DELINEATION OF RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS IN CROSSBRED CATTLE

By
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Certified that the thesis entitled **"Delineation of Random Amplified Polymorphic DNA markers in crossbred cattle "** is a record of research work done independently by **Dr. K.Anilkumar**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Introduction

1.INTRODUCTION

The bovine genome consists of 29 pairs of acrocentric autosomes and one pair of sex chromosomes apart from a small fragment of DNA present in mitochondria. It is estimated that between 50.000 and 1.00.000 genes are encoded in the genome and only a small fraction of these have been mapped to their respective sites. The average bovine genome is approximately 100 million base pairs long. About 5% of the genome is thought to actually code for protein, rRNA and tRNA. The position of a gene in the genome is the locus of the gene. Efforts to complete the bovine gene map are in progress in various laboratories.

Each and every locus of the genome has numerous variants. These variations constitute the genetic polymorphism. Genetic polymorphism can be defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Detection of genetic polymorphism can be done through a battery of techniques. Earlier methods to detect the genetic polymorphism were based on phenotypes, blood groups, serum proteins and enzymes. But the efficiency of these methods is low and the accuracy doubtful. DNA fingerprinting is the combined use of several single locus detection systems that are used as tools for investigating various aspects of genomes.

A genetic marker is described as the stable and inherited variation that can be measured or detected by a suitable method, and can be used subsequently to detect the presence of a specific genotype or phenotype other than itself, which is otherwise non measurable or very difficult to detect. Those genetic markers used to reveal the polymorphism at DNA level are known as molecular markers. Molecular markers have the advantage over morphologic markers in being more numerous and also that they do not disturb the physiology of the organism. Of the various types of molecular markers used for detecting the DNA polymorphism, Polymerase Chain Reaction (PCR) based markers are more popular and in general involve *in vitro* amplification of particular DNA sequences or loci with the help of specifically

or arbitrarily chosen oligonucleotide sequences and a thermostable DNA polymerase enzyme. PCR is extremely sensitive and operates at a very high speed.

Randomly Amplified Polymorphic DNA (RAPD) markers are arbitrary nucleotide sequences used to detect nucleotide sequence polymorphisms in DNA at a low stringency amplification reaction. They amplify pieces of DNA which are flanked by two inverted copies of the primer and having the length between 200 to 3500 base pairs. For the average sized genome, 5 to 15 fragments will be amplified to produce the bands. Polymorphisms occur when there are sequence variations in the primer binding sites.

Compared to the other molecular markers RAPD analysis requires only low quantities of template DNA (5 to 50 ng). It is easy, quick and quite inexpensive. The primer binding sites are abundant throughout the genome and are randomly distributed. The technique generates multiple bands and is amenable for automation. The disadvantages of this method include low reproducibility and requirements of high molecular weight purified DNA. Contamination of DNA can create non specific bands and it can sabotage the experiment. Because of the dominant nature of bands, the band profiles cannot be interpreted in terms of alleles.

One of the objectives of this study was to assess the feasibility of using RAPD markers for parentage identification. Prior to the 1980's, parentage determination was based on blood group data and protein polymorphisms. The accuracy of blood tests is reported to be high, but their usefulness was limited due to the finite number of blood group systems and the low level of polymorphisms in serum proteins. Introduction of DNA technologies have lead to the identification of markers far more efficient than erythrocyte antigens and serum proteins.

Microsatellite markers are the most specific of the various molecular markers. They are dinucleotide or trinucleotide repeat sequences usually found in

non coding regions of the DNA. Some of them which are linked to various genes are of importance in molecular genetic studies. High levels of polymorphisms and the relative ease of processing the test samples make them suitable in forensic medicine. Microsatellites are widely used for characterization at species, breed, population and individual levels. Considerable progress has been made in automated typing using fluorescent labelled microsatellite markers.

More than 80% of the cattle of Kerala are crossbreds of Jersey, Holstein Friesian and Brown Swiss. The exotic inheritance of these animals ranges from 25% to 100%. As per 1996 census the pure indigenous animals in the state is only 1.1 lakh out of 33.9 lakh cattle of Kerala . These cattle are dwarf type and four different groups of native animals namely Vechur, Vatakara, Highrange dwarf and Kasargode were identified and described by different researchers based on physical characters. Of these four, only Vechur finds a place among the 30 recognised cattle breeds of India.

The studies on genetic polymorphism of cattle using molecular markers were not attempted till date in the state. The major objective of the study was to delineate the RAPD marker polymorphisms in crossbred cattle of Kerala. The second objective of the project was to study the possibility of application of RAPD technique in parentage determination. Characterisation of dwarf cattle of Kerala with microsatellite markers was also attempted using allelic frequencies.

The results of this study will pave the way for applying RAPD markers for genetic characterisation, marker assisted selection and parentage identification. The characterisation of the dwarf cattle of Kerala using Microsatellite markers will be helpful in prioratising the future developmental plans for these valuable animals.

Review of Literature

2.REVIEW OF LITERATURE

2.1 CATTLE OF KERALA

The first report available on the local cattle of Kerala was that of Aiya (1906). He described the cattle of Kerala as low milk producers and poor workers. They were very small in size and black in colour. He was the first to mention about the small Vechur cow with comparatively better milk production.

Iyer (1937), the then Director of Agriculture, Government of Travancore, reported that there was no specific breed of cattle in Kerala and the animals available in the state were very much smaller in build and were poorer milk producers. The deterioration of the cattle of the state was attributed to improper and insufficient feeding apart from the adverse climatic conditions.

According to Velupillai (1940), though official records classified the Travancore cattle as nondescript, they could divided into different groups according to the locality to which they belonged. He mentioned the Vechur cattle as better producer.

Chacko and George (1984) described the development of Sunandini breed in Kerala as a result of grading up of local zebu cattle with SwissBrown bulls upto a limitof 62.5% inheritance. They recommended that for better performance the exotic inheritance of these animals should be between 50% and 62.5%.

Chacko (1985) opined that breeding programme for milkpriduction of cows was started in Kerala in 1960s and was implemented through out the state in 1970s.

The committee constituted to recommend the breeding policy of livestock in Kerala recommended the use of Jersey and Brown Swiss breeds extensively and

Holstein Friesian to a limited extent for improvement of the cattle. The exotic inheritance should not be more than 62.5%. (Anon.1992)

The first scientific study on Vechur cattle was that of Girija (1994), who stated that Vechur cattle had unique characteristics which made their position as a breed possible. She stressed the necessity of conservation of dwarf cattle of Kerala, which is the smallest variety available in India and perhaps in the world.

According to Iype *et al* (1993), in Kerala Jersey bulls were used initially for crossbreeding of local cattle. Subsequently Brown Swiss bulls were used and lately Holstein Friesian. This has resulted in a mosaic inheritance among present cattle.

Chacko (1994) discussed the origin and development of Sunandini breed and stated that 2 million female calves of Sunandini type are produced every year in Kerala.

The recommendations of the committee constituted for formuating the livestock breeding policy of the state was to use Jersey and Holstein breeds as exotic germplasm for improvement of cattle of the state. The use of Brown swiss need be discontinued (Anon.1998).

Iype and Venkatachalapathy (2001) prepared a breed descriptor for Vechur breed. The other dwarf cattle of Kerala namely Highrange dwarf (Anilkumar, 2002), Vatakara cattle (Anilkumar, 2003) and Kasargode cattle (Anilkumar and Raghunandanan, 2003) have also been described.

2.2.ISOLATION OF DNA

The first report on isolation of DNA from eukaryotic blood can be traced back to Blin and Stafford (1976). They were able to obtain high molecular weight DNA from eukaryotes by extraction with phenol.

The phenol chloroform method was fine tuned by Beckman *et al.* (1986) and Andersson *et al.* (1986). Even though the methods suggested involved use of toxic and corrosive phenol and repeated extractions with phenol and chloroform, the yield was superior to that obtained by many other methods.

A method to isolate DNA from human blood was described where instead of phenol, guanidine hydrochloride was used. But the technique was found unsuitable for the isolation of DNA from the blood of cattle and buffalo (Jeanpierre, 1987).

Miller *et al.* (1988) developed high salt method for extraction of DNA from blood samples. It was claimed to be inexpensive, safe and rapid because hazardous organic solvents like phenol and chloroform were not used in this method. Instead, in high salt method, the cellular proteins were salted out by dehydration followed by precipitation with saturated solution of Sodium Chloride.

Bahnak *et al* (1988) could obtain DNA yield of 200 µg/ 1 X 10^8 sperms in cattle. Isolated DNA samples were found suitable for PCR based RFLP studies.

A comparative evaluation of the three methods of DNA extraction namely high salt method, phenol chloroform method and guanidine hydrochloride method was performed in the blood of sheep (Montgomery and Sise,1990) and cattle(Aravindakshan *et al.* 1998a) . They found that phenol chloroform method and high salt method were suitable for DNA isolation from blood but not guanidine hydrochloride method. High salt method was found suitable to isolate DNA from cattle blood by Van Ejik *et al.*, 1992, Gwakisa *et al.*, 1994 and Gelhaus *et al.*,1995.

Lien *et al.* (1990) proposed a method to isolate DNA from frozen semen. The yield obtained from 10 doses of semen (2.5 X 10^8 sperms / ml) was between 300-500 µg.

Apparao *et al.* (1994) described a modified phenol chloroform extraction procedure for isolation of DNA from blood of cattle, sheep, goats, buffaloes and pigs in 1994.

Kantanen *et al.* (1995) and Nagaraja (1998) used high salt method with an additional chloroform extraction method to isolate DNA from cattle blood .

Phenol chloroform method and high salt method for isolation of DNA were compared by Senthil *et al.* (1996). They found that high salt method was quick and easy and yielded better quality and quantity of DNA from blood.

2.2.1.Yield and Purity

Andersson *et al.* (1986) obtained a yield of 150 to 250µg DNA from 10 ml of cattle blood. The yield of DNA obtained from 10 ml of human blood was 200 µg (Jeanpierrie,1987)

The average yields of DNA obtained from 20 ml blood each from 100 sheep were 640±0.26 µg and 500±0.19 µg by high salt method and phenol method respectively. The OD ratio (OD_{260}/OD_{280}) was in between 1.6 and 2.0(Montgomery and Sise, 1990).

Apparao *et al.* (1994) could obtain 250 to 300µg DNA from 15 ml of whole blood of different species of domestic animals.

The yield of DNA from 15 ml cattle blood samples varied from 450 µg to 800 µg with an average yield of 625 µg (Senthil, 1995). Annapoorni (1996) could obtain DNA in the range of 210 µg to 602 µg with an average yield of 400 µg per 10 ml of buffalo blood.

Aravindakshan (1997) observed the mean yield of DNA from 10 ml of buffalo blood as 432 ± 19.34 µg. The mean OD ratio (OD_{260}/OD_{280}) of the samples was found to be 1.81 ± 0.01.

