out at 65ºC for 5 minutes in a final volume of 30 µl in a dry bath. Following the digestion, the enzyme was inactivated by incubating the tubes at 80^{0}C for 20 min and the digested products were stored at 4^{0}C till analyzed.

3.6.4 Separation of restriction fragments

The digested DNA fragments of *PIT* 1 gene was separated by electrophoresis in 4 per cent agarose gels in 1 x TAE buffer containing ethidium bromide at 55 V for 3 hrs. A 100bp size DNA marker was electrophoresed with the samples in a separate well. The gels were visualized and the images were documented in a gel documentation system (Biorad Laboratories, USA).

3.7 PCR-RFLP ANALYSIS OF PITUTARY SPECIFIC TRANSCRIPTION FACTOR-1 GENE (*PIT* 1 gene)

3.7.1 Primers for PCR

The primers for *PIT* 1 gene were selected from published reports of Woollard *et al* (1994) and were custom synthesised to amplify a 451 bp fragment from exon-six and intron five of pitutary specific transcription factor-1 gene which is composed of the transition from adenine to guanine in nucleotide 1256.

Forward primer	: 5´AAACCATCATCTCCCTTCTT3´
Reverse primer	: 5´AATGTACAATGTGCCTTCTGAG3´

The primers obtained in lyophilised form were centrifuged at 10,000 rpm for 10 min and were reconstituted in sterile distilled water to a concentration of 200 pM/ µl. At the time of use, it was further diluted 20 fold.

3.7.2 Setting up of PCR

PCR was carried out in a final volume of 25 µl with concentrations of reagents as given in the Table 3.2. A peltier thermal cycler was pre-programmed for temperature and cycling conditions specified in Table 3.4 and used for amplification. The PCR products were cooled down to 4ºC and stored at ⁻20ºC till further analysis.

The amplification was checked by agarose gel electrophoresis. Two microliter of the product was subjected to electrophoresis using 2% agarose gel in 1X Tris Acetic Acid EDTA (TAE) buffer. The product size was confirmed using 100 bp DNA size marker.

3.7.3 Restriction digestion of amplified products

15 µl of the amplified product was digested with 0.6 µl of restriction enzyme *HinfI* at 37^{O}C. The composition of reaction mixture in a final volume of 25 µl was as follows

PCR product	-	15.0 µl
10X assay buffer	-	2.5 µl
HinfI (10U/µl)	-	0.6 µl
Distilled water	-	6.9 µl
Total	-	25.0 µl

Digestion was carried out at 37ºC for 12 hrs in a final volume of 15 µl in a dry bath. Following the digestion, the enzyme was inactivated by incubating the tubes at 80^{0}C for 20 min and the digested products were stored at 4^{0}C till analyzed.

3.7.4 Separation of restriction fragments

The digested DNA fragments of *PIT* 1 gene was separated by electrophoresis in 3 per cent agarose gels in 1 x TAE buffer containing ethidium bromide at 55 V for 3 hrs. A 100bp size DNA marker was electrophoresed with the samples in a separate well. The gels were visualized and the images were documented in a gel documentation system (Biorad Laboratories, USA).

3.8 ALLELIC AND GENOTYPE FREQUENCIES

The allelic and genotype frequencies at *ESR*/*TspRI* and PIT/*HinfI* locus were calculated by direct counting method for Vechur and crossbred cattle separately. The variation of the allelic frequencies among the two populations was analyzed by the *Chi*-square test of significance as described by Snedecor and Cochran (1994) considering the allelic frequencies in a 2×2 table using the formula,

$$\chi^2 = \frac{(ad - bc)^2 N}{(a + c)(b + d)(a + b)(c + d)}$$

where a, b, c and d are allelic frequencies in different populations and N is the total number of alleles observed.

The distribution of ESR/*TspRI* and PIT/*HinfI* genotypes in Vechur and crossbred cattle population was checked for Hardy-Weinberg equilibrium by *Chi*-square test comparing the observed and expected frequencies.

3.9 MILK PRODUCTION TRAITS

The information regarding the daily milk yield, 305 day milk yield and lactation length were collected from the records maintained in the farms. The lactation milk yield was standardized for 305 days to obtain 305 day milk yield (Rice *et al*., 1970).

3.10 EFFECT OF ESTROGEN RECEPTOR GENE AND PITUTARY SPECIFIC TRANSCRIPTION FACTOR 1 GENE POLYMORPHISMS ON MILK PRODUCTION TRAIT

The effect of ESR/*TspRI* and PIT/*HinfI* and on 305 day milk yield, daily milk yield and lactation length were determined by t- test and univariate analysis of variance (ANOVA) in Vechur and crossbred cattle.

Table 3.1. Optimized concentrations of PCR reagents for *ESR* gene

Sl. no.	PCR reagents	*ESR*		Final concentration in 10 µl
		Concentration	Volume (µl)	
1	Template DNA	50 ng/µl	1.00	50 ng
2	Deoxynucleotide triphosphates	25 m*M*	0.16	200 µ*M*
3	Magnesium chloride	15 m*M*	2.0	1.5 m*M*
4	Forward primer	20 pmol/µl	1	20 pmol
5	Reverse primer	20 pmol/µl	1	20 pmol
6	10X PCR buffer	200m*M* Tris-HCl & 500m*M* KCl	2.00	1X
7	*Taq* DNA polymerase	5 U/µl	0.2	1 U
8	Distilled water		12.64	
9	Total volume		20	

Table 3.2. Optimized concentrations of PCR reagents for *PIT* 1 gene

Sl. no.	PCR reagents	*PIT* 1		Final concentration in 10 µl
		Concentration	Volume (µl)	
1	Template DNA	50 ng/µl	2.00	100 ng
2	Deoxynucleotide triphosphates	25 m*M*	0.2	200 M*m*
3	Magnesium chloride	15 m*M*	2.5	1.5 m*M*
4	Forward primer	10 pmol/µl	1.5	15 pmol
5	Reverse primer	10 pmol/µl	1.5	15 pmol
6	10X PCR buffer	200m*M* Tris-HCl & 500m*M* KCl	2.5	1X
7	*Taq* DNA polymerase	5 U/µl	0.2	1 U
8	Distilled water		14.8	
9	Total volume		25	

Table 3.3 Standardized temperature and cycling conditions for amplification of *ESR/TspRI* loci

Locus	Initial denaturation	36 Cycles of			Final extension
		Denaturation	Primer annealing	Primer extension	
TspRI	94 ºC for 5 min	94 ºC for 1 min	60 ºC for 1 min	72 ºC for 1 min	72 ºC for 5 min

Table 3.4 Standardized temperature and cycling conditions for amplification of *PIT* 1/*HinfI* loci

Locus	Initial denaturation	35 Cycles of			Final extension
		Denaturation	Primer annealing	Primer extension	
HinfI	94 ºC for 5 min	94 ºC for 1 min	56 ºC for 1 min	72 ºC for 1 min	72 ºC for 5 min

4. RESULTS

4.1 ISOLATION OF GENOMIC DNA

Good quality DNA samples were obtained from Vechur and crossbred cattle using phenol chloroform extraction procedure. Single, clear band without shearing on agarose gel upon electrophoresis indicated the presence of good quality high molecular weight DNA (Plate1).

4.2 PCR-RFLP ANALYSIS OF ESTROGEN RECEPTOR GENE

Exon one of estrogen receptor gene could be amplified without any major non-specific amplified products in Vechur and crossbred cattle of Kerala (Plate 2). In all the animals studied, the size of the amplified product was 276 bp, indicating conservation of exon one of estrogen receptor gene in *Bos indicus* and their crossbreds with *Bos taurus*.

Digestion of DNA with restriction enzyme *TspRI* and further electrophoresis revealed three restriction digestion patterns in Vechur and crossbred cattle (Plate 3) with the identification of two alleles namely A and C. A allele was indicated by the presence of two bands of size 232 and 44 bp while C allele by a single band of size 276 bp.

The allele and genotype frequencies of ESR/*TspRI* polymorphism in Vechur and crossbred are presented in Table 4.1. The frequencies for A and C alleles in Vechur were noted as 0.43 and 0.57, respectively and for crossbreds the frequencies were recorded as 0.53 and 0.47, respectively.

In Vechur cattle the genotype frequencies of ESR/*TspRI* polymorphism were observed as 0.33 (CC), 0.47 (AC) and 0.20 (AA) whereas in crossbred cattle the frequencies were 0.24 (CC), 0.46 (AC) and 0.30 (AA).

A comparison of the ESR/*TspRI* alleles among Vechur and crossbred cattle populations, carried out using the *Chi*-square test, showed that C and A alleles were homogenously distributed in both the populations (Table 4.2). The Vechur and crossbred cattle populations were found to be in Hardy Weinberg equilibrium (Table 4.3) by comparing the observed and expected frequencies by *Chi*-square test.

4.3 PCR-RFLP ANALYSIS OF PITUTARY SPECIFIC TRANSCRIPTION FACTOR 1 GENE

The DNA samples of Vechur and crossbred cattle could amplify intron five and exon six of pituitary specific transcription factor 1 gene without any major non-specific amplified products (Plate 4). In all the animals studied, the size of the amplified product was 451 bp, indicating conservation of size of the amplicon of the pituitary specific transcription factor 1 gene in *Bos indicus*, and their crossbreds with *Bos taurus*.