Nagaraja (1998) extracted 400 µg of good quality DNA from 10-15 ml of cattle blood. Miller *et al* (1998) obtained OD ratios in between 1.8 and 2.0 for cattle DNA samples isolated by high salt method.

Nagarajan (1998) could obtain 292.39 ± 25. 95 µg DNA from 5 ml of sheep blood with OD ratio of 1.81 ± 0.01. Saravanan (1999) reported a DNA yield of 228 ± 5.3 µg from 10 ml of sheep blood.

Appannavar (2001) got the DNA yield of 350 µg from 10 ml of blood of Deoni breed of cattle with an OD ratio in between 1.70 and 1.90.

2.3.RAPD-PCR TECHNIQUE

The technique of Random Amplified Polymorphic DNA was first described by Williams *et al.* (1990). They used random oligonucleotide primers and applied a low annealing temperature for amplification of DNA by PCR. The technique was useful for development of fingerprints.

According to Sodhi *et al.* (2001) the efficiency, sensitivity and specificity of the PCR assay depends upon several parameters like primer concentration, dNTPconcentration, concentration of template DNA, $MgCl_2$ concentration and annealing temperature. They opined that optimization is essential to minimize production of incompletely amplified products, or extraneous primer dimer artifacts.

2.3.1.Buffer

The standard PCR buffer contains 50 m*M* KCl, 10 m*M* Tris-HCl (Ph 8.3) and 1.5 m*M* $MgCl_2$ (Sambrook *et al.*, 1989). Innis and Gelfand (1990)

recommended 150 mM Tris Hydrochloride with pH in between 8.3 and 8.8 at 20°C for PCR buffer.

2.3.2.Concentration of Magnesium chloride

Williams *et al.* (1990) suggested a concentration of 1.5 mM to 2.0 mM $MgCl_2$ in their studies on RAPD-PCR. This was accepted by other workers (Mosseler *et al.*, 1992; Scott *et al.*, 1992; Kangfu and Pauls, 1992; Gwakisa *et al.*, 1994 and Kemp Teale, 1994).

Welsh and McClelland (1990) used 4mM level of magnesium chloride. The concentration of $MgCl_2$ used by Riedy *et al.* (1992) was 0.4 m*M*, where as the concentration used by Welsh *et al.* (1991) was 4 m*M* and Kubota *et al.* (1992) used a concentration of 5 m*M* $MgCl_2$.

Rothuizen and Wolferen used 2.5 m*M* $MgCl_2$ in RAPD-PCR analysis. According to Kataria *et al.* (2000), the concentration of magnesium was critical for getting specific product and needs to be titrated for each system.

2.3.3.dNTP concentration

According to Innis and Gelfand (1990) the concentration of each dNTP should be 20 µM for synthesis of 2.5 µg of DNA for PCR reactions. Williams *et al.* (1993) suggested a dNTP concentration of 100 µ*M* in RAPD-PCR. The amount of dNTP used by Gwakisa *et al.* (1994) was 200 µM each.

2.3.4.Template DNA

Baily and Lear (1994) used 50 ng of template DNA isolated from Arabian horses for RAPD- PCR reactions. The Amount of template DNA used by Gwakisa *et al.* (1994) was 20 ng.

2.3.5.Amount of Taq DNA polymerase

According to Lawyer *et al.* (1989) the recommended concentrations of *Taq* DNA polymerase ranged between 1 unit and 2.5 unit per 100 µl reaction mixture.

William *et al.* (1990) suggested 0.5 units of Taq DNA polymerase in 25 µl reaction mixtures. Kangfu and Pauls (1992) used 2 units of Taq DNA polymerase per reaction of 25 µl.

Meunier and Grimont (1993) established that there is difference in the amplified products between three brands of *Taq* DNA polymerases used. They found that *Taq* DNA polymerase can cause variation in RAPD results.

Schierwater and Ender (1993) reported that there is no substantial variation due to commercial preparations of the enzyme. Bowditch *et al.* (1994) concluded that 0.02 units / µl *T*aq DNA polymerase was sufficient for 0.2 to 0.6 ng / µl template DNA with the primer DNA concentration of 0.24 p*M* / µl in RAPD-PCR reactions.

In another study Rothuizen and Wolferen (1994) suggested a concentration of 1.25 units *T*aq DNA polymerase in 35 µl reactions. There were differences in fingerprints developed from the same template amplified with two commercial brands of Taq DNA polymerase (Jayasankar and Dharmalingam, 1997).

2.3.6.Concentration of Primer

William *et al.* (1993) suggested primer concentration of 0.1 to 2 µ*M* for RAPD-PCR reactions. Gwakisa *et al.* (1994) and Kemp and Teale (1994) used a concentration of 6p*M* primer in their 20 µl reactions. The concentration used by Aravindakshan and Nainar (1998) was 15 ng primer in 20 µl reactions. Ramesha *et al.*(2002), used 40 p*M* primer in 30 µl reactions.

2.3.7.Volume of reaction mixture

Gwakisa *et al* (1992) used 10µl reactions for RAPD-PCR reactions. The volume of reaction mixture used by Geng *et al* (2002) was 20 µl.

2.3.8. Denaturation

According to Erlich *et al.* (1991) and Haff (1993), the denaturation of DNA for PCR can be at 94°C to 98°C.

Kangfu and Pauls (1992) fixed the denaturation step at 94°C for five seconds in 40 cycle reactions. They found that denaturing for five seconds gave better results compared with the denaturation at ten seconds or thirty seconds. Gwakisa *et al.* (1994) also used five second denaturation phase at 94°C in his study.

Kemp and Teale (1994) followed a 30 second denaturation at 95°C followed by ten second denaturation for the cycles at 96°C. Nagaraja (1998) used initial denatuaration at 95°C for two minutes followed by 10 second denaturation step for repeated cycles at 94oC.

2.3.9.Annealing temperature

William *et al.* (1993) opined that annealing temperature above 40°C prevented the annealing of most 10 base primers. Erlich *et al.* (1991) used an annealing temperature of 37°C.

Kangfu and Pauls (1992) used annealing temperature of 36°C for five seconds for RAPD-PCR amplification. Gwakisa *et al.* (1992) used the annealing temperature of 35°C.

Haff (1993) reviewed the trends in PCR procedure and suggested that primer binding can be done at temperatures between 37°C to 65°C. The annealing temperature used by Yu and Pauls (1994) was 36°C. Nagaraja (1998) used an annealing temperature of 35°C for10 seconds.

According to Joshi *et al.* (1998) higher annealing temperature during initial cycles of RAPD-PCR amplification increases specificity of the products.

Aravindakshan and Nainar (1998) used 35°C as annealing temperature in their studies on RAPD of cattle and buffaloes.

The annealing temperature used by Yoon and Park (2002) was 37°C. Yeo *et al.* (2002) also used the same annealing temperature. Ramesha *et al.* (2002) used an annealing temperature of 35°C.

2.3.10. DNA synthesis

Erlich *et al.* (1991) suggested the optimum temperature for DNA synthesis in PCR cycles to be 72°C. 72°C universally accepted primer extension temperature since the enzyme Taq DNA polymerase shows maximum activity at this temperature. DNA synthesis step of 72 °C for 2 minutes was used by Geng *et al* (2002).

2.3.11. Number of cycles

Number of cycles in RAPD PCR used by Kangfu and Pauls (1992) and Gwakisa *et al* (1992) was 40. Kanfu and Pauls (1992) could not find any difference in PCR products from 35, 55 or 75 cycles.

Bowditch *et al.* (1993) could not get any amplified products in 20 cycle reactions and some products formed in 25 and 30 cycle reactions were not reproducible.

Baily and Lear (1994) used PCR reactions having 40 cycles with 11 primers for comparison of Arabian and Throughbred horses. Number of cycles used for RAPD-PCR reactions carried out by Kemp and Teale (1994) was also 40.

Geng *et al.* (2002) used 45 cycles for RAPD-PCR on DNA samples of goats of Tibetian plateau of China.

2.3.12.Final extension

Nagaraja (1998) used a final cycle of 2 minute denaturation at 94°C, 2 minutes annealing at 35°C and 10 minutes extension at 72°C. Most of the researchers used the final extension at 72°C for 10 minutes (Geng *et al.*, 2002, Ramesha *et al.*, 2002)

2.3.13. Reproducibility of RAPD fingerprinting

It has been reported that reproducible results could be obtained in spite of several limitations in the RAPD procedure. (Welsh and McClelland, 1990; Carlson *et al.*, 1991, Hu and Quiros., 1991, Klein *et al.*, 1991, Paran *et al.*, 1991, Halward *et al.*, 1992, Mqsseler *et al.*, 1992, Uphoff and Wricke, 1992, Rothuizen and Wolferen, 1994).

But many other scientists questioned the reproducibility of RAPD amplification due to the low annealing temperature (Hedrick 1992, Scott *et al.*, 1992, Ellsworth *et al.*, 1993, Macpherson *et al.*, 1993, Meunier and Criment, 1993, Micheli *et al.*, 1994; Ayliffe *et al.*, 1994).

In a multilocational study of RAPD analysis in seven laboratories in North America, Penner *et al.* (1993) found the influence of temperature profiles of different steps in the reaction to be crucial for reproducibility of the results.

Joshi *et al.* (1999) opined that due to stoichastic nature of DNA amplification with random sequence primers, it is important to optimize conditions for reproducible results. Ahn *et al.* (1999) conducted a study on Holstein, Hereford and Hanwoo cattle and found that the band patterns were not affected by slight changes in PCR conditions.

2.4.APPLICATIONS

Antoniou and Skidmore (1995) identified a bovine Y specific marker of 3100 bp based on RAPD technique. Cushwa and Medrano (1996) suggested that RAPD assay has a useful role in genetic analysis of livestock.

For measurements of genetic variability and genetic distances between population Payne and Hodges (1997) recommended highly polymorphic molecular markers. Adan *et al.* (1997) developed a quick, sensitive and accurate method for determining the sex of sheep embryos using RAPD marker derived form sheep Y chromosome. The test was 100% accurate in all the samples studied.

2.4.1.Conservation Of Genetic Resources

Development of efficient methods for conservation of domestic animal germplasm depends on molecular variation between and within populations (Balakrishna, 1996).

RAPD was proved as a useful method by Parejo *et al.* (2002) for evaluating polymorphism in the endangered Blanca Cacerena breed of cattle. The results of the study guided to plan more adequate mating in order to maintain genetic diversity and to improve the efficiency of conservation of the breed.

Eding *et al.* (2002) concluded that in conservation schemes the contribution of breed to genetic diversity is very high.

2.4.2.Species Characterisation.

Kantanen *et al.* (1995) carried out RAPD analysis of five Finnish cattle breeds and two Finnish sheep breeds and observed that two of the ten RAPD fragments tested namely 0.75 kb fragment from bovine DNA amplified with primer 4 and 0.6 kb fragment from ovine DNA amplified with primer 6 hybridised to both sheep and cattle samples.

Cushwa and Medrano (1996) suggested that RAPD assay has a useful role in genetic analysis of livestock species.

Rao *et al.* (1996) calculated Jaccard's similarity coefficients based on the RAPD patterns to compare four species of domestic animals namely cattle, buffaloes, sheep and goats. Distinct RAPD patterns with higher level of polymorphism were detected between different species using 14 arbitrary primers. Wagner Parsimony analysis revealed very low similarity among the four species.

Glazko *et al.* (1997) prepared dendrograms of species relationships between three species of antelope, cattle, bison, gayal, horse, Przewalski horse and Kulan. They observed variations between these dendrograms and the currently accepted phylogenetic relationships of the species. This was attributed to the selection procedure in the populations of some of these species.

Joshi *et al.* (1998), opined that RAPD profile of DNA samples from goats, sheep, buffaloes , cattle and dogs were unique for each species in terms of number and size of bands.

Koh *et al.* (1998) concluded from a study with 29 oligonucleotide primers that RAPD method could not be used to differentiate between beef and Kara beef and between wild boar and domestic pig meat.

Along with different variants of DNA fingerprinting techniques, Semenova *et al.* (2000) used three arbitrary primers for assessing genetic variability and population diversity in domesticated populations of American bison (*Bison bison*), Aurochs (*Bison bonasus*) and gray Ukranian cattle (B*os taurus*). The relatedness between aurochs was higher than that of of bison and gray Ukranian cattle.

Huang *et al.* (2003) in their study on different species of poultry, pigs and cattle found that distribution of major bands emphasizes the existence of

recognizable genetic similarity with species. They concluded that RAPD markers could be useful in distinguishing and identifying different species.

2.4.3.Parentage Identification

Riedy *et al.* (1992) reported a high frequency of non parental bands from baboons and humans by use of five primers. They concluded that presence of non parental bands, whether due to mutation or PCR artifact makes the technique unsuitable for paternity analysis.

Mendelian dominance of the RAPD markers and the reduction this caused in exclusionary power were main disadvantages of RAPD technique (Lewis and Snow, 1992). However, for species producing large clutches or broods of offspring, the effect of artifactual bands that could lead to false exclusions in an offspring-by-offspring analysis could be minimized by a pooled "synthetic offspring" approach that allowed testing of proportionate representation of different potential parents (Hadrys *et al.* 1993).

Scott *et al.* (1992) opined that even a high frequency of non parental bands did not prelude its use for parentage analysis. They proposed that all bands considered for parentage exclusion must be reproducible and the bands unique to one of the putative parents should be used.

Hadrys *et al.* (1993) and Milligan and McMurray (1993) supported the use of RAPD in parentage identification.

According to Newton and Graham (1994), the RAPD technique was not a method of choice for paternity testing where unequivocal results were essential.

Carpio *et al.* (1996) assessed 24 bulls selected for progeny testing and 10 cows with 10 base oligonucleotide primers using RAPD-PCR technique. The

polymorphic bands among animals and between groups were identified and the similarity index among animals varied from 0.76 to 1.00.

In a study with 131 oligonucleotide primers, Cushwa *et al.* (1996) demonstrated that RAPD assay was a powerful approach for identifying polymorphisms. They could identify 85 RAPD polymorphisms between each parental pair.

Chung *et al.* (1995) established in their study on Holstein dairy cattle using twelve RAPD primers on three families of sire, dam ond one offspring established that the amplified products in offspring were always present in one or both parents.

Application of RAPDs and their related modified markers in variability analysis and individual specific genotyping was less popular due to poor reproducibility, faint or fuzzy products and difficulty in scoring bands (Joshi *et al* 1999)

2.4.4.Genetic Characterisation

The process of breed characterisation using molecular markers required the knowledge of genetic polymorphism within and between populations (Hetzel and Drinkwater, 1992)

Bardin *et al.* (1992) used three random primers of 25 bases length to study the genetic variation in bovine populations.

Clark and Lanigan (1993) described the prospects of using RAPD for estimating nucleotide divergence. They suggested that for haploid data fragments generated by RAPD-PCR can be treated like that for restriction fragment data. But the diploid samples require the consideration that presence of a band is dominent to absence of the band. They summerised the restrictions and other criteria that must be met when RAPD data are used for estimating population genetic parameters.

Kemp and Teale (1994) were able to observe that the RAPD primer ILO 526 could produce a 480bp fragment, which is specific for *Bos indicus* cattle.

Lear *et al.* (1994) constructed a phylogenetic tree based on the RAPD data of equidae. Gwakisa *et al.* (1994) identified two RAPD markers from 141 random primers, capable of distinguishing three *Bos indicus* cattle breeds.

Baily and Lear (1994) identified a 1000 bp marker (UBC127) using RAPD which was present in all the 31 Arabian horses and absent in 20 Throghbred horses studied.

Chung *et al.* (1995) conducted RAPD analysis using 10 primers which produced 78 RAPD marker bands. They were able to identify Holstein specific RAPD markers by use of combination of two primers.

The genetic variation and relationship between seven Italian breeds of cattle were studied by Bardin *et al.* (1994). They used RAPD markers and restriction enzymes for analysis of the results.

Gwakisa *et al.* (1994) used 141 short random primers for characterisation of zebu cattle breeds of Tanzania. They could establish that within breed variation found out by band sharing and average percentage difference was lower than variation found between breeds.

In a study on five Finnish cattle namely native Eastern, Northern Finn cattle, Western Finn cattle, Finnish Ayshire and Finnish Holstein as well as white and grey Finn sheep, Kantanen *et al.* (1995) reported that two of the ten RAPD fragments tested by cross hybridization showed homology between species. They used 11 arbitrary primers and intrabreed and interbreed indices constructed did not show any significant difference between Finnish breeds of cattle.

Teale *et al.* (1995) described a convenient, reliable and effective means of detecting introgression of zebu genes in *Bos taurus* cattle populations using RAPD method. They could identify a 1.1 kb product by amplification with the primer ILO 1065. Caprio *et al.* (1996) used RAPD patterns to compare between and within group variations in cattle.

Band sharing and mean average percentage differences generated by RAPD technique were used by Aravindakshan and Nainar (1998) to analyse the genetic variation in cattle and buffalo breeds. They could establish greater homogeneity within breeds studied and the divergence between breeds was measurable.

Genetic distances between Holstein , Korean native, Charolais, Aberdeen-Ancus and Hereford cattle were calculated using RAPD method with 8 primers by Jeon-Gi *et al.* (1998). The lowest genetic distance was found between Korean native and Holstein breeds and the highest was observed between Hereford and Charolais breeds.

Nagaraja (1998) used RAPD technique for genetic characterisation of seven *Bos indicus* and two *Bos taurus* breeds. He was able to describe two *Bos indicus* specific products by RAPD analysis.

Breed marker fragments of DNA were identified by Parejo *et al.* (1998) in four breeds of cattle namely Charolais , White caceres, Avilena and Retinta.

Ahn *et al.* (1999) were able to identify breed specific RAPD banding patterns for Hanwoo cattle of Korea as compared to Holstein and Hereford cattle.

Rincon *et al.* (2000) could identify a total of 215 loci of size ranging from 300 to 2500bp in Uruguayan Creole cattle using RAPD markers. They were able to identify breed specific RAPD bands for Creole, Hereford and Holstein breeds.

Thiyagarajan (2000) was able to describe breed specific bands in Umblachery, Kangayam, RedSindhi and Ongole breeds of cattle using various RAPD primers.

Ganai *et al.* (2000) used four RAPD primers of 10 bases size to fingerprint cattle and buffaloes. They could obtain amplification products in two of these primers. They observed that the probability of two animals of buffalo to have identical fingerprints was lower than two animals of cattle if the individual primers and their combination are used for analysis.

Operon kits were used as random primers by Choy *et al.* (2001) to identify the beef breeds from the beef samples. They opined that the primer OPB-11 resolved well between breeds. Combination of two or more primers was better to improve the resolution of breeds.

Molecular genetic polymorphism of Simmental, Ukrainian red motley lactescent and Golshtine was studied by Zubets *et al.* (2001) and prepared a dendrogram reflecting the relationship of these animals.

Serrano *et al.* studied the genetic relationship and population structure of five Brazilian native cattle breeds in 2002. They established that RAPD technique was capable of distinguishing native breeds.

Appannavar (2001) characterized Deoni breed of cattle using RAPD technique. He was able to differentiate three types of Deoni cattle namely Wannera, Balankya and Waghya with RAPD technique.

The genetic differentiation coefficient and genetic similarity coefficient in three goat populations were studied by Geng *et al.* (2002) using RAPD analysis. They found that the genetic relationship between Chaidamu Cashmere goat (CCG)

and Liaoning Cashmere goat (LCG) were closest compared with that of Chaidamu goat with CCG or LCG.

A breed specific DNA marker of 519 bp was identified by Yeo *et al.* (2002) for Hanwoo breed of cattle.

Ramesha *et al.,* (2002) prepared a dendrogram of relationship among seven South Indian cattle breeds, namely Amrithmahal, Krishnavally, Malanad Gidda, Ongole, Khillari, Deoni and Hallikar using genetic distances as estimated by Distance Wagner procedure using seven RAPD markers. They found that Malanad Gidda was distinct from all the other breeds.

2.5.MICROSATELLITE ANALYSIS

Microsatellites are repetitive elements containing simple sequence motifs, mostly dinucleotide repeats or trinucleotide repeats, usually less than 100 bp and found to be embedded in unique DNA sequences. They are distributed through out the eukaryotic genome but not in the prokaryotic genome (Miesfeld *et al.* 1981., Hamada *et al.* 1982)

Microsatellite markers are highly polymorphic markers and are devoid of most of the disadvantages of the other markers for detection of DNA polymorphisms (Weber and May, 1989, Litt and Luty, 1989, Tautz, 1989, Fries *et al.,* 1991). They can be amplified by PCR (Saiki *et al.* 1988) and visualized by poly acrylamide gel electrophoresis.

Weber (1990) classified the microsatellite regions into three categories namely perfect repeat sequences without interruption in the dinucleotide repeat (65%), imperfect repeat sequences with one or more interruptions in the repeat (25%) and compound repeat sequences with adjacent tandem simple repeats of different sequences(10%).

Preliminary chromosome maps with micorsatellites were constructed by different workers (Weber *et al.,* 1991, Hazen *et al.,* 1992, Kwiatkowski *et al.,* 1992). Microsatellites were found to occur once in every 10 kb of DNA sequence (Tautz, 1989). The average spacing between $(CA)_n$ / $(GT)_n$ sites was estimated as 30 kb in human, 20 kb in rat and 18 kb in mouse (Stallings *et al.,* 1991).