Digestion of 451 bp size PCR product with restriction enzyme *HinfI* and further electrophoresis revealed three restriction digestion patterns in Vechur and crossbred cattle (Plate 5) with the identification of two alleles namely A and B. B allele was indicated by the presence of two bands of size 244 and 207 bp while A allele by a single band of size 451 bp.

The allele and genotype frequencies of PIT/*HinfI* polymorphism in Vechur, crossbred population are presented in Table 4.4. The frequencies for A and B alleles in Vechur were noted as 0.25 and 0.75, respectively and for crossbreds the frequencies were recorded as 0.24 and 0.76, respectively.

In Vechur cattle the genotype frequencies of PIT/*HinfI* polymorphism were observed as 0.57 (BB), 0.37 (AB) and 0.06 (AA) whereas in crossbred cattle the frequencies were 0.56 (BB), 0.40 (AB) and 0.04 (AA).

A comparison of the PIT/*HinfI* alleles among Vechur and crossbred cattle populations, carried out using the *Chi*-square test, showed that A and B alleles were homogenously distributed in both the populations (Table 4.5). The Vechur and crossbred cattle populations were found to be in Hardy Weinberg equilibrium (Table 4.6) by comparing the observed and expected genotypic frequencies by *Chi*-square test.

4.4 MILK PRODUCTION TRAITS

The average values for 305 day milk yield, daily milk yield and lactation length of Vechur and crossbred cattle are presented in Table 4.7.

4.4.1 305 Day Milk Yield

The average milk yield standardized for 305 days were 310.27±19.98 and 2106.78±56.57 kg, respectively for Vechur and crossbred cattle.

4.4.2 Daily Milk Yield

The average daily milk yields for Vechur and crossbred cattle were recorded as 1.18 ± 0.06 and 7.10 ± 0.19 kg, respectively.

4.4.3 Lactation length

The average lactation length for Vechur and crossbred cattle were 184.30±17.98 and 292.42±6.15 days, respectively.

4.5 ESR/*TspRI* POLYMORPHISM AND MILK PRODUCTION TRAITS

The average values for milk production traits for Vechur and crossbred cattle carrying different ESR/*TspRI* genotypes are presented in Table 4.8.

4.5.1 ESR/*TspRI* Polymorphism and 305 Day Milk Yield

Genotypes of ESR/*TspRI* polymorphism had no significant effect on 305 day milk yield in the cattle populations studied. The 305 day milk yield for CC, AC and AA genotyped Vechur animals were 303.10±35.41, 319.33±32.10 and 301.08183±38.58 kg, respectively while for crossbreds the corresponding values were 2115.04±89.07, 2066.21±91.24 and 2164.33±106.56 kg, respectively.

4.5.2 ESR/*TspRI* Polymorphism and Daily Milk Yield

The mean values for daily milk yield were not significantly different in Vechur and crossbred cattle. The average daily milk yield for AA, AC and CC genotypes of Vechur cattle were 1.1114 ± 0.08, 1.2247 ± 0.10 and 1.1633 ± 0.09 kg, respectively while for crossbreds, 7.29±0.38, 7.00±0.29 and 7.07± 0.29 kg, respectively.

4.5.3 ESR/*TspRI* Polymorphism and first lactation length

First lactation lengths for AA, AC and CC genotyped Vechur cattle were 198.50± 38.53, 183.14± 31.23 and 177.40± 25.26, respectively and for crossbred 296.74± 9.37, 288.70± 9.96 and 294.40± 12.98, respectively. No significant difference is seen between the various genotypes.

4.6 PIT/*HinfI* POLYMORPHISM AND MILK PRODUCTION TRAITS

The average values for milk production traits for Vechur and crossbred cattle carrying different PIT/*HinfI* genotypes are presented in Table 4.9.

4.6.1. PIT/*HinfI* Polymorphism and 305 Day Milk Yield

Genotypes of *HinfI* polymorphism had significant effect on 305 day milk yield in the cattle populations studied. The 305 day milk yield for AA, AB and BB genotyped Vechur animals were 462.10±66.37, 380±34.78 and 246.99±11.65 kg, respectively while for crossbreds and 2834.61±152.86, 2392.03±40.52 and 1766.21±57.46 kg, respectively.

4.6.2 PIT/*HinfI* Polymorphism and Daily Milk Yield

The mean values for daily milk yield were significantly higher in Vechur and crossbred cattle carrying A allele of PIT/*HinfI* polymorphism. The average daily milk yield for AA, AB and BB genotyped Vechur animals were 1.55±0.14, 1.34±0.12 and 1.03±0.03 kg, respectively while for crossbreds, 9.43±0.65, 7.91±0.15 and 6.11±0.22kg, respectively.

4.6.3 PIT/*HinfI* Polymorphism and first lactation length

First lactation lengths for AA, AB and BB genotyped Vechur cattle were 305.50 ± 57.50, 250.45 ± 22.09 and 127.24 ± 17.99, respectively and for crossbred 273.25 ± 23.31, 303.32 ± 7.32 and 285.28 ± 9.99. No significant difference is seen between the various genotypes in crossbred cattle but in Vechur, AB and AA had a longer lactation period.

Table 4.1 Genotype and allele frequencies of ESR/*TspRI* polymorphism in Vechur and crossbred cattle of Kerala

Population	Genotype frequency			Allele frequency	
	AA	AC	CC	A	C
Vechur cattle (30)	0.20 (6)	0.47 (14)	0.33 (10)	0.43 (26)	0.57 (34)
Crossbred cattle (100)	0.30 (30)	0.46 (46)	0.24 (24)	0.53 (106)	0.47 (94)

Table 4.2 Comparison of frequencies of ESR/*TspRI* alleles in Vechur and crossbred cattle of Kerala

Population	A allele	C allele	χ^2 value
Vechur	0.43 (26)	0.56 (34)	0.001487^{NS}
Crossbred cattle	0.53 (106)	0.47 (94)	

^{NS} – Not significant

Figures in parenthesis are number of observations

Table 4.3 Testing of genotypes of ESR/*TspRI* polymorphism for Hardy –Weinberg equilibrium in Vechur and crossbred cattle

Population	Source	Genotypes			χ^2 value (df=1)
		AA	AC	CC	
Vechur	Number Observed	6 (0.2)	14 (0.4667)	10 (0.334)	0.06838^{NS}
	Number Expected	5.65	14.7	9.64	
Crossbred cattle	Number Observed	30 (0.30)	46 (0.46)	24 (0.24)	0.05879^{NS}
	Number Expected	28.09	49.82	22.09	

^{NS} – Not significant,
df – degrees of freedom
Figures in parenthesis are genotype frequencies

Table 4.4 Genotype and allele frequencies of PIT/*HinfI* polymorphism in Vechur and crossbred cattle of Kerala

Population	Genotype frequency			Allele frequency	
	BB	AB	AA	A	B
Vechur cattle (30)	0.57 (17)	0.37 (11)	0.06 (2)	0.25 (15)	0.75 (45)
Crossbred cattle (100)	0.56 (56)	0.40 (40)	0.04 (4)	0.24 (48)	0.76 (152)

Figures in parenthesis are actual numbers

Table 4.5 Comparison of frequencies of PIT/*HinfI* alleles in Vechur and crossbred cattle of Kerala

Population	A allele	B allele	χ^2 value
Vechur	0.25 (15)	0.75 (45)	0.0251^{NS}
Crossbred cattle	0.24 (48)	0.76 (152)	

^{NS} – Not significant
Figures in parenthesis are number of observations

Table 4.6 Testing of genotypes of PIT/*HinfI* polymorphism for Hardy – Weinberg equilibrium in Vechur and crossbred cattle

Population	Source	Genotypes			χ^2 value (df=1)
		BB	AB	AA	
Vechur	Number Observed	17 (0.5667)	11 (0.3667)	2 (0.0667)	0.0148^{NS}
	Number Expected	16.875	11.25	1.875	
Crossbred cattle	Number Observed	56 (0.56)	40 (0.4)	4 (0.04)	0.093^{NS}
	Number Expected	57.76	36.48	5.76	

^{NS} – Not significant, df – degrees of freedom
Figures in parenthesis are genotype frequencies

Table 4.7 Milk production traits in Vechur and crossbred cattle

Sl. no.	Milk production traits	Population (Mean±SE)	
		Vechur cattle (30)	Crossbred cattle (100)
1	305 day milk yield (kg)	310.27±19.98	2106.78±56.57
2	Daily milk yield (kg)	1.18 ± 0.06	7.10±0.19
3	Lactation length (days)	184.30±17.98	292.42±6.15

Number of observations in parenthesis

4. DISCUSSION

With the advent of molecular techniques and its application in selection, breeding programmes can be more efficient. The DNA markers offer two possible future applications in animal selection; the combination of best alleles of two or more breeds, or the selection of best alleles within a breed (Haley, 1995)[32]. Markers can be used for selection of young dairy bulls prior to progeny testing as a selection criterion for dairy sires (Soller and Beckmann, 1983)[102]. This genetic marker assisted selection acts as an initial step to increase the frequency of favorable alleles and decrease the frequency of unfavorable alleles within a population (Brascamp *et al.*, 1993[9]). With the use of PCR-RFLP method, the conventional selection methods are complemented by determining the potentials of an animal even before being expressed phenotypically by determining the genetic variants of any gene at the DNA level, independent of age and sex.