Beckman and Weber (1992) found one AC repeat for every 30 kb of human genome. Buchanan and Crawford (1993) reported that on an average, ovine genome contained one AC repeat for every 65kb of genome.

Buchanan *et al.* (1994) concluded from a study on four sheep breeds with eight microsatellites that they were a better indicator of evolutionary relationships within a species. According to Mullis and Fries (1994), the set of eight microsatellites used by them is an extremely powerful system for parentage identification in cattle.

The use of microsatellite markers for genetic differentiation and assessing genetic relationships among different livestock breeds and populations were stressed by various researchers (MacHugh *et al.*, 1998 ; Martinez *et al.*, 2000; Pandey *et al.*, 2002).

The capacity of microsatellite markers to distinguish individuals was used for assigning breed identities to anonymous samples (Diez-Tascon *et al.*, 2000, Bjrnstad and Roed, 2001).

Dorji *et al.* (2003), used 20 microsatellite markers to study the genetic diversity and relationship of populations of Siri breed of cattle of Bhutan. They compared the breed with Tibetian Golleng, Nepal Hill cattle, Holstein Friesian, Indian Jaba and Mithun. The phylogenic analysis separated Bhutanese Siri population from Holstein Friesian and Goleng cattle suggesting that if a taurine influence was present in Siri cattle, it was likely to be small.

2.5.1. DRB3

DRB3 microsatellite was expressed, highly polymorphic, and encodes a functional restriction element (Burke *et al.*, 1991, Fraser *et al.*, 1994). The gene coding for the bovine major histocompatability complex class II antigen, DRB3 was found to contain a microsatellite within 2nd intron (Muggli-Cockett and Stone., 1988). The DRB3 microsatellite was composed of three repeat motifs, a stretch of at least 10 uninterrupted $(TG)_n$ dinucleotides, and a few $(CAGA)_n$ tetranucleotides (Ellegren *et al.* 1993).

2.5.2. ETH 131

The microsatellite ETH 131 was mapped to the syntenic group U_3 and was assigned to the chromosome 5 (Steffen *et al.*, 1993). The microsatellite was described as a $(CA)_{23}$ dinucleotide repeat with higher polymorphism.

2.5.3.FSHβ

Beta subunit of follicle stimulating hormone (FSHβ) was also reported to contain a microsatellite sequence (Kemp and Teale. 1991). The stretch of $(AT)_{20}$ repeats was found to be highly polymorphic. The number of alleles reported in bovines with BOVFSH microsatellite was 11 and heterozygosity was 88.9% by Moore *et al.* (1992). Frequencies of these alleles were also found out in different cattle reference families.

2.5.4.HEL-6

It is a microsatellite described by Kaukinen and Varvio (1993). HEL-6 is a dinuleotide repeat of $(GT)_{23}$. In a study on cattle and sheep genotyping, HEL6 was found to map to nonhomologous chromosomes (Maddox *et al.*, 2001).

2.6.STATISTICAL ANALYSIS

Nei (1972), proposed the concept of the genetic distance between populations. Nei (1978) gave a formula for calculating heterozygosity based on frequency of alleles. A method to construct a phylogenetic tree with neighbor joining algorithm was presented by Saitou and Nei, (1987) and to calculate heteorzygosity was proposed by Ott (1992).

Botstein *et al.* (1980) suggested a formula to calculate the PIC value of the primers. Reynold *et al.* (1983) presented a method for calculation of coancestory identity / distance.

Construction of phylogenetic tree could be done using UPGMA algorithm (Swofford and Olsem, 1990.) Software programmes like software Biosys 1 are now available on internet for statistical analysis of the data for finding out observed heteozygosity, expected heterozygosity and mean number of alleles per loci (Swofford and Selander, 1989), software Dispan for constructing phylogenetic tree with neighbour joining algorithm (Otta, 1993), GENEPOP for equilibrium expectations (Raymond and Rousset, 1995), Treeview to visualize the phylogenetic tree (Page, 1996) and POPGENE for genetic analysis(Yeh *et al.* 1999). Wimmers *et al.* (2000) opined that the use of highly variable and less variable microsatellite reduces the risk of overestimating genetic variability, which may occur if only highly variable loci were used.

Materials and Methods

3.MATERIALS AND METHODS

3.1 SOURCE OF SAMPLES

DNA samples from crossbred cattle of Kerala formed the material for this study. Blood was collected from unrelated animals of University Livestock Farm, Mannuthy, Cattle Breeding Station, Thumburmuzhi, and farmers premises. For dam-progeny pairs blood was collected immediately after calving to avoid misidentification.

Five milliliter of blood was collected from the jugular vein of animals for DNA isolation. The blood was collected using sterile disposable syringes and 18G needles. Immediately after the collection blood was transferred to sterile disposable polypropylene tubes containing Ethylene Di-amine Tetra Acetic Acid (EDTA) as anticoagulant (1mg / ml of blood).

In case of bulls, semen was used to isolate DNA. Frozen semen of the bulls under the ICAR Progeny Testing Scheme and the fresh semen of the bulls of Kerala Agricultural University Bull station, Mannuthy were used for DNA isolation. Fresh semen was obtained on the day of ejaculation itself and was transported to the laboratory in a chilled condition. Around 300–500 million sperms (2-3 ml) were used for the isolation of DNA. In case of frozen semen, 5 straws of 24 million sperms each were used for isolation of DNA.

3.2 ISOLATION OF DNA

3.2.1. Isolation of DNA from Whole Blood

The DNA samples were isolated from blood using the standard phenol chloroform extraction procedure with modifications. The procedure used for isolation of DNA from blood was as follows

1. Five milliliter of blood was taken in a centrifuge tube and centrifuged at 3500 rpm for 10 minutes to remove the plasma.

2. The cell pellet was suspended in 10 ml of ice cold RBC lysis solution (150m*M* NH_4Cl. 10 m*M* KCl. 0.1 m*M* EDTA) and mixed well and incubated in ice for 10 minutes with occasional mixing followed by centrifugation at 3500 rpm for 10 minutes to separate the pellet. The pellet was then washed 2-3 times.

3. The cell pellet was washed twice in 10 ml of Tris buffered saline (TBS- 140 m*M* NaCl. .0.5 m*M* KCl. 0.25m*M* Tris), by centrifugation at 3000 rpm for 10 minutes.

4. The washed pellet was suspended in 5 ml of SE buffer (Nacl 75m*M*. EDTA 35 m*M*), 20µl of Proteinase K (20 mg / ml) and 0.25 ml of 20% sodium dodecyl sulphate were added and mixed gently.

5. The samples were incubated overnight at 65°C in a water bath. On cooling to room temperature. 300µl of 5*M* sodium chloride was added and mixed.

6. To the mixture equal volume of saturated phenol (pH 7.8) was added and mixed followed by centrifugation at 3500 rpm for 15 minutes to collect the aqueous supernatant.

7. To the aqueous phase equal volume of phenol: chloroform : isoamyl alcohol (25:24:1) was added. The contents were mixed and centrifuged at 3500 rpm for 15 minutes.

8. To the aqueous phase, collected in fresh tubes, equal volume of chloroform : isoamyl alcohol (24:1) was added and mixed for 10 minutes. The tubes were then centrifuged at 3500 rpm for 15 minutes.

9. The chloroform extraction was repeated and the aqueous phase was transferred to a sterile 50 ml beaker. To this one tenth volume of 3 *M* sodium acetate (pH 5.5) was added and mixed well.

10. To the beaker equal volume of chilled isopropyl alcohol was added and the precipitated DNA was spooled using a fresh micropipette tip on a glass rod.

11. The spooled out DNA was washed with 70% ethanol and it was air dried. The air dried DNA was suspended in 500µl TE buffer (Tris 10m*M*, EDTA 0.1 m*M*) and stored at –20°C.

3.2.2.Isolation of DNA from Semen

The difference procedure for isolation of DNA from fresh semen and frozen semen.

1. Fresh semen containing around 300-500 million sperms (2-3 ml) was used for isolation of DNA. It was taken in a centrifuge tube and was washed twice with 10 ml of Phosphate buffered saline (PBS- 138m*M* NaCl, 4 m*M* NaH_2PO_4, 6 m*M* $Na_2H\ PO_4$) by centrifugation at 3500 rpm for 10 minutes. In case of frozen semen the contents of five straws of frozen semen was thawed in water and the semen was collected in centrifuge tubes for washing with PBS.

2. The sperm pellet was suspended in 2ml of PBS by vortexing.

3. Six ml of warm sperm lysis buffer (100mM Tris, 500mM NaCl, 10 mM EDTA, 1% SDS, 2% Mercaptoethanol) was added to the centrifuge tube, mixed well and incubated at 50°C in a water bath for 30 minutes with occasional mixing.

4. 100 µl of Proteinase K (20 mg / ml) was added and incubation at 50°C was continued for three hours.

5. Complete digestion of all the material was ensured by clarity of the solution.

6. Equal volume of saturated phenol (pH 7.8) was added to the mixture and was mixed thoroughly by gentle repeated inversion of tubes for 10 minutes followed by centrifugation at 3500 rpm for 15 minutes and the upper aqueous phase was collected in fresh centrifuge tubes.

7. To the aqueous phase collected equal volume of phenol: chloroform : isoamyl alcohol (25:24:1) was added and was mixed for 10 minutes. The tubes were then centrifuged at 3500 rpm for 15 minutes and the aqueous supernatant was collected in fresh centrifuge tubes.

8. To the aqueous phase collected equal volume of chloroform : isoamyl alcohol (24:1) was added and mixed for 10 minutes. The tubes were then centrifuged at 3500 rpm for 15 minutes.

9. The chloroform extraction procedure was repeated once as in step 8 and the aqueous phase sample was transferred to a sterile 50 ml beaker. To this, one tenth volume of 3 *M* sodium acetate (pH 5.5) was added and mixed well.

10. Equal volume of chilled isopropyl alcohol was added and the precipitated DNA was spooled using a fresh micropipette tip on a glass rod.

11. The spooled out DNA was immediately washed with 70% ethanol and was air dried. The air dried DNA was resuspended in 500µl TE buffer (Tris 10m*M*, EDTA 0.1 m*M*) and stored at -20°C.

3.2.3. Determination of Yield and Purity of DNA

Twenty microliters of the stock solution of DNA was diluted to 2 ml with sterile distilled water. Optical density was measured at 260 and 280 nm using a 2 ml cuvette in an UV spectrophotometer (Genvay UK). Sterile water was used as blank. Concentration of the DNA sample was calculated as follows

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

Purity of DNA sample was assessed as OD ratio which is given by OD_{260} / OD_{280}. The total yield of DNA obtained from 5 ml blood was calculated and recorded. The quality of DNA samples was first assessed electrphoretically on 0.7% agarose gel in TAE buffer.

3.3.RAPD-PCR ANALYSIS

3.3.1. DNA Samples

The DNA samples prepared were grouped into two sets. The first set was of 84 unrelated animals and the second set was that of 52 offspring, their dams and sires. The assessment of RAPD polymorphism was done with the samples of unrelated crossbred animals. DNA samples of offspring, dam and sire combination were used for assessing the possibility of using RAPD technique for parentage verification.

3.3.2. Primers Used

Commercially available primer kit (Operon Technologies Inc., USA) and custom synthesized primers (Life Technologies India Pvt. Ltd.) were used in this study. Twenty six random 10 base oligonucleotide primers were used in this study. They were OPA 1 to OPA 20 , ILO 1127, ILO 876, ILO526, OPAV15, G_1 and G_2. Of these 20 primers were selected for use in panel of DNA of unrelated crossbred cattle. Name and sequence of the primers chosen to find out RAPD polymorphism of unrelated crossbred cattle are shown below.

Sl. No.	Name of Primer	Sequence of the primer
1	OPA 1	5`CAGGCCCTTC3`
2	OPA 2	5`TGCCGAGCTG3`
3	OPA 4	5`AATCGGGTTG3`
4	OPA 7	5`GAACGGGCTG3`
5	OPA 8	5`GTGACGTAGG3`
6	OPA 9	5`GGGTAACGCC3`
7	OPA 10	5`GTGATCGCAG3`
8	OPA 12	5`TCGGCGATAG3`
9	OPA 14	5`TCTGTGCTGG3`
10	OPA 15	5`TTCCGAACCC3`
11	OPA 16	5`AGCCAGCGAA3`
12	OPA 17	5`GACCGCTTGT3`
13	OPA 18	5`AGGTGACCGT3`
14	OPA 19	5`CAAACGTCGG3`
15	OPA 20	5`GTTGCGATCC3`
16	PRG 1	5`GTGACGTAGG3`
17	ILO 526	5`GCCGTCCGAG3`
18	ILO 876	5`GGGACGTCTC3`
19	ILO 1127	5`CCGCGCCGGT3`

Twelve primers were selected based on the polymorphism to evaluate the banding patterns in offspring, dam and sire combinations. The primers selected were OPA1, OPA2, OPA7, OPA9, OPA 14, OPA15, OPA 18, OPA 19, OPA 20, G1 and ILO 1127.

The primers were suspended in sterile triple distilled water to make the stock solution of 200 p*M* /µl concentration. The tubes were incubated at room temperature for one hour. The stock solution was stored at –20°C. Working solution of the primers were prepared from the stock solution by diluting it to get a final concentration of 5 p*M* /µl.

3.3.3. Template DNA

The working solution of DNA was prepared from stock solution of DNA by diluting with distilled water to get a final concentration of 25ng /µl. One microlitre of this working solution was used in every 10 µl reaction.

3.3.4. PCR Conditions

For all the PCR reactions 10 µl volume was used. Each reaction tube had 1 µl template DNA, 1 µl 10X PCR buffer (10 m*M* Tris, pH 8.3; 50 m*M* KCl, 1.5 m*M* $MgCl_2$), 0.5 unit of *Taq* DNA polymerase and 200 µ*M* each of the four dNTP solutions.

Modified PCR conditions and a programmable Thermal Cycler (Techme) were used in this study. Following an initial denaturation at 94°C for three minutes, 35 cycles were carried out with denaturation at 94°C for one minute, annealing at 38°C for one minute and extension at 72°C for one minute followed by a final extension at 72°C for 10 minutes. The amplified products were stored at -20°C till use.

3.3.5. Agarose Gel Electrophoresis

The horizontal submarine electrophoresis was done using electrophoretic apparatus (Amersham Pharmacia, USA). The gel was cast in custom synthesized gel trays for RAPD-PCR products. The open ends of the gel tray were sealed with cello tape to form the mould. The combs were with a well size of 0.8 mm X 1.5 mm and the thickness of the gel was 5 mm. Volume of the well was approximately 6 mm^3.

Analysis of the PCR products was done in 1.5% agarose in 1X TAE buffer (0.04 *M* Tris acetate, 0.001*M* EDTA). This mixture was then boiled in microwave oven for 3 minutes. The agarose solution thus prepared was allowed to cool to 50°C. To the cooled solution, 7µl of ethidium bromide (10 mg / ml) solution was added and mixed well to get a final concentration of 0.5 µg / ml. The agarose solution was then poured into a gel tray carefully without forming any bubbles and was allowed to set the gel. Combs were removed after gel formation is completed.

3.3.6. Sample Loading

Sample volume used for electrophoresis was 4.7 µl with approximately 1.0 µl of gel loading buffer (0.25% Brom phenol blue.0.25% Xylene cyanol.40% sucrose). The markers employed were φX 174 / HaeIII digest or 1 kb ladder. 0.7 µl of the marker was mixed with 2 µl of gel loading buffer and loaded into the well.

3.3.8. Electrophoresis

Electrophoresis was done at 100 volts for two hours. TAE was used as the electrophoresis buffer. The gel was taken out and viewed under UV transilluminater and were analysed in gel documentation system with quantity one programme(Biorad)

The bands were scored as present or absent. The frequency of different bands, their polymorphism and other characters were noted. The polymorphic pattern of bands produced by each primer was assessed.

3.4. MICROSATELLITE ANALYSIS

3.4.1. Animals

Four genetic groups of dwarf cattle available in Kerala, namely Vechur, Highrange dwarf, Vatakara and Kasargode formed the subjects for the microsatellite analysis. The DNA samples were isolated from the Highrange dwarf animals of Chinnar, Elappara, Vandiperiyar, Menmala, Pattumala, Fairfield and Glenmary estates of Idukki district in Highranges of Kerala. Vatakara animals were from Memunda and Ayanchery and Theekuni areas of Calicut district and Kasargode animals were from Vorkadi and Meenja areas of Kasargode district. DNA samples of the Vechur animals of the Kerala Agricultural Univeristy, Vechur conservation unit was collected for the study.

3.4.2. Loci Studied

The studies on polymorphisms of DRB3, ETH-131, HEL-6 and FSHβ microsatellite loci were done. The sequence of forward and reverse primers used for each locus with the position of loci and approximate size of alleles reported are presented below.

Micro-Satellite	Primer sequence	Position of locus	Size range
DRB3	Forward 5'GAGAGTTTCACTGTGCAG3' Reverse 5'CCAGAGTGAGTGAAGTATCT3'	D1S4	150-200
FSH	Forward 5'TGGGATATAGACTTAGTGGC3' Reverse 5' CAGTTTCTAAGGCTACATGGT3'	15q22/24-qter	150-200
ETH-131	Forward 5'GTGGACTATAGACCATAAGGT3' Reverse 5'GCTGTGATGGTCTACGAATGA3'	D21S4	141-161
HEL-6	Forward 5'GGACACGACTGAGCAAGTTA3' Reverse 5'AGGCAGCATACATTACCACTA3'	D1S21	260-290

3.4.3. Sequencing M13 Bacteriophage

The sequencing kit supplied by M/s Amersham Biosciences Corporation. USA was used for sequencing of the M13 as per the directions of the manufacturers.

1. Preparation of annealing mixture .

The composition of the mixture was as follows

DNA (M13 phage200 µl/ml))	5 µl
Water	2 µl
Sequence reaction buffer	2 µl
Primer (0.5p*M*/ µl)	1 µl
Total	10 µl

The mixture was spun briefly. This was followed by incubation at 65°C for two minutes. The mixture was allowed to cool slowly to room temperature over 15 to 30 minutes and then stored in ice.

2. Four tubes were labeled G, A, T and C. To each of these tubes 2.5 µl of respective termination mixture were added.

3. Preparation of diluted labeling mixture.

The labeling mix available in the sequencing kit was diluted five folds to prepare the diluted labeling mixture.

Labeling mixture	0.5 µl
Water	2.0 µl

4. Dilution of the enzyme.

The enzyme available in the sequencing kit was diluted eight fold with enzyme dilution buffer.

Enzyme	0.5 µl
Enzyme dilution buffer	3.5 µl

5. Labeling reaction

The components were added to the icecold annealing mixture prepared.

Dithiothreitol (DTT) 0.1 *M*	1	µl
Diluted labeling mixture	2	µl
α^{35}Sd ATP(10µCi/ml)	2	µl
Diluted enzyme mixture	2	µl

The contents were mixed well and incubated at room temperature for five minutes.

6. To each of the termination tubes 3.5 of labeling reaction mixture was added. mixed well and incubated at 37°C for five minutes. The reaction was stopped by adding 4µl of the stop solution available in the kit.

3.4.4. End Labeling of Primers

The end labeling of the microsatellite primers was done with γ^{32} P d*CTP. The process include preparation of the end labeling mix. The composition of the mix was as follows

Polynucleotidekinase (PNK) buffer	1	µl
T4 Polynucleotidekinase (PNK)(10 units/ µl)	0.5	µl
Forward primer (200 p*M* / µl)	1	µl
γ^{32} P d*CTP	2	µl
Nuclease free water	5.5	µl

The mixture was incubated at 37°C for 30 minutes and stored at 4°C.End labeled primer was diluted in distilled water to a final concentration of 5 p*M* and 1 µl of the primer was used per reaction in PCR. Radioactive safely procedures were followed for handling of radioactive material.

3.4.5. Preparation of PCR Mixture

PCR was done for forty samples at a time. The PCR conditions for different loci were standardised separately. The standardization was done for four loci namely HEL-6, ETH-131, FSH β and DRB3 locus.

3.4.6. Denaturing Poly Acrylamide Gel Electrophoresis

Denaturing PAGE was performed on the Vertical Sequencer (BIORAD USA). The gel was cast between two glass plates whose sides were sealed before casting of the gel. The upper and lower tanks were filled to the required level with electrode buffer.

The glass plates of the sequencer were cleaned with soap solution. After drying the glass plates were cleaned with chloroform followed by acetone to remove greasy dirt. The spacers were kept on either side of the bigger notched glass plate before casting the gel. The other glass plate was kept over this, with the cleaned sides facing each other. The sides of the two plates were then sealed with packing tape.

Analysis of the PCR products was done in Urea PAGE (denaturing Poly Acrylamide Gel Electrophoresis) by the method suggested by Hughes (1994) with modifications. 60 ml of the 6% gel mix was taken in a beaker and 125 µl each of 10% ammonium per sulphate solution and TEMED were added and mixed. The mixture was then poured into the space between the glass plates kept, carefully avoiding air bubbles. The plates were clamped on three sides and shark tooth comb was placed on top to form the sample loading channel.

The gel was allowed to set for one hour, the tape was removed and the plates were cleaned and kept in the sequencer. The upper and lower chambers of the apparatus were filled with freshly prepared 0.5X TBE buffer (0.045 *M* Tris

borate, 0.001 *M* EDTA) to the required level. The comb was removed and the sample loading channel was cleaned to remove the pieces of gel or other materials if present using the buffer solution. The comb was then inserted to form the wells for sample loading.