5.1 POLYMORPHISM OF ESR/*TspRI*

5.1.1 Amplification of Estrogen receptor gene

The polymorphism of estrogen receptor gene was investigated in Vechur and crossbred cattle of Kerala by PCR-RFLP using restriction enzyme, *TspRI*. In all the animals tested, the size of the amplified product was 276 bp indicating conservation of exon 1 of the estrogen receptor gene in *Bos indicus* and their crossbreds with *Bos taurus*. The 276 bp sized PCR product is within the 294 bp nucleotide sequence that was reported earlier by Szreder and Zwierzchowski, (2004)[109] in Polish Friesian, Charolaise, Hereford, Simmental, Polish Red cattle. Research in polymorphism study of *ESR* gene and its association with milk production traits is scanty.

5.1.2 **PCR RFLP analysis of estrogen receptor gene**

Analysis of amplified DNA by *TspRI* revealed three restriction digestion patterns indicative of two alleles namely C (uncut 276 bp fragment) and A (232 and 44bp fragments), the names of alleles corresponding to the nitrogenous bases (Cytosine or Adenine) at position 503 of exon one of estrogen receptor gene which created *TspRI* polymorphism (Szreder and Zwierzchowski, 2004) [109].

In Vechur cattle the frequencies of A and C alleles of ESR/*TspRI* polymorphism were observed as 0.43 and 0.57, respectively. The present findings are in close agreement with Szreder and Zwierzchowski. (2004)[109] who reported the allele frequencies in Hereford as 0.54 for C and 0.46 for A allele.

Crossbred cattle of Kerala, used in the present study have a mosaic genetic makeup of Jersey, Brown Swiss, Holstein Friesian and local cattle. The A and C allele frequencies of ESR/*TspRI* polymorphism for crossbred cattle of Kerala were obtained as 0.53 and 0.47, respectively indicating the predominance of A allele in the population. Szreder and Zwierzchowski. (2004)[109] made similar findings in Polish Friesian and reported C and A allele frequencies as 0.44 and 0.56, respectively.

Difference in allele frequency was not significant between Vechur and crossbred cattle.

The genotype frequencies of ESR/*TspRI* polymorphism in Vechur cattle were recorded as 0.33 (CC), 0.47 (AC) and 0.20 (AA). In crossbred cattle of Kerala the genotype frequencies of ESR/*TspRI* polymorphism were found to be 0.24 for CC, 0.46 for AC and 0.30 for AA.

Chi-square test was used to assess the homogeneity of two populations. The two populations are in Hardy-Weinberg Equilibrium

5.2 POLYMORPHISM OF PIT/*HinfI*

5.2.1 Amplification of Pitutary specific transcription factor 1 gene

In all the animals tested, the size of the amplified product was 451bp indicating conservation of the amplicon of the pituitary specific transcription factor 1 gene in *Bos indicus* and their crossbreds with *Bos taurus*. Similar sized PCR products were first reported by Woollard *et al.* (1994)[123] using the same primers.

5.2.2 PCR RFLP analysis of Pitutary specific transcription factor 1 gene

Analysis of amplified DNA by PIT/*HinfI* revealed three restriction digestion patterns indicative of two alleles namely A (uncut 451bp fragment) and B (244 and 207 bp fragments). The above findings are in close agreement with the reports of Woollard *et al.* (1994) [123] in Holstein, Zakizadeh *et al.* (2007) [128] in Iranian Breeds and Nassiri *et al.* (2010) [71] in Najdi cattle.

Parmentier *et al*. (1999) [77] analyzed the gene sequence and reported that A to G transition in nucleotide 1256 led to A allele as the enzyme *HinfI* could not recognize the cutting site in this sequence.

In Vechur cattle the frequencies of A and B alleles of PIT/*HinfI* polymorphism were observed as 0.25 and 0.75, respectively indicating the predominance of B allele in the population. A much higher frequency of A allele is seen in Vechur when compared with the previous reports in Indian cattle viz Gir, Kankrej, Sahiwal, Rathi, Tharparkar by Mukesh *et al*. (2008)[69] who observed an average frequency of 0.063 for A allele.

Crossbred cattle of Kerala had A and B allele frequencies of 0.24 and 0.76, respectively for PIT/*HinfI* polymorphism which is similar to the reports of Dybus *et al*. (2004)[25] who found the same allele frequencies in Polish Black and white breeds.

Study of literature revealed that the allele frequencies of PIT/*HinfI* polymorphism were different in various breeds of *Bos taurus*, *Bos indicus* and their crossbreds. But in the present study, no difference was observed in the frequencies of A and B allele in Vechur (*Bos indicus*) and their crossbred cattle with *Bos taurus*.

The present finding is in close agreement with the reports of Moody *et al*. (1995) [68], Sabour *et al.* (1996)[91], Klauzinska *et al*. (2000)[49], Di Stasio *et al*. (2002) [21], Oprzadek *et al*. (2003) [75] and Zakizadeh *et al*. (2007)[128] in Hereford, Canadian Holstein, Polish Black and White, Piedmontese, Polish Black and White and Sarabi breed, respectively.

Most of the exotic cattle had a higher proportion of B allele except for Taleshi and Canchim breed which was reported by Javanmard *et al*.(2010)[44] and Carrijo *et al*. (2003) [11], respectively. They observed a lower frequency of 0.14 and 0.23 in Canchim and Taleshi breed, respectively.

Higher frequency of B allele (0.95) was reported by Moody *et al*. (1995)[68] in Brahman cattle, De Mattos *et al*. (2004)[20] in Brazilian Gyr cattle and Beauchemin *et al*. (2006) [7] in Brahman steers.

No significant difference in A and B allele of *PIT*-1 gene was observed by Renaville *et al*. (1997)[82a] in Belgian Blue cattle.

The genotype frequencies of PIT/*HinfI* polymorphism in Vechur cattle were recorded as 0.57 (BB), 0.37 (AB) and 0.06 (AA). In contradiction to the present findings in Vechur cattle with regard to the genotype frequencies of

PIT/*HinfI* polymorphism, absence of AA genotype was reported in 15 breeds of *Bos indicus* cattle by Mukesh *et al* .(2008)[69].

In case of crossbred cattle, the genotype frequencies for AA, AB and BB were 0.04, 0.40 and 0.56. This result is in close agreement with the reports of Moody *et al*. (1995)[68] in Holstein Friesian and these breeds were extensively used for crossbreeding programmes in Kerala.

BB genotype was the most frequent in all the population studied, followed by heterozygotic AB, whereas the AA was the least frequent.

Deviation of observed from the expected genotype frequencies under Hardy Weinberg equilibrium were not significant ($p<0.05$), suggesting that the Vechur and crossbred cattle population are in equilibrium for *PIT*-1 locus and there is no selective advantage for any of the genotypes.

5.3 MILK PRODUCTION TRAITS

5.3.1 305 Day Milk Yield

The recorded average 305 day milk yield of Vechur cattle was 310.27±19.98kg in the present study as against 471Kg reported by Girija (1994), in the same breed.

In crossbred cattle of Kerala the average 305 day milk yield was recorded as 2106.78±56.57 kg. Similar result was reported by Kannan *et al*. (2000)[46] in crossbreds of Kerala (2113±54.98 kg). A higher average value was reported by Chandran (2007) [13] for 305 day milk yield of cattle from different zones of Kerala (2406±16.6 kg).

5.3.2 Daily Milk Yield

The average daily milk yield of Vechur cattle was recorded as 1.18±0.06 kg in the present study. Girija (1994)[29] and Iype and Venkatachalapathy (2001)[42] reported higher averages for daily milk yield in Vechur cattle as 2.17 kg and 2.2 kg, respectively.

In crossbred cattle of Kerala the daily milk yield was recorded as 7.10±0.19kg. The above finding is in close agreement with Thirumurugan and Saseendran (2006) [114], who reported the average daily milk yield (7.78±0.12 to 8.86±0.13 kg) of crossbred cattle maintained in various housing systems in Kerala Agricultural University livestock farms.

5.4. ESR/*TspRI* POLYMORPHISM AND MILK PRODUCTION TRAITS

The association of various ESR/*TspRI* genotypes in Vechur and crossbred cattle of Kerala with milk production traits like 305 day milk yield and daily milk yield were analyzed in the present study.

5.4.1 ESR/*TspRI* Polymorphism and 305 Day Milk Yield

There was no significant difference in the 305 day milk yield between the various genotypes of ESR/*TspRI.* The 305 day milk yield for CC, AC and AA genotyped Vechur animals were 303.10±35.41, 319.33±32.10 and 301.08±38.58 kg, respectively while for crossbreds 2115.04±89.07, 2066.21±91.24 and 2164.33±106.56kg, respectively.

5.4.2 ESR/*TspRI* Polymorphism and Daily Milk Yield

The average daily milk yield for AA, AC and CC genotyped Vechur animals were 1.11±0.08, 1.22±0.10 and 1.16±0.09 kg, respectively while for crossbreds, 7.29±0.38, 7.00±0.29 and 7.07± 0.29 respectively. No association of average daily milk yield with the genotypes of ESR/*TspRI* could be observed.

5.4.3 ESR/*TspRI* Polymorphism and first lactation length

First lactation lengths for AA, AC and CC genotyped Vechur cattle were 198.50±38.53, 183.14±31.23 and 177.40±25.26 days, respectively and for crossbred 296.74± 9.37, 288.70± 9.96 and 294.40± 12.98 days, respectively. No significant difference is seen between the various genotypes with regard to this trait.