3.4.7. Sample Loading

The PCR products were mixed with 3.5 µl formamide dye and spun briefly. The bromophenol dye in the loading buffer had a mobility equivalent to 25 bp fragment and cyanol dye had a mobility equivalent to 100 bp DNA fragment. The samples were denatured at 95°C for five minutes, snap cooled on ice and 4µl of the dye mixed PCR product was used for loading each well. The samples were kept in ice till loading is complete. The sequenced G,A, and C markers were also denatured simultaneously and loaded in the gel. The markers were loaded in the central wells of the gel and the volume used was 2.5µl.

3.4.8. Electrophoresis

Electrophoresis was done at 30W for three hours. Sufficient care was taken to see that the temperature is maintained between 45°C to 55°C . The glass plates were then taken from the sequencer and the spacers were removed first and the glass plates were separated, carefully keeping the gel on one of the plates.

The gel was then transferred to a filter paper by spreading it on top of the gel and pressing it to make it adhere with the gel. Then it was taken carefully without breaking the gel. Excess filter paper from all the sides were then cut off and covered the gel surface with a Klin film.

3.4.9. Gel Drying

The gel was dried in the gel drier at 82 °C and the drying was continued for two to three hours. Once drying was completed the gel was taken out, and put in an autoradiography cassette with the X ray film and an intensifying screen. X ray film was developed after 48 to 72 hours depending on the primer used and the intensity of radioactive signal.

3.4.10. Development of X ray Film

The X ray film was taken out and developed in the dark room. The film was first placed in 1X developer solution (Kodak) for 3 to 5 minutes and then in 1% acetic acid for one minute. The film was washed in distilled water for three minutes. The film was then transferred to the fixer solution (Kodak) and kept for 7 to 10 minutes and finally washed in running tap water for 30 minutes.

3.4.11. Microsatellite Typing

The alleles were identified with the help of an X ray viewer. The exact size of the alleles were determined by comparing with the M-13 sequencing ladder and the frequencies of different alleles were obtained.

3.5.STASTISTICAL ANALYSIS

3.5.1. RAPD Analysis of Unrelated Crossbred Cattle

The mean values of the number of bands and size of bands for each primer were calculated. Frequency of bands and the allelic frequency of the bands and the non bands were calculated. The allelic frequency of the non band was calculated as the square root of the frequency of the non band. The allelic frequency of the band was calculated by subtracting the frequency of the non band from one.

Allelic frequency of non band $q_i = \sqrt{Q_i}$

Where Q_i is the frequency of the non band and where q_i is the allelic frequency of i^{th} non band

Allelic frequency of band $p_i = 1 - q_i$

Where q_i is the allelic frequency of the non band and p_i is the allelic frequency of i^{th} band.

3.5.2. RAPD Analysis of Offspring, Dam and Sire Combinations

The presence of non parental bands were calculated as the ratio of number of non parental bands to total bands identified in the offspring. Because of the presence of non parental bands the statistical analysis for computation of probability of sire exclusion was not attempted.

3.5.3. Microsatellite Analysis of DwarfCattle

3.5.3.1. Direct Count Heterozygosity

The usefulness of a marker for applications in parentage studies or any other study depends on its heterozygosity. The Heterozygosity is calculated by the method of Ott.1992.

$$\text{Heterozygosity} = 1 - \left[\sum_{i=1}^{k} p_i^2 \right]$$

where is the frequency of i^{th} allele

3.5.3.2. Unbiased Heterozygosity

The unbiased heterozygosity was calculated using the formula of Pandey *et al* 2002.

$$\text{Heterozygosity} = \left[2n/(2n-1)\right] 1 - \left[\sum_{i=1}^{k} p_i^4 \right]$$

where p_i is the frequency of i^{th} allele and N is the number of observations

3.5.3.3.Polymorphic Information Content (PIC)

The PIC value of the primers were calculated using the formula suggested by Botstein *et al* (1980).

$$\text{Polymorphic Information Content} = 1 - \left[\sum_{i=1}^{k} p_i^4 \right] - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2 p_i^4 p_j^4$$

Results

4.RESULTS

4. 1. RAPD-PCR STUDIES

4.1.1. Samples Collected

Two panels of DNA samples were prepared for the study. The first panel contained samples of 84 unrelated crossbred cattle collected from different parts of the state. The second panel contained 52 samples of offspring, dam and sire. Combinations.

4.1.2 Yield and Quality of DNA

The average yields of DNA obtained from different sources are provided in Table 4.1. Presence of a single high molecular weight band on agarose gel electrophoresis suggested that the DNA extracted was intact.

The ratio of optical density at 260 nm and 280 nm was taken as a measure of purity of the DNA isolated. Concentration of the DNA samples were calculated from the assumption that an optical density of 1 at 260 nm is equivalent to 50µg double stranded DNA. The average OD ratios obtained in this study were 1.72 ± 0.14, 1.81 ± 0.26 and 1.61 ± 0.34 for the DNA obtained from whole blood, frozen semen and fresh semen respectively.

4.1.3. Optimization of PCR Parameters

RAPD-PCR conditions were optimized for efficient amplification and to minimize the production of incomplete and nonspecific amplified products. The standardized reaction conditions are presented in Table 4.2.

4.1.4. Primers Used

Twenty six random oligonucleotide primers were used for the amplification of randomly selected DNA samples of the two panels. PCR products were obtained only for twenty four primers. Based on intensity, clarity and polymorphism of bands, nineteen primers were selected for analysing the RAPD polymorphic patterns in crossbred cattle of Kerala.

4.1.5. Number of Bands

The product size obtained ranged from ~230 bp to~3580 bp, the average number of bands produced by different primers ranged from 3.78 ± 0.15 in OPA 4 to 8.15 ± 0.25 in ILO1127 (Table 4.3). Number of bands produced by different primers ranged from 7 in OPA18 to 16 in ILO1127. The percentage of polymorphism represented by individual primer varied from 66.66 to 100 in different groups (Table 4.4).

4.1.6. Frequency of Bands

Frequency of each band was determined from the ratio of the number of animals carrying finge rprints (n) to the total number of animals screened (N). The frequencies of different bands and their allelic frequencies were found out for different primers.

4.1.7. Allelic Frequency of Bands

Allelic frequency of different bands were calculated from the band frequency. Since RAPD markers are dominant markers both the dominant homozygote and heterozygote are represented by the presence of bands. The blood samples collected represent a large population and hence it is assumed to be in Hardy- Weinberg equilibrium. Accordingly the allelic frequency of the band and

the other allele are calculated based on the assumption that there are only two alleles at the same locus.

4.1.8. Primer wise results

The results of amplification of DNA samples of the unrelated crossbred cattle with the nineteen selected primers are presented. The classification of the bands based on their frequency is presented in Table 4.5.

4.1.8.1. Primer OPA 1

Amplification with the primer OPA 1 is presented in Fig 4.1, which revealed the presence of 10 amplified products. The approximate size of products ranged from ~290 bp to ~2240 bp (Table 4.6.). Three of the bands namely OPA01b, OPA01e and OPA01f were present in all the animals studied. The rare bands with frequencies less than 0.25 were four namely OPA01a, OPA01h, OPA01i and OPA01j.

4.1.8.2. Primer OPA 2

The primer OPA 2 was highly polymorphic and yielded 11 products. The approximate size and frequency of the bands produced are provided in Table 4.7. The bands OPA02a, OPA02d, OPA02g, OPA02i and OPA02k had frequencies less than 0.25. None of the bands showed the frequency of one. The polymorphic pattern obtained for the primer is shown in Fig 4.2.

4.1.8.3.Primer OPA 4

The RAPD banding pattern obtained with the primer OPA 4 is shown in Fig 4.3. The primer was capable of producing 12 polymorphic bands. Four of them namely OPA04a, OPA04i, OPA04j and OPA 04l were with frequencies less than 0.25 and none of the bands had the frequency of one. The approximate size of the bands and their frequencies are provided in Table 4.8.

4.1.8.4.Primer OPA 7

The approximate size and name of bands produced by the primer OPA 7 are presented in Table 4.9. The size of the bands ranged between ~290 bp and ~2350 bp. The maximum frequency was obtained for the band OPA07d (1.000) and three bands (OPA07a,OPA07f and OPA07g) were having frequency less than 0.25.

Banding pattern produced by the primer is shown in Fig.4.4.

4.1.8.5. Primer OPA 8

Name, frequency and allelic frequencies of bands obtained by the amplification of the primer OPA 8 are presented in Table 4.10. Of the twelve bands identified, the band OPA08j was present in all the animals screened. The lowest frequency of 0.105 was obtained for the band OPA08l. The bands OPA08a, OPA08b and OPA08k were also having frequency less than 0.25. Various bands produced are presented in Fig 4.5.

4.1.8.6. Primer OPA 9

The primer OPA 9 revealed nine polymorphic bands and one common band (Fig 4.6). Four of the bands namely OPA09a, OPA09d, OPA09h and OPA09j were having frequencies below 0.25. The allelic frequencies and sizes of different bands are presented in Table 4.11.

4.1.8.7. Primer OPA 10

The banding pattern produced by the primer OPA 10 is presented in Fig 4.7. Size of the amplified products ranged from ~340 bp to ~2590 bp. The approximate size and frequency of the bands and their allelic frequency are presented in table 4.12. The band OPA10c had a frequency of 1 while OPA10h had frequency less than 0.25.

4.1.8.8. Primer OPA 12

Name, frequency and allelic frequencies of the bands produced by the primer OPA 12 are presented in Table 4.13. The sizes of the bands ranged from ~390 bp to ~1490 bp. The size range of the bands was narrow compared to the products of all other primers used. Banding pattern obtained for the primer OPA 12 is shown in Fig 4.8. There were two common bands (OPA12h and OPA12i) and one band with frequency less than 0.25 (OPA12a).

4.1.8.9. Primer OPA 14

The bands obtained with the primer OPA14 is shown in Fig 4.9. Approximate size and frequency of the different bands are presented in Table 4.14. The product OPA14h was present in all the animals screened. Of the 13 bands

identified only three were having frequency less than 0.25 namely OPA14b, OPA14g and OPA14j.

4.1.8.10. Primer OPA 15

Thirteen bands were observed with the primer OPA 15 (Fig 4.10). The band OPA15g was present in all the animals and the bands OPA15f, OPA15h, OPA15l and OPA15m were having very low frequencies (less than 0.1). The band size and frequencies are provided in Table 4.15.

4.1.8.11. Primer OPA 16

The name, band size, frequency and allelic frequency of different bands produced by the primer OPA 16 are presented in Table 4.16. The size of bands ranged from ~430 bp to ~3580 bp. The variability in size of bands were greater compared with the other primers used in this study. The bands OPA16a, OPA16b, OPA16i and OPA16k were having frequencies less than 0.25. The band OPA16f was observed in all the animals screened (Fig 4. 11).

4.1.8.12. Primer OPA 17

OPA 17 primer produced 9 products on RAPD- PCR (Fig 4.12). The approximate sizes and frequencies of these bands are presented in Table 4.17. None of the bands were common in the animals studied. The bands OPA17b, OPA17f and OPA17i were having frequencies less than 0.25.

4.1.8.13. Primer OPA 18

Lowest numbers of bands were obtained for the primer OPA 18 (Fig 4.13). The size and frequency of different bands are provided in Table 4.18. The sizes of the seven bands ranged from ~450 bp to ~1840 bp. None of the bands was common and OPA18a was the only band with a frequency less than 0.25.

4.1.8.14. Primer OPA 19

Thirteen bands of sizes ranging from ~310 bp to ~2090 bp were observed by amplification with the RAPD primer OPA 19 (Fig 4.14). Of these bands only the band OPA19g was common for all the animals studied. The approximate size and frequencies of these bands are provided in Table 4.19. Five bands OPA19c, OPA19h, OPA19i, OPA19j and OPA19k were having frequencies less than 0.25.

4.1.8.15. Primer OPA 20

The RAPD–PCR products with the primer OPA 20 are shown in Fig 4.15. The size and frequency of the eight bands obtained are provided in Table 4.20. None of the bands were common to all the animals studied and the band OPA20f was the only one with frequency less than 0.25.

4.1.8.16. Primer G_1

Name, frequency and approximate band size of products obtained with the primer G_1 are provided in Table 4.21. The band sizes ranged from 460 bp to 2270 bp. Rare bands having frequency less than 0.25 were absent and there was only one common band (PRG01h) as shown in Fig 4.16.

4.1.8.17. Primer ILO 526

Name, approximate size and frequency of the bands produced by the primer ILO 526 are provided in Table 4.22. The sizes of the bands ranged from ~300 bp to ~2250 bp. Three of the bands namely ILO526b, ILO526c and ILO526d were present in all the animals screened (Fig 4.17). Frequency of two bands (ILO526e and ILO526i) was less than 0.25.

4.1.8.18. Primer ILO 876

The primer ILO 876 produced 12 bands as shown in Fig 4.18. The approximate size and frequency of these bands are presented in Table 4.23. Three bands namely ILO876a, ILO876i and ILO876j were having frequencies less than 0.25. None of the bands were common.

4.1.8.19. Primer ILO 1127

The maximum number of bands (16) was obtained for the primer ILO 1127 (Fig 4.19). The approximate size and frequency of the bands are provided in Table 4.24. None of these bands were present in all the animals studied. The bands ILO1127c, ILO1127f, ILO1127k and ILO1127n were having frequencies less than 0.25. The sizes of bands ranged from ~340 bp to ~3030 bp.

4.2.Offspring, Dam and Sire comparisons

The comparisons between the bands of offspring with their dam and sire were done for different primers with the objective of using the RAPD-PCR technique for parentage identification. Twelve primers showing highly polymorphic DNA bands were selected for analysis of offspring. their dam and sire. Non parental bands were observed in some of the primers (Table 4.25).

4.2.1 OPA 1

The banding pattern obtained for the offspring. dam and sire combinations with the primer OPA1 are provided in Fig 4.20. The non parental bands noticed in offspring were OPA01b, OPA01d and OPA01f.

4.2.2. OPA 2

The only non parental band observed in one of the offspring was OPA02i. All the other offspring were having bands present in either sire or dam or both. The products are shown in Fig 4.21.

4.2.3. OPA 4

Three non parental bands were observed in offspring namely OPA04d. OPA04j and OPA04l. The results are presented in Fig 4.22.

4.2.4 OPA 7

Two bands OPA07g and OPA07f were observed as non parental bands in some of the offspring. The results are presented in Fig 4.23. The frequency of these bands was very low (2.43%).

4.2.5. OPA 9.

The bands OPA09c and OPA09h were the non parental bands observed in offspring. (Fig 4.24.) The frequency of the band OPA09h was very low in the samples of Offspring. dam sire combinations.

4.2.6. OPA 14.

OPA 14 was one of the primers where no non parental bands were observed in offspring. The results are shown in Fig 4.25.

4.2.7. OPA 15

The frequency of non parental bands was very high (14.6%) in OPA 15. The non parental bands noticed were OPA15b, OPA15d, OPA15j. and OPA15k. The amplified products of offspring, dam and sire combination with OPA 15 primer are presented in Fig 4.26.

4.2.8. OPA 18

The presence of non parental bands was observed for this primer also. The band OPA18c was observed as non parent band in 2% of offspring. The results are presented in Fig 4.27.

4.2.9. OPA 19

The primer OPA 19 produced three non parental bands in different offspring. They were OPA19c, OPA19e and OPA19g. The percentage of non parental bands for the primer was 13.6. Results are presented in Fig 4.28.

4.2.10.OPA 20

Amplified products of the primer OPA 20 on offspring, dam and sire combinations are shown in Fig 4.29. Only one non parental band namely OPA20e was observed in the offspring.

4.2.11. G 1

No non parental bands were observed for the primer G 1. The results are presented in Fig 4.30.

4.2.12. ILO 1127.

The presence of non parental bands were observed to the extent of 6.2% in offspring. The band ILO1127m, ILO1127 b and ILO1127 k were the non parental bands observed (Fig 4.31).

4.3.MICROSATELLITE ANALYSIS

4.3.1. Animals

The study was conducted on local dwarf cattle of Kerala. A total of 108 DNA sample were isolated from whole blood of four different groups of cattle comprising 24 Vechur, 25 Highrange dwarf, 24 Vatakara and 35 Kasargode animals using phenol: chloroform extraction procedure.

4.3.2. Yield and Quality of DNA

The average yield of DNA obtained from 5 ml of blood samples was 110.1 ± 9.4 µg. The ratio of optical density at 260 nm and 280 nm was taken as a measure of purity of the DNA isolated. The average value was 1.86 ± 0.23. DNA samples appeared as single bands on agarose gel electrophoresis, indicated the presence of intact DNA in the isolated samples.

4.3.3. End-labelling of PCR primers

Four microsatellite loci were chosen for the study. Forward primers of all the four loci used in the study were end labelled with γ^{32}P ATP using the enzyme polynucleotide kinase (PNK). The reaction was carried out in a total volume of 10µl containing 1µl of 10 X PNK buffer (700 mM Tris, 100mM M, 50 MM DTT) , 1µl primer (200p*M*), 0.5 units of PNK, 1µl of γ^{32} P dATP (10µCi/ µl) and 7µl water. The mixture was mixed well and incubated at 37°C for 30 minutes. The end labelled primer was diluted with 30µl water and used at the rate of 1µl per reaction.

4.3.4. PCR Conditions

4.3.4.1. Primer DRB3

The PCR conditions standardized for the microsatellite locus DRB3 are presented in Table 4.26. The template DNA used was 25ng and the concentrations of the primers were 5 p*M* each. Concentration of $MgCl_2$ was found to be very critical and it was standardized at 1.5 m*M*. 35 PCR cycles of denaturation at 94°C for one minute, annealing at 60°C for 1 minute, extension of 72°C for one minute with a final extension at 72°C for 10 minutes were used in the study. The results are presented in Fig 4.32.

4.3.4.2.Primer ETH 131

The PCR reaction mix was prepared as above except for the concentration of $MgCl_2$ which was fixed at 1.25 mM. In cycle parameters the primer annealing temperature was changed to 55°C for one minute. The PCR conditions for the locus ETH 131 are presented in Table 4.27. The results of the analysis of the locus ETH131 in dwarf cattle of Kerala are presented in Fig 4.33.

4.3.4.3.Primer HEL 6

The PCR programme used was same as that of DRB3 except for the $MgCl_2$ concentration which was fixed at 1.0 m*M*. Fig 3.34 shows the results of analysis of the locus Hel-6 in dwarf cattle of Kerala. The PCR conditions for the primer are shown in Table 4.28.

4.3.4.4. Primer FSHβ

The PCR conditions fixed for the locus FSHβ are presented in Table 4.29. The concentration of $MgCl_2$ was fixed at 1.25 m*M* for reaction. Number of cycles and other PCR conditions were same as that of DRB3 and HEL 6 loci.

4.3.5.Sequencing using Sequenase kit

The results of sequencing of M13 phage with Sequenase version 2.0 sequencing kit (United States Biochemical, USA) is presented in Fig 4.32. The reaction was carried out in three steps namely annealing, elongation and termination as per manufacturer's instructions.

1. Annealing

5µl of control DNA (M13 phage), 2µl of 5X reaction buffer (200 m*M* Tris, 100 m*M* $MgCl_2$, 250 m*M* NaCl), 1µl of primer and 2 µl of deionised water were mixed and heated for 2 minutes at 65°C and cooled slowly to less than 37°C within a period of 30 minutes.

2. Elongation

After annealing, 1 µl of 0.1*M* DTT, 2µl of diluted (8 fold) labelling mix (7.5 µ*M each of* dGTP, dATP, dTTP and dCTP), 0.5 µl α^{35}SdATP and 2µl of diluted (6 fold) sequenase version 2 enzyme (diluted in enzyme dilution buffer, 10 mM Tris, 5 mM DTT, 0.5 mg/ ml BSA) were added to the tube and incubated at room temperature for 3-5 minutes.

3. Termination

3.5 µl of this labeled reaction mix were dispensed into each of the four tubes (Labelled A,G,T and C) containing 2.5 µl of one of the four termination mixes (ddNTPs) respectively and incubated at 37°C for 5 minutes. Reactions were stopped by addition of 4 µl stop solution (Formamide containing dye).

4.3.6.Size and Frequency of alleles

The size of the different alleles and their frequencies were found out for different loci.

4.3.6.1. DRB3 locus

The different alleles identified with their frequencies in different genetic groups of dwarf cattle and average of the whole population of dwarf cattle are also presented are presented in Table 4.30. The size of the alleles ranged from 176 to 236 bp.

4.3.6.2. ETH 131 locus

The size range of the alleles of ETH 131 locus was from 160 bp to 184 bp. The frequencies of different alleles of different dwarf cattle of Kerala namely Vechur, Highrange dwarf, Vatakara and Kasargode with the allelic frequency of the whole population are presented in Table 4.31.

4.3.6.3. HEL 6 locus

Allelic frequencies of different dwarf cattle of Kerala are shown in Table 32. The allelic size of HEL-6 locus varies from 263 to 295 bp.

4.3.7.Genetic group wise results

The three microsatellite loci were typed in four different dwarf cattle, Vechur, Highrange dwarf, Vatakara and Kasargode. The results of the whole population are also provided.

4.3.7.1. Highrange dwarf cattle

Number of alleles, size of alleles, values of unbiased heterozygosity, direct count heterozygosity and Polymorphic Information Content value of the DRB3 locus and ETH 131 locus in Highrange dwarf cattle are presented in Table 4.33.