Polymorphism was noticed at the ESR/*TspRI* due to the A to C transversion found at position 503 within the proline codon CCA. But this resulted in silent mutation as both triplets CCA and CCC code for proline. Szreder and Zwierzchowski (2004)[109] postulated that the silent mutation could link to causative mutations located in the vicinity, so it has the potential for a genetic marker for production and functional traits in cattle. Present study showed no significant association of ESR/*TspRI* polymorphism with either 305 day milk yield or daily milk yield. This result could be due to the small sample size. For accuracy, further research has to be done with more number of animals.

5.5 PIT/*HinfI* POLYMORPHISM AND MILK PRODUCTION TRAITS

Various PIT/*HinfI* genotypes in Vechur and crossbred cattle of Kerala were associated with milk production traits like 305 day milk yield and daily milk yield.

5.5.1 PIT/*HinfI* Polymorphism and 305 Day Milk Yield

There was significant difference in the 305 day milk yield between the various genotypes of PIT/*HinfI* . The 305 day milk yield for BB, AB and AA genotyped Vechur animals were 462.10±66.37, 380±34.78 and 246.99±11.65 kg respectively while for crossbreds it was 2834.61±152.86, 2392.03±40.52 and 1766.21±57.46 kg, respectively.

The present study shows that the A allele is associated with increased milk production and it is in close agreement with the reports of Sabour *et al*.(1996) [91], Renaville *et al*. (1997a) [81], Parmentier *et al*. (1999) [77] and De Mattos *et al*.(2001) [20] who got similar results.

5.5.2 PIT/*HinfI* Polymorphism and Daily Milk Yield

The average daily milk yield for AA, AB and BB genotyped Vechur animals were 1.55±0.14, 1.34±0.12 and 1.03±0.03 kg, respectively while for crossbreds, 9.43±0.65, 7.91±0.15 and 6.11±0.22 kg, respectively. Positive effect of A allele was observed in both Vechur and crossbred cattle.

5.5.3 PIT/*HinfI* Polymorphism and first lactation length

First lactation lengths for AA, AB and BB genotyped Vechur cattle were 305.50± 57.50, 250.45± 22.09 and 127.24± 17.99 days, respectively and for crossbred 273.25± 23.31, 303.32± 7.32 and 285.28± 9.99 days, respectively. No significant difference is seen between the various genotypes in crossbred cattle but in Vechur, AB and AA had a longer lactation period.

The findings of this work generate the knowledge related to PIT/*HinfI* allele and genotypic distribution pattern in naturally evolved Vechur cattle wherein systematic information in terms of frequency of known variants at this important candidate gene locus was lacking.

Significant positive effect of PIT/*HinfI* A allele and AA genotype for milk yield necessitate the formulation of suitable breeding strategies to perpetuate the distribution of desirable A alleles in Vechur and crossbred cattle.

6. SUMMARY

Variations at DNA level contribute to the genetic characterization of livestock populations and also open new possibilities for accurate evaluation of economically important traits in dairy cattle. Milk production traits are typical quantitative characteristics controlled by a number of genes. But the knowledge about the existing genotypic profile at such important functional loci in cattle breeds of Kerala is lacking. In the present study the associations of polymorphisms of ESR/*TspRI* and PIT/*HinfI* with milk production traits were analyzed in Vechur and crossbred cattle of Kerala.

DNA was isolated from blood samples of 30 Vechur and 100 crossbred cattle of Kerala using phenol chloroform extraction procedure. All DNA samples obtained were suitable for further analysis.

In PCR-RFLP analysis of estrogen receptor gene, a 276bp fragment of exon one of the gene was amplified with specific primers. The PCR products were digested with restriction enzyme, *TspRI* at 65ºC for 5 minutes. On agarose gel electophoresis, three restriction digestion patterns in Vechur and crossbred cattle were revealed with the identification of two alleles namely C (uncut 276 bp fragments) and A (232 and 44bp fragment). The allele sizes were confirmed using 100bp DNA size marker.

The A/C allele frequencies in Vechur and crossbred cattle population were 0.43/0.57 and 0.53/0.47, respectively indicating the predominance of C allele in Vechur. *Chi*-square test revealed that the C and A alleles were homogenously distributed in both populations.

The genotype frequencies of ESR/*TspRI* polymorphism were 0.33 (CC), 0.47 (AC) and 0.20 (AA) in Vechur, and 0.24 (CC), 0.46 (AC) and 0.30 (AA) in crossbreds. The genotypes in Vechur and crossbred cattle populations were according to Hardy-Weinberg equilibrium at estrogen receptor gene locus.

Vechur and crossbred cattle of Kerala were genotyped for the PIT/*HinfI* and it revealed three restriction digestion patterns indicative of two alleles namely A (uncut 451bp fragment) and B (244 and 207 bp fragments). The genotypes of PIT/*HinfI* polymorphism were distributed according to Hardy-Weinberg equilibrium with frequencies 0.57 (BB), 0.37 (AB) and 0.06 (AA) in Vechur and 0.56 (BB), 0.40 (AB) and 0.04 (AA) in crossbred cattle.

The average milk yield (kg) standardized to 305 days was recorded as 310.27±19.98 in Vechur and 2106.78±56.57 in crossbred cattle. The average daily milk yield (kg) and lactation length (days) in Vechur cattle were 1.18±0.06 and 232.67±13.11, while 7.10±0.19 and 292.42±6.10 in crossbred cattle.

The association of ESR/*TspRI* and PIT/*HinfI* with milk production traits like 305 day milk yield, daily milk yield and lactation length was studied using student’s t-test and analysis of variance in Vechur and crossbred cattle population.

No significant association was observed for the various genotypes of ESR/*TspRI* and the milk production traits.

A positive effect was observed for Vechur and crossbred cattle bearing A allele of PIT/*HinfI* with the milk production traits. There was significant difference in the 305 day milk yield and daily milk yield between the various genotypes of PIT/*HinfI* . The 305 day milk yield for BB, AB and AA genotyped Vechur animals were 462.10±66.37, 380±34.78 and 246.99±11.65 kg, respectively while for crossbreds the values were 2834.61±152.86, 2392.03±40.52 and 1766.21±57.46 kg, respectively. The average daily milk yield for AA, AB and BB genotyped Vechur animals were 1.55±0.14, 1.34±0.12 and 1.03±0.03 kg, respectively, and for crossbreds, 9.43±0.65, 7.91±0.15 and 6.11±0.22 kg, respectively. No significant difference was seen between the various genotypes and lactation period in crossbred cattle but in Vechur, AB and AA groups had a longer lactation period.

While going for marker assisted selection for milk production in cattle population of Kerala, PIT/*HinfI* could be made use of to a greater degree. The research on estrogen receptor gene polymorphism should be extended to a larger cattle population of different geographical regions of the state to accurately determine the association on milk production traits.

REFERENCES

1. Anderson, B. and Rosenfeld, M.G. 1994. Pit-1 determines cell types during development of the anterior pituitary gland. *J. Biol. Chem*. 269: 29335-29338.

2. Anilkumar, K. and Raghunandanan, K. V. 2003. *The Dwarf Cattle and Buffalo of Kerala*. Kerala Agricultural University, Thrissur, 44p.

3. Aravindakshan, T. V., James, S. P. and Raghunandanan, K. V. 2006. Screening for bovine leukocyte adhesion deficiency (BLAD) in Vechur cattle. *Indian J. Anim. Sci.* 76: 712-715.

4. Aravindakshan, T. V., James, S. P. and Raghunandanan, K. V. 2007. RELP at the growth hormone gene locus in Vechur and Kasargode cattle of Kerala. *Indian Vet. J.* 84: 1247-1250.

5. Aravindakshan, T. V., Venkatachalapathy, R. T., Iype, S. and James, S. P. 2004. Polymorphism at the β-lactoglobulin locus in Vechur and dwarf cattle of Kerala using PCR-RFLP. *Indian J. Dairy Sci.* 56: 163-165.

6. Aravindakshan, T. V., Venkatachalapathy, R. T., James, S. P., Kurien, E. and Iype, S. 2003. Polymorphism of the prolactin gene locus in Vechur and other dwarf (Kassargode) cattle of Kerala detected by PCR-RFLP. *Indian J. Dairy Sci.* 57: 246-249.

7. Beauchemin, V.R., Thomas, M.G., Franke, D.E. and Silver, G.A. 2006. Evaluation of DNA polymorphisms involving growth hormone relative to growth and carcass characteristics in Brahman steers. *Genet. Mol. Res*. 5: 438–447.

8. Bodner, M., Castrillo, J.L., Theill, L.E., Deerinck, T., Ellisman, M. and Karin, M. 1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell*. 55:505-518.

9. Brascamp, E. W., Van Arendonk, J.A.M. and Groen, A. F. 1993. Economical apprasial of utilization of genetic markers in dairy cattle breeding. *J. Dairy Sci*. 76: 1204-1213.

10. Byers, M., Kuiper, G.G.J.M., Gustafsson, J.A. and Park-Sarge, O.K. 1997. Estrogen receptor-β mRNA expression in rat ovary: down-regulation by gonadotropins. *Mol. Endocrinol.* 1997.11: 172–182.

11. Carrijo, S.M, Regitano, L.C.A, Alencar, M.M, Toral, F.L.B, 2003. Influence of genetic variants of the gene features on PIT1 meat production in Canchim. Proceedings of the 49th National Congress of Genetics, Lindenow Waters, SP, Brazil.

12. Chacko, C. T. and Jose, T. K. 1988. Crossbreeding for milk production enhancement in Kerala. *Indian Dairyman.* 40: 697-709.

13. Chandran, R. R. 2007. Evaluation of lactation milk yield and polymorphism of alpha-lactalbumin gene in crossbred cattle of Kerala. M. V. Sc. Thesis, Kerala Agricultural University, Thrissur, 50p.

14. Chen,K.F., Huang, L.S., Li, N., Zhang, Q., Luo, M. and Wu,C.X. 2000. The genetic effect of estrogen receptor (ESR) on litter size traits in pig. *Yi Chuan Xue Bao*. 27: 853–857

15. Chen, R., Ingraham, H.A., Treacy, M.N., Albert, V.R., Wilson, L. and Rosenfeld, M.G. 1990. Autoregulation of the Pit-1 gene expression mediated by two cis-active promoter elements. *Nature.* 346: 583-586.

16. Clerc, R.G., Corcoran, L.M., LeBowitz, J.H., Baltimore, D. and Sharp, P. A. 1988. The B-cell specific Oct-2 protein contains POU box and Homeo box-type domains. *Genes. Dev*. 2: 1570-1581.

17. Cohen, L.E., Wondisford, F.E., Salvatoni, A., Maghnie, M., Brucker-Davis, F., Weintraub, B.D. and Radovick, S. 1995. A “hot spot” in the pit-1 gene responsible for combined pituitary hormone deficiency: Clinical and molecular correlates. *J. Clin. Endocrinol. Metab*. 80:679-684.

18. Cohen, L.E., Wondisford, F.E. and Radovick, S. 1997. Role of pit-1 in the gene expression of growth hormone, prolactin, and thyrotropin. *Endocrinol. Metabol. Clin. N. America*. 25:523-540.

19. Delhase, M., Vila, V., Hooghe-Peters, E.L. and Castrillo, J.L. 1995. A novel pituitary transcription factor is produced by alternative splicing of the human GHF-1/Pit-1 gene. *Gene*. 155:273-275.

20. De Mattos, K.K., Del Lama, S.N., Martinez, M.L. and Freitas, A.F. 2004. Association of β-GH and Pit-1 gene variants with milk production traits in dairy Gyr bulls. Braz. *J. Agric. Res*. 39, 147–150.

21. Di Stasio., Sartore, S., Albera, A.2002.Lack of association of GH1 and POU1F1 gene variants with meat production traits in Piemontese cattle. *Anim Genet*. 33: 61-64

22. DiMattia, G.E., Rhodes, S.J., Krones, A., Carriere, C., O’Connell, S., Kalla, K., Arias, C., Sawchenko, P. and Rosenfeld, M.G. 1997. The Pit-1 gene is regulated by distinct early and late pituitary-specific enhancers. *Dev. biol*. 182: 180-190.

23. Dollé, P., Castrillo,J.L., Theill, L.E., Deerinck,T., Ellisman, M. and Karin,M. 1990. Expression of GHF-1 protein in mouse pituitaries correlates both temporally and spatially with the onset of growth hormone gene activity. *Cell.* 60: 809-820.

24. Drogemuller, C., Thieven, U. and Harlizius, B. 1997. An AvaI and MspA1I polymorphism at the porcine oestrogen receptor (ESR) gene. *Anim Genet.* 28: 59.

25. Dybus, A., Szatkowska, I., Czerniawska-Piątkowska, E., Grzesiak, W., Wójcik, J., Rzewucka, E. and Zych, S. 2004. PIT1-Hinf I gene polymorphism and its associations with milk production traits in polish Black-and-White cattle. *Arch. Tierz. Dummerstorf* .47: 557–563.

26. Enmark,E., Pelto-Huikko,M. and Grandien, K. 1997. Human estrogen receptor β – gene structure, chromosomal localization and expression pattern. *J. Clin. Endocrinol. Metab*.82: 4258-4265.

27. Fnney, M., Ruvkun, G. and Horvitz. H.R. 1988. The *C. elegans* cell lineage and differentiation gene unc-86 encodes a protein with a homeodomain and extended similarity to transcription factors. *Cell*. 55: 757-769.

28. Gibson, J.P, Jiang, Z.H., Robinson, J.A., Archibald, A.L. and Haley, C.S. 2002. No detectable association of the ESR *PvuII* mutation with sow productivity in a Meishan · Large White F2 population. *Anim Genet*. 33: 448–450.

29. Girija, C. R. 1994. Characterization and evaluation of dwarf cattle of Kerala, Ph. D. Thesis, Kerala Agricultural University, Thrissur. 112p.

30. Goliasova, E. and Wolf, J. 2004. Impact of the ESR gene on litter size and production traits in Czech Large White pigs. *Anim Genet*. 35: 293-297.

31. Gordon, D.F., Haugen, B.R., Sarapura, V.D., Nelson, A.R., Wood, W.M. and Ridgway, E.C. 1993. Analysis of Pit-1 in regulating mouse TSH beta promoter activity in thyrotropes. *Mol. Cell. Endocrinol*. 96: 75-84.

32. Haley, C. S. 1995. Livestock QTLS Bringing home the bacon? *Trends Genetics.* 11: 488-492.

33. Haugen, B.R., Wood, W.M., Gordon, D.F. and Ridgway, E.C. 1993. A thyrotrope specific variant of Pit-1 transactivates the thyrotropin beta promoter. *J. Biol. Chem*. 268: 20818-20824.

34. Haugen, B.R., Gordon, D.F., Nelson, A.R., Wood, W.M. and Ridgway, E.C. 1994. The combination of Pit-1 and Pit-1T have a synergistic stimulatory effect on the thyrotropin-subunit promoter but not the growth hormone or prolactin promoters. *Mol. Endocrinol*. 8: 1574-1582.

35. Herr W., Sturm, R.A., Clerc, R.G., Corcoran, L.M., Baltimore, D., Sharp, P.A., Ingraham, H.A., Rosenfeld, M.G., Finney, M. and Ruvkun, G. 1988. The POU domain: A large conserved region in the mammalian pi1, oct-1, oct-2, and *Caenorhabditis elegans* unc86 gene products. *Genes Dev*. 2:1513-1516.

36. Holloway, J.M., Szeto, D.P., Scully, K.M., Glass, C.K. and Rosenfeld, M.G. 1995. Pit-1 binding to specific DNA sites as a monomer or dimmer determines gene-specific use of a tyrosine dependent synergy domain. *Genes Dev*. 9: 1992-2006.

37. Hori-Oshima, S. and Barreras-Serrano, L. 2002. A Relationships between DGAT1 and Pit-1 genes polymorphism and milk yield in Holstein cattle. *J. Anim. Sci.* 81: 252.

38. Humpoliček, P., Urban,T., Matoušek,V. and Tvrdoň, V. 2007. Effect of estrogen receptor, follicle stimulating hormone and myogenin genes on the performance of Large White sows. *Czech J. Anim. Sci*. 52: 334–340

39. Ingraham, H.A., Chen, R.P., Mangalam, H.J., Elsholtz, H.P., Flynn, S.E., Lin, C.R., Simmons, D.M., Swanson, L. and Rosenfeld. M.G. 1988. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* .55:519-529.

40. Ingraham, H.A., Flynn, S.E., Voss, J.W., Albert, V.R., Kapiloff, M.S., Wilson, L. and Rosenfeld, M.G. 1990. The POU-specific domain of Pit-1 is essential for sequence specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interactions. *Cell*. 61: 1021-1033.

41. Isler,B.J., Irvin, K.M., Neal, S.M., Moeller, S.J. and Davis, M.E. 2002. Examination of the relationship between the estrogen receptor gene and reproductive traits in swine. *J. Anim. Sci*. 80: 2334-2339.

42. Iype, S. and Venkatachalapathy, R. T. 2001. *Vechur cattle of Kerala*, Kerala Agricultural University, Thrissur, 37p.

43. Jacobson, E.M., Li, P., Leon-del-Rio, A., Rosenfeld, M.G. and Aggarwal, A.K. 1997. Structure of Pit-1 POU domain bound to DNA as a dimmer: unexpected arrangement and flexibility. *Genes. Dev*. 11: 198-212.

44. Javanmard Comparison of DNA Polymorphism of Bovine Pituitary-Specific Transcription Factor and Leptin Gene Between Iranian Bos indicus and Bos taurus Cattle Using PCR-RFLP

45. Kamin´ski, S., Rusc, A. and Brym, P. 2004 Relation between *AvaI* polymorphism within the estrogen receptor gene (ESR) and meatiness in Polish Large White boars. *J. Appl. Genet* .44:521– 524.

46. Kannan, A., Vijayakumar, P., Venkatachalapathy, T. and Rajagopalaraja, C. A. 2000. Effect of season and parity on production performance of crossbred dairy cattle. Proceedings of the Small Holder Livestock Production Systems in Developing Countries, November 24-27, 2000 (eds. Thomas, C. K. and Sasty, N. S. R.) Kerala Agricultural University, Thrissur, pp. 154-158.

47. Kant, N. and Prasad, R. B. 1997. Milk production efficiency of crossbred cattle at ten years of age. Indian *J. Anim. Sci***.** 67: 1997.

48. Kinghorn, B.P., Smith, C. and Dekkers, J.C.M. 1991. Potential genetic gains in dairy cattle with gamete harvesting and in vitro fertilization. *J. Dairy .Sci*. 74: 611 - 622.

49. Klauzińska,M., Zwierzchowski, L., Siadkowska, E., Szymanowska, M., Grochowska, R. and Żurkowski,M. 2000. Comparison of selected gene polymorphisms in Polish Red and Polish Black-and-White cattle. *Anim. Sci. Pap. Rep*. 18:107–116.

50. Klemm, J.D. and Pabo, C.O. 1996. Oct-1 POU domain-DNA interactions: Cooperative binding of isolated subdomains and effects of covalent linkage. *Genes. Dev.* 10:27-36.

51. Klemm, J.D., Rould, M.A., Auroro, R., Herr, W. and Pabo, C.O. 1994. Crystal structure of the Oct-1 POU domain bound to an octomer site: DNA recognition with tethered DNA-binding modules. *Cell*. 77:21-32.

52. Kmieć, M., Dvořák, J. and Vrtková, I. 2002 .Study on a relation between estrogen receptor (ESR) gene polymorphism and some pig reproduction performance characters in Polish Landrace breed. *Czech J. Anim. Sci*. 47:189–193.

53. Ko, H.S., McBride, W. and Staudt, L.M. 1988. A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeobox domain. *Cell*. 55:135-144.

54. Koh, J.M., Kim, D.J., Hong, J.S., Park, J.Y., Lee, K.U., Kim, S.Y. and Kim, G.S. 2002. Estrogen receptor alpha gene polymorphisms *PvuII* and *XbaI* influence association between leptin receptor gene polymorphism (Gln223Arg) and bone mineral density in young men. *Eur J. Endocrinol*. 147:777–783.

55. Konzak, K.E. and Moore, D. 1992. Functional isoforms of Pit-1 generated by alternative messenger RNA splicing. *Mol. Endocrinol*. 6:241-247.

56. Korach,S.K. 1994. Insights from the study of animals lacking functional estrogen receptor. *Science.* 266:1524–1527.

57. Kos, M., Reid, G., Denger, S. and Gannon, F. 2001. Minireview: genomic organization of the human ER-alpha gene promoter region. *Mol Endocrinol* .15 : 2057-2063.

58. Lehrer,S., Sanchez, M., Song, H.K., Dalton, J., Levine,E., Savoretti,P., Thung,S.N. and Schachter, B. 1990. Oestrogen receptor β-region

polymorphism and spontaneous abortion in women with breast cancer. *Lancet* .335:622–624.

59. Li, S., Crenshaw III, E.B., Rawson, E.J., Simmons, D.M., Swanson, L.W. and Rosenfeld, M.G. 1990. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene Pit-1. *Nature*. 347:528-533.

60. Lin, C., Lin, S.C., Chang, C.P. and Rosenfeld, M.G. 1992. Pit-1 dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. *Nature*. 360:765-768.

61. Lin, S.C., Li, S., Drolet, D.W. and Rosenfeld, M.G. 1994. Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development*. 120: 515-522.

62. Linville, R.C., Pomp, D., Johnson, R.K. and Rothschild, M.F. 2001. Candidate gene analysis for loci affecting litter size and ovulation rate in swine. *J.Anim Sci*. 79:60-67.

63. Mangalam, H.J., Albert, V.R., Ingraham, H.A., Kapiloff, M., Wilson, L., Nelson, C., Elsholtz, H. and Rosenfeld, M.G. 1989. A pituitary POU-domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. *Genes .Dev*. 3:946-958.

64. Mason, M.E., Friend, K.E., Copper, J. and Shupnik, M.A. 1993. Pit-1/GHF-1 binds to TRH-sensitive regions of the rat thyrotropin beta gene. *Biochemistry*. 32:8932-8938.

65. McCormick, A., Brady, H., Theill, L.E. and Karin, M. 1990. Regulation of the pituitary specific homeobox gene GHF1 by cell-autonomous and environmental cues. *Nature*. 345:829-832.

66. McEvilly, R.J. and Rosenfeld, M.G. 1999. The role of POU domain proteins in the regulation of mammalian pituitary and nervous system development. *Prog Nucl Acid Research Mol Biol.* 63:223-55.

67. Metivier, R., Petit, F.G., Valotaire, Y. and Pakdel, F. 2000. Function of N-terminal transactivation domain of the estrogen receptor requires a potential a-helical structure and is negatively regulated by the A domain. *Mol Endocrinol*. 14:1849– 1871.

68. Moody, D. E., Pomp, D. and Barendse, W. 1995. Restriction fragment length polymorphism in amplification products of the bovine Pit1 gene and assignment of Pit1 to bovine chromosome 1. *Anim .Genet.* 26:45-47.

69. Mukesh, M., Sodhi, M., Sobti, R. C., Prakash, B., Kaushik, R., Aggarwal, R.A.K. and Mishra, B.P. 2008. Analysis of bovine pituitary specific transcription factor-HinfI gene polymorphism in Indian zebuine cattle. *Livest Sci.* 113:81-86.

70. Muñoz, G., Ovilo, C., Amills, M. and Rodríguez, C. 2004. Mapping of the porcine oestrogen receptor 2 gene and association study with litter size in Iberian pigs, *Anim. Genet*. 35 : 242−244.

71. Nassiri, M.T.B., Biranvand, Z., Hartatik, T., Fayazil, J. and Tavakoli, S. 2010. The study of PIT1 gene polymorphism in the Najdi cattle using PCR-RFLP method. *J. Anim. Vet. Advances*. 9:2001-2003.

72. Nelson, C., Albert, V.R., Elsholtz, H.P., Lu, L.I. and Rosenfeld, M.G. 1988. Activation of cell-specific expression of rat growth hormone and prolactin gene by a common transcription factor. *Science*. 239:1400-1405.

73. Niemann-Sorenson, A. and Robertson, A. 1961. The association between blood groups and several production characteristics in three Danish cattle breeds. *Acta Agriculture Scandinavica.* 11:163-196.

74. Ohta, K., Nobukuni, Y., Mitsubuchi, H., Ohta, T., Tohma, T., Jinno, Y., Endo, F. and Matsuda, I. 1992. Characterization of the gene encoding human pituitary-specific transcription factor, Pit-1. *Gene.* 122:387-388.

75. Oprzadek, J., Flisikowski, K., Zwierzchowski, L. and Dymnicki, E. 2003. Polymorphism at loci of leptin (LEP), Pit1 and STAT5A and their association with growth, feed conversion and carcass quality in Black-and-White bulls. *Anim. Sci. Pap. Rep*. 21, 135–145.

76. Pfaffl, M.W., Lange, I.G., Daxenberger, A. and Meyer, H.H.D. 2001. Tissue-specific expression pattern of estrogen receptors (ER): Quantification of ERα and ERβ mRNA with real-time RT-PCR. *APMIS.* 109 : 345-355.

77. Parmentier, I., Portetelle, D., Gengler, N., Pradi, A., Bertozzi, C., Vleurick, L., Gilson, R. and Renaville, R. 1999. Candidate gene markers associated with somatotropic axis and milk selection. *Domest. Anim. Endocrinol.* 17, 139–148.

78. Radhika, G. 1997. Evaluation of Holstein crossbred bulls based on milk composition of progeny. M. V. Sc. Thesis, Kerala Agricultural University, Thrissur, 97p.

79. Raghunandanan, K. V. 2006. Vechur cattle of Kerala. *Indian Cow* 7: 48-49.

80. Rajeev, M. 2007. Molecular cloning and characterization of alpha lactalbumin gene in Vechur cattle. M. V. Sc. Thesis, Kerala Agricultural University, Thrissur, 96p.

81. Renaville,R., Gengler, N., Parmentier, I., Mortiaux, F., Massart, S.,Bertozzi, C., Burny, A., Portetelle, D., 1997a. Pit1 gene Hinf I RFLP and growth traits in double-muscled Belgian Blue cattle. *J. Anim. Sci*. 75: 146.

82. Renaville,R., Gengleer, N., Vrech, E., Prandi, A., Massart, S., Corradini, C., Bertozzi, C., Mortiaux, F., Burny, A. and Portetelle, D. 1997. Pit-1 gene polymorphism, milk yield, and conformation traits for Italian Holstein-Friesian bulls. *J. Dairy Science.* 80:3431-3438.

83. Rhodes, S.J., Chen, R., DiMattia, G.E., Scully, K.M., Kalla, K.A., Lin, S.C., Yu, V.C. and Rosenfeld, M.G. 1993. A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation on the Pit-1 gene. *Gene Dev*. 7:913-932.

84. Rice, V. A., Andrews, F. N., Warwick, E. J. and Legates, J. E. 1970. *Breeding and Improvement of Farm Animals.* Sixth edition. Tata Mc Graw-Hill, Aroon Purie at Thomas press, India. 721p.

85. Rohrer, G.A., Ford, J.J., Wise, T.H., Vallet, J.L. and Christenson, R.K. 1999. Identification of quantitative trait loci affecting female reproductive traits in a multigeneration Meishan-White composite swine population. *J. Anim. Sci*. 77 (1999) 1385−1391.

86. Rollerova, E. and Urbancikowa, M. 2000. Intracellular estrogen receptors, their characterization and function (review). *Endocr Regul*. 34:203–218.

87. Rosenfeld, C.S., Ganjam, V.K., Taylor, J.A., Yuan, X., Stiehr, J.R., Hardy,M.P. and Lubahn,D.B. 1998. Transcription and translation of estrogen receptor-b in the male reproductive tract of estrogen receptor-a knockout and wild type mice. *Endocrinology*.139:2982–2987.

88. Rosenfeld, M.G. 1991. POU-domain transcription factors: POU-er-ful developmental regulators. *Genes. dev*. 5:897-907.

89. Rothschild,M., Jacobson, C., Vaske, D., Tuggle, C., Wang, L., Short, T., Eckardts, G., Sasaki, S., Incent, A., McLaren, D., Southwood, O., van der Steen, H., Mileham, A. and Plastov, G. 1996. The estrogen receptor locus is associated with a major gene influencing litter size in pigs. *Proc. Nat. Acad. Sci, USA.* 93: 201-205.

90. Ryan, A.K. and Rosenfeld, M.G. 1997. POU domain family values: flexibility, partnerships, and developmental codes. *Genes Dev*. 11:1207-1225.

91. Sabour, M.P., Lin, C.Y., Lee, A.J. and Mcallister, A.J. 1996. Association between milk protein variation and genetic value of Canadian Holstein bulls for milk yield traits. *J. Dairy Sci*. 79: 1050-1056.

92. Sambrook and Russell (2001). Molecular Cloning: A Laboratory Manual (3rd ed.). Cold Spring Harbor Laboratory Press.

93. Santana, B.A.A.., Fernando, H.B., Robson,C.A., Mauricio,B., Mauricio, M.F. and Luiz R.G. 2006. Association of the estrogen receptor gene *Pvu II* restriction polymorphism with expected progeny differences for reproductive and performance traits in swine herds in Brazil. *Genet. Mol. Biol*. 29: 45-51

94. Sathian, C. T. 2001. Somatic cell count and its influence on the quality of milk in crossbred cows. Ph. D. Thesis, Kerala Agricultural University, Thrissur, 109p.

95. Short, T.H., Rothschild, M.F., Southwood, O.I., McLaren, D.G., de Vries, A., van der Steen, H., Eckardt, G.R., Tuggle, C.K., Helm, J., Vaske, D.A., Mileham, A.J. and Plastow, G.S. 1997. Effect of the estrogen receptor locus on reproduction and production traits in four commercial pig lines. *J Anim Sci*. 75:3138-3142.

96. Simmons, D.M., Voss, J.W., Ingraham, H.A., Holloway, J.M., Broide, R.S., Rosenfeld, M.G. and Swanson, L.W. 1990. Pituitary cell phenotypes involve cell-specific Pit- 1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev*. 4:695-711.

97. Sinha, Y.N., Salocks, C.B. and Vanderlaan, W.B 1975. Pituitary and serum concentrations of prolactin and GH in Snell dwarf mice. *Proc. Soc. for Exp. Bio.Med*. 150:207-213.

98. Smith, K. L., Muir, L. A., Ferguson, L. C., and H. R. Conrad. 1971. Selective transport of IgG1 into the mammary gland: Role of estrogen and progesterone. *J. Dairy Sci.* 54:1886.

99. Smith, K. and Schanbacher, F. L. 1973. Hormone induced lactation in the bovine lactational performance following injections of 17/β-estradiol and progesterone. *J. Dairy Sci*. 56:738.

100. Smith, K.P., Liu, B., Scott, C. and Sharp, Z.D. 1995. Pit-1 exhibits a unique promoter spacing requirement for activation and synergism. *J. Bio. Chem.* 270:4484-4491.

101. Snedecor, G. W. and Cochran, W. G. 1994. *Statistical Methods*. Eighth edition. Iowa State University Press, Ames, Iowa, 564p.

102. Soller, M. and Beckmann, J.S. (1983).Restriction fragment length polymorphism in genetic improvement. *Theor. Appl. Genet*. 67:25 - 33.

103. Sornson, M.W., Wu, W. and Dasen, J.S. 1996. Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. *Nature*. 384:327-333.

104. Speirs, V., Adams, I.P., Walton, D.S. and Atkin, S.L. 2000. Identification of wild-type gene influencing and exon 5 deletion variants of estrogen receptor beta in normal human mammary gland. *J Clin Endocrinol Metab*. 85:1601–1605.

105. Stancekova, K., Vasicek, D., Peskovicova, D., Bulla, J. and Kubek, A. 1990. Effect of genetic variability of the porcine pituitary-specific transcription factor (Pit-1) on carcass traits in pigs. *Anim. Genetics*. 30:313-315.

106. Steinfelder, H.J., Hauser, P., Nakayama, Y., Radovick, S., McClaskey, J.H., Taylor, T., Weintraub, B.D. and Wondisford, F.E. 1991. Thyrotropin-releasing hormone regulation of human TSH-β expression: role of a pituitary-specific transcription factor (Pit-1/GHF- 1) and potential interaction with a thyroid hormone-inhibitory element. *Proc. Nat. Acad. Sci. USA*. 88: 3130-3134.

107. Sturm, R.A., Das, G. and Herr. W. 1988. The ubiquitour octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. *Genes Dev*. 2:1582-1599.

108. Suprabha, P., Anilkumar, K., Aravindakshan, T. V. and Raghunandanan, K. V. 2005. Assessment of bandsharing values in RAPD-PCR analysis of dwarf cattle of Kerala. *Asian-Aust. J. Anim. Sci.* 18: 1217-1220.

109. Szreder, T. and Zwierzchowski, L. 2004. RFLP-TspRI polymorphism within exon 1 of the bovine estrogen receptor—α (ERα) gene. *Anim Sci Pap Rep* 22:543-549

110. Tanaka, M., Yamanoto, I., Ohkubo, T., Wakita, M., Hoshino, S. and Nakashima, K. 1999.cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary. *Gen. Comp. Endocrinol*. 114:441-448.

111. Terman. 2006. Estrogen receptor gene (ESR) and semen characteristics of boars. *Arch. Tierz. Dummerstorf.* 49 :1, 71-76

112. Theill, L.E., Castrillo, J.L., Wu, D. and Karin, M. 1989. Dissection of functional domains of the pituitary transcription factor GHF-1. *Nature*. 342:945-948.

113. Theill, L.E., Hattori, K., Lazzaro, D., Castrillo J.L. and Karin, M. 1992. Differential splicing of the GHF1 primary transcript gives rise to two functionally distinct homeodomain proteins. *Embo. J*. 11:2261-2269.

114. Thirumurugan, P. and Saseendran, P. C. 2006. Effect of housing systems and sprinkling water on production and reproduction performances of crossbred dairy cow. *Intl. J. Cow Sci.* 2: 18-22.

115. Tkaczyk, M. and Kalita, K. 2001. Estrogen receptor β – structure, regulation and function. *Adv. Biochem*. 47:72-79.

116. van Rens, B.T.T.M., Hazeleger, W. and van der Lende, T. 2000. Periovulatory hormone profiles and components of litter size in gilts with different estrogen receptor (ESR) genotypes. *Theriogenology*. 53:1375.

117. Verrijzer, C.P., Kal, A.J. and Van der Vliet, P.C. 1990. The Oct-1 homeo domain contacts only part of the octamer sequence and full Oct-1 DNA-binding activity requires the POU-specific domain. *Genes Dev*. 4:1964-1974.

118. Venkatachalapathy, R. T. 1996. Milk protein polymorphism and milk composition in Vechur cattle. M. V. Sc. Thesis, Kerala Agricultural University, Thrissur, 97p.

119. Venkatachalapathy, R. T. and Iype, S. 1997. Fat globule size and distribution in milk and iodine value of milk fat of Vechur cattle of Kerala. *Indian J. Anim. Sci*., 67: 910-912.

120. Voss, J.W., Wilson, L. and Rosenfeld, M.G. 1991a. POU domain proteins Pit-1 and Oct-1 interact to form a heteromeric complex and can cooperate to induce expression of the prolactin promoter. *Genes Dev*. 5:1309-1320.

121. Voss, J.W., Yao, T.P., Rosenfeld, M.G. 1991b. Alternate translation initiation site usage results in two structurally distinct forms of Pit-1. *J. Biol. Chem.* 266:12832-12835.

122. Wegner, M., Drolet, D.W. and Rosenfeld, M.G. 1993. POU domain proteins: Structure and function of developmental regulators. *Cell Biol*. 5:488-498.

123. Woollard, J., Schmitz, C.B., Freeman, A.E. and Tuggle, C.K. 1994. Rapid communication: Hinf I polymorphism at the bovine Pit-1 locus. *J. Anim. Sci.* 72:3267.

124. Xu, L., Lavinsky, R.M., Dasen, J.S., Flynn, S.E. , McInerney, E.M., Mullen, T.M., Heinzel, T., Szeto, D., Korzus, E., Kurokawa, R., Aggarwal, A.K., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. 1998. Signal-specific co-activator domain requirements for Pit-1 activation. *Nature.* 395:301-306.

125. Xu, N.Y., Zhang, S.Q. and Peng, S.H. 2003. Investigation on the distribution and their effects on reproduction traits of three major genes in Jinhua pigs. *Yi Chuan Xue Bao*. 30:1090–1096.

126. Yu, T.P., Tuggle, C.K., Schmitz, C.B. and Rothschild, M.F. 1995. Association of Pit-1 polymorphism with growth and carcass traits in pigs. *J. Anim. Sci*. 73:1282-1288.

127. Yu, T.P., Rothschild, M.F., Tuggle, C.K., Haley, C., Archibald, A., Marklund, L. and Anderson, L. 1996. Pit-1 genotypes are associated with birth weight in three unrelated pig resource families. *J. Anim. Sci. 74*:22.

128. Zakizadeh, S., Reissmann, M. and Rahimi, G. 2007. Polymorphism of the bovine POU1F1 gene: allele frequencies and effects on milk production in three Iranian native breeds and Holstein cattle of Iran. *Pak. J. Biol. Sci*. 10:2575-2578.

129. Zhang, S.J., Xiong, Y.Z., Deng, C.Y., Zeng, F.T., Qiu, X.P., Xiao, S.M., Xia, Y., Xu, J.X. and Ruan, Z. 2002. Study of molecular genetics of the size of reproductive organs and litter size in the sow. *Yi Chuan Xue Bao.* 29:1073–1077.

130. Zhao, Q., Davis, M. E. and Hines, H. C. 2004. Associations of polymorphisms in the Pit-1 gene with growth and carcass traits in Angus beef cattle. *J. Anim. Sci*. 82: 2229-2233.

ANNEXURE – 1

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

Agarose (0.8%)

Weighed 0.8 g of agarose powder and mixed with 100 ml of 1X TBE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

Agarose (3%)

Weighed 3 g of agarose powder and mixed with 100 ml of 1X TAE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

EDTA (0.5*M*, pH 8.3)

Dissolved 18.61 g of EDTA (disodium, dihydrate) in 80 ml of distilled water by bringing the pH to 8.3 with NaOH solution and volume made upto 100 ml. Stored at room temperature after filtration and autoclaving.

Ethidium Bromide (10 mg/ml)

Dissolved 100 mg ethidium bromide in 10 ml of distilled water. Solution stored at 4°C in a dark coloured bottle.

Gel loading buffer

Bromophenol blue	0.25 %	50 mg

Xylene cyanol	0.25 %	50 mg
Sucrose	40 %	8 g

Components stirred well in 20 ml distilled water and stored at 4°C.

Phenol (Saturated, pH 7.8)

Commercially available crystalline phenol melted at 65°C in a water bath. Hydroxyquinolone added to a final concentration of 0.1 percent. To the molten phenol, 0.5 *M* Tris HCl (pH 8.0) was added in equal volume and stirred for 30 minutes in a magnetic stirrer. The contents were transferred into a separating funnel. Lower phenolic phase was collected and mixed with equal volume of 0.1 *M* Tris HCl (pH 8.0) and stirred again for 30 minutes. The phenolic phase was collected and extraction repeated with 0.1 *M* Tris HCl (pH 8.0) until the pH of phenolic phase was more than 7.8. Finally 0.1 volume of 0.01 *M* Tris HCl (pH 8.0) added and stored in a dark bottle at 4°C.

RBC lysis buffer

Ammonium chloride	150 m*M*	8.0235 g
Potassium chloride	10 m*M*	0.7455 g
EDTA	0.1 m*M*	0.0372 g

Dissolved the contents in distilled water and volume made up to 1000 ml. Stored at 4°C after filtration and autoclaving.

Sodium acetate

Dissolved 40.824 g of Sodium acetate in 70 ml of distilled water and pH adjusted to 5.5 with glacial acetic acid. Volume made up to 100 ml, autoclaved and stored at room temperature.

Sodium chloride (5 *M*)

Dissolved 29.22 g of sodium chloride in 80 ml distilled water and volume made up to 100 ml. Solution filtered and stored at room temperature.

Sodium chloride- EDTA (SE) buffer (pH, 8.0)

Sodium chloride	75 m*M*	4.383 g
EDTA	35 m*M*	9.306 g

Dissolved the contents in 900 ml distilled water and pH adjusted to 8.0. Made up the volume to 1000 ml, filtered, autoclaved, stored at 4°C.

Sodium dodecyl sulphate (SDS) 20 %

SDS	20 g
Distilled water make up to	100 ml

Stirred, filtered and stored at room temperature.

Tris Acetate EDTA (TAE) buffer (50X)

Tris base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA (pH 8.0)	20 ml
Distilled water up to	1000 ml

Autoclaved and stored at room temperature.

Tris-Borate (TBE) buffer pH 8.3) 10X

Tris base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g

Dissolved in 700 ml of distilled water and pH adjusted to 8.3. Volume made up to 1000 ml, autoclaved and stored at room temperature.

Tris Buffered Saline (TBS) pH 7.4

Sodium chloride	140 m*M*	8.18 g
Potassium chloride	0.5 m*M*	0.0373 g
Tris base	0.25 m*M*	0.0303 g

Dissolved in 900 ml of distilled water and pH adjusted to 7.4. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4°C.

Tris EDTA (TE) buffer (pH 8.0)

Tris base	10 m*M*	1.2114 g
EDTA	0.1 m*M*	0.3722 g

Dissolved in 900 ml of distilled water and adjusted the pH to 8.0. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4°C.

Tris 1M (pH 8.0)

Tris base	121.14 g

Added distilled water up to 1000 ml, pH adjusted to 8.0, filtered and stored at room temperature.

ANNEXURE – II

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

(A) CHEMICALS

Agarose (Low EED)	-	Bangalore Genei Pvt. Ltd.
Ammonium chloride	-	SRL, Bombay
Boric acid	-	SRL, Bombay
Chloroform	-	Merck
Crystalline phenol	-	Merck
dNTPs	-	Finn Enzymes
EDTA	-	SRL, Bombay
Ethanol	-	Merck
Ethidum bromide	-	BDH lab, England
6X gel loading buffer	-	Bangalore Genei Pvt. Ltd.
Glacial acetic acid	-	BDH-E, Merck (India) Ltd.
Hydroxy quinolone	-	Qualigens Chemicals, Mumbai
Isoamyl alcohol	-	Merck
Potassium chloride	-	SRL, Bombay
Sodium acetate	-	SRL, Bombay
Sodium chloride	-	SRL, Bombay
Sodium dodecyl sulphate (SDS)	-	SRL, Bombay
Tris base	-	SRL, Bombay

(B) PRIMERS

Sigma Aldrich chemicals, Bangalore

(C) MOLECULAR MARKERS

100 bp ladder	-	Bangalore Genei Pvt. Ltd.

(D) ENZYMES

Restriction endonuclease, *TspRI* - Fermentas

Restriction endonuclease, *HinfI* - Bangalore Genei Pvt. Ltd.

Taq DNA polymerase - Sigma-Aldrich chemicals, Bangalore

Proteinase-K - Bangalore Genei Pvt. Ltd.

ANNEXURE – III

ABBREVIATIONS

ESR	Estrogen Receptor gene
PIT 1	Pitutary specific transcription factor – 1 gene
dw mice	Genetically dwarf mice
RFLP	Restriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
DNA	Deoxyribo Nucleic Acid
MAS	Marker Assisted Selection
QTL	Quantitative Trait Loci
EDTA	Ethylene Diamine Tetra acetic Acid
He	Heterozygosity
µl	microlitres
µg	microgram
mg	milligram
m*M*	millimolar
cm	centimeter
nm	nanometer
pmol	picomols
mCi	millicurie
kb	Kilo basepair
bp	base pair
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate

ABSTRACT

Estrogen receptor gene and pituitary specific transcription factor 1 gene was studied as candidates for genetic markers of milk production traits. The study was aimed to reveal the known variants at ESR/*TspRI* and PIT/*HinfI* locus in Vechur and crossbred cattle of Kerala and to analyze their association with milk production. The analysis of ESR/*TspRI* polymorphism revealed existence of three digestion patterns (CC, CA and AA) in Vechur and crossbred cattle. The A/C allele frequencies were 0.43/0.57 and 0.53/0.47, respectively in Vechur and crossbred cattle. The genotypes of ESR/*TspRI* polymorphism were distributed according to Hardy-Weinberg equilibrium with frequencies 0.33(CC), 0.47 (AC) and 0.20 (AA) in Vechur and 0.24 (CC), 0.46 (AC) and 0.30 (AA) in crossbred cattle under study. PCR-RFLP analysis of 451 bp fragment of *PIT*-1 gene revealed the predominance of BB genotype and B allele with a mean frequency of 0.57 and 0.75, respectively. AB genotype was the next most frequent with a mean frequency of 0.39. Only six individuals out of the 130 screened were observed to have AA genotype. Allele frequency at PIT/*HinfI* was the same in Vechur and its crossbred with *Bos Taurus*. Association studies revealed the significant influence of PIT/*HinfI* polymorphism on milk production traits. The A allele bearing Vechur and crossbred cattle showed an increase in 305 day milk yield and average daily milk yield. Lactation length in Vechur was significantly high for AA and AB genotype. ESR/*TspRI* showed no significant difference in milk yield. The selection of Vechur and crossbred cattle for A allele of PIT/*HinfI* polymorphism can significantly improve milk yield. This study validates the utility of PCR-RFLP as efficient means of exploring the distribution pattern of known variants at important candidate genes in the cattle population of Kerala for future breeding plans.