4.3.7.2. Vechur cattle

The unbiased heterozygosity, direct count heterozygosity, PIC values, number of alleles and sizes of alleles of three microsatellite loci namely DRB3, ETH131 and HELl-6 in Vechur cattle are presented in Table 4.34.

4.3.7.3. Vatakara cattle

Table 4.35. shows the information on three loci of Vatakara cattle namely DRB3, ETH 131 and HEL 6. The values of direct count heterozygosity, unbiased heterozygosity and PIC are also presented in the table.

4.3.7.4. Kasargode cattle

The number of alleles for DRB3 locus in Kasargode cattle was 11. The size of the alleles ranged from 186 to 236 bp. The information on the number of alleles, their size, direct count heterozygosity, unbiased heterozygosity and PIC values are presented Table 4.36.

4.3.7.5. Dwarf cattle

The information on analysis of whole population of dwarf cattle with the three microsatellite loci are presented in Table 4.37. Size range of different alleles, number of alleles, unbiased heterozygosity, direct count heterozygosity and PIC values are also presented.

Table 4.1 Yield and purity of DNA samples extracted from different sources.

Sl No		Whole Blood	Frozen Semen	Fresh semen
1	Yield	256±0 2.12 µg / 5 ml	91.32 ± 6.01 µg/ 0.25 ml straw	241.23 ± 8.42 µg/ 400 million sperms
2	OD ratio	1.72 ± 0.14	1.81 ± 0.26	1.61 ± 0.34

Table 4.2. The variables fixed for RAPD-PCR analysis.

	Parameter / variable	Level
	REACTION MIX	
1	Template DNA	25 ng
2	Primer concentration	5 p*M*
3	dNTP concentration	200 µ*M*
4	Concentration of $MgCl_2$	1.5 m*M*
5	*Taq* DNA polymerase	0.5 unit
6	Reaction volume	10 µl
	CYCLE PARAMETERS	
7	Initial denaturation	94°C for 10 minutes
8	Denaturation for PCR cycles	94°C for 1 minute
9	Primer annealing	38°C for 1 minute
10	Primer extension	72°C for 1 minute
11	Number of cycles	35
12	Final extension	72°C for 10 minutes

Table 4.3. Name, sequence and approximate size of bands amplified by different RAPD primers

Sl No	Primer	Sequence	Approximate size of bands	
			Range (Kb)	Average
1	OPA 1	5'CAGGCCCTTC3'	0.29 - 2.24	1.14 ± 0.27
2	OPA 2	5'TGCCGAGCTG3'	0.37 – 2.34	1.78 ± 0.20
3	OPA 4	5'AATCGGGTTG3'	0.28 – 2.24	0.95 ± 0.17
4	OPA 7	5'GAACGGGCTG3'	0.29 – 2.35	1.13 ± 0.29
5	OPA 8	5'GTGACGTAGG3'	0.33 – 2.31	1.14 ± 0.20
6	OPA 9	5'GGGTAACGCC3'	0.40 – 2.10	1.05 ± 0.18
7	OPA 10	5'GTGATCGCAG3'	0.34 – 2.59	1.19 ± 0.27
8	OPA 12	5'TCGGCGATAG3'	0.39 – 1.50	0.87 ± 0.15
9	OPA 14	5'TCTGTGCTGG3'	0.30 – 2.40	1.14 ± 0.18
10	OPA 15	5'TTCCGAACCC3'	0.23 – 2.66	0.93 ± 0.20
11	OPA 16	5'AGCCAGCGAA3'	0.43 – 3.58	1.62 ± 0.31
12	OPA 17	5'GACCGCTTGT3'	0.28 – 2.74	1.19 ± 0.28
13	OPA 18	5'AGGTGACCGT3'	0.45 – 1.84	1.12 ± 0.21
14	OPA 19	5'CAAACGTCGG3'	0.31 – 2.09	0.99 ± 0.15
15	OPA 20	5'GTTGCGATCC3'	0.50 – 2.26	1.20 ± 0.20
16	G 1	5'GTGACGTAGG3'	0.46 – 2.27	1.20 ± 0.22
17	ILO 1127	5'CCGCGCCGGT3'	0.34 – 3.03	1.39 ± 0.29
18	ILO 876	5'GGGACGTCTC3'	0.23 – 2.02	0.96 ± 0.17
19	ILO 526	5'GCCGTCCGAG3'	0.30 – 2.25	1.01 ± 0.22

Table 4.4. Name. average number of bands identified and percentage polymorphism of the different RAPD primers

Sl No	Primer	GC content of primers (%)	Number of bands		Percentage polymorphism
			Range	Average	
1	OPA 1	70%	3 - 8	6.29 ± 0.11	70.00
2	OPA 2	70%	3 - 8	5.81 ± 0.14	100.00
3	OPA 4	60%	2 - 7	3.78 ± 0.15	100.00
4	OPA 7	60%	2 – 6	4.19 ± 0.12	87.50
5	OPA 8	60%	4 - 9	6.70 ± 0.16	91.66
6	OPA 9	70%	3 - 7	5.23 ± 0.15	90.00
7	OPA 10	60%	3 - 8	5.72 ± 0.17	85.00
8	OPA 12	60%	2 - 9	6.58 ± 0.16	77.77
9	OPA 14	60%	2 - 9	6.00 ± 0.20	91.66
10	OPA 15	60%	2 - 7	4.08 ± 0.21	92.31
11	OPA 16	60%	3 - 7	5.03 ± 0.21	90.91
12	OPA 17	60%	3 - 6	4.15 ± 0.12	100.00
13	OPA 18	60%	2 - 7	4.44 ± 0.16	100.00
14	OPA 19	60%	3 - 10	6.66 ± 0.41	92.31
15	OPA 20	60%	4 - 8	5.89 ± 0.12	100.00
16	G 1	60%	3 - 8	6.46 ± 0.16	85.00
17	ILO 1127	90%	3 – 10	8.15 ± 0.25	100.00
18	ILO 876	70%	4 - 10	6.81 ± 0.16	100.00
19	ILO 526	80%	4 – 7	5.81 ± 0.12	66.66

Table 4.5. Classification of bands identified by amplification with different RAPD primers based on their frequency.

Sl No	Primer	Number of bands with allelic frequencies				Total Number of bands
		< 0.25	0.25 to 0.50	0.50 to <1	1	
1	OPA 1	4	0	3	3	10
2	OPA 2	5	0	6	0	11
3	OPA 4	4	6	2	0	12
4	OPA 7	3	1	3	1	8
5	OPA 8	4	2	5	1	12
6	OPA 9	4	1	4	1	10
7	OPA 10	1	1	5	1	8
8	OPA 12	1	1	5	2	9
9	OPA 14	3	4	4	1	12
10	OPA 15	7	2	3	1	13
11	OPA 16	4	3	3	1	11
12	OPA 17	3	2	4	0	9
13	OPA 18	1	2	4	0	7
14	OPA 19	5	1	6	1	13
15	OPA 20	1	1	6	0	8
16	G 1	0	1	6	1	8
17	ILO 1127	2	2	2	3	9
18	ILO 876	3	1	8	0	12
19	ILO 526	4	5	7	0	16

Table 4.6. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA1 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA01a	2240	0.131	0.068	0.932
2	OPA01b	1890	1.000	1.000	0
3	OPA01c	1570	0.908	0.697	0.303
4	OPA01d	1160	0.961	0.803	0.197
5	OPA01e	980	1.000	1.000	0
6	OPA01f	750	1.000	1.000	0
7	OPA01g	640	0.934	0.743	0.257
8	OPA01h	470	0.224	0.119	0.881
9	OPA01i	400	0.145	0.075	0.925
10	OPA01j	290	0.079	0.040	0.960

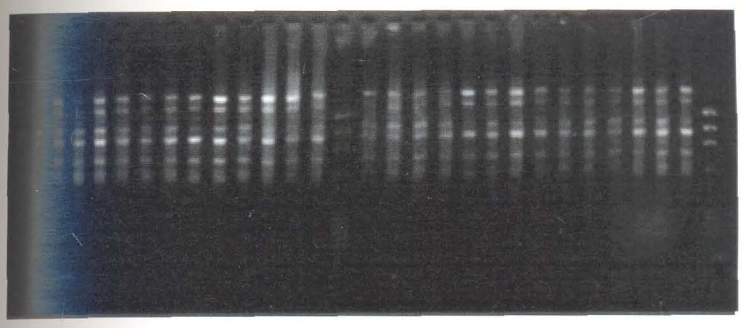

Fig.4.1. Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 1.
Line 1-28 DNA samples,
Line 29 PhiX 174 Hae III digest

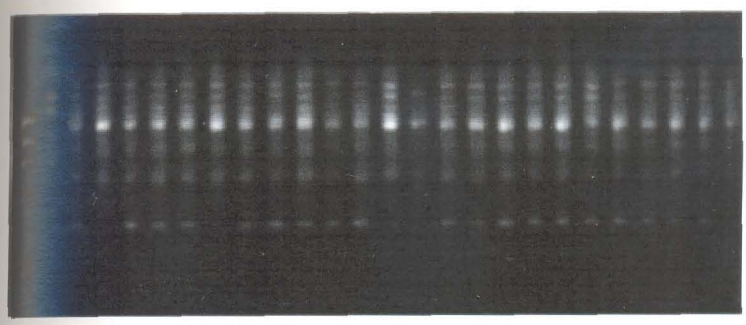

Fig.4.2. Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 2.
Line 2-25 DNA samples,
Line 1 PhiX 174 Hae III digest

Table 4.7. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA2 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA02a	2340	0.088	0.045	0.955
2	OPA02b	1980	0.860	0.626	0.374
3	OPA02c	1700	0.614	0.379	0.621
4	OPA02d	1570	0.123	0.064	0.936
5	OPA02e	1290	0.983	0.870	0.130
6	OPA02f	990	0.737	0.487	0.513
7	OPA02g	940	0.211	0.112	0.888
8	OPA02h	740	0.965	0.813	0.187
9	OPA02i	580	0.193	0.102	0.896
10	OPA02j	460	0.895	0.676	0.324
11	OPA02k	370	0.228	0.121	0.879

Table 4.8. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA4 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA04a	2240	0.036	0.018	0.982
2	OPA04b	1650	0.346	0.191	0.809
3	OPA04c	1320	0.927	0.730	0.270
4	OPA04d	1160	0.307	0.168	0.832
5	OPA04e	990	0.291	0.158	0.842
6	OPA04f	900	0.273	0.147	0.853
7	OPA04g	750	0.655	0.413	0.587
8	OPA04h	710	0.291	0.158	0.842
9	OPA04I	600	0.146	0.076	0.924
10	OPA04j	450	0.073	0.037	0.962
11	OPA04k	350	0.255	0.137	0.863
12	OPA04l	280	0.164	0.086	0.914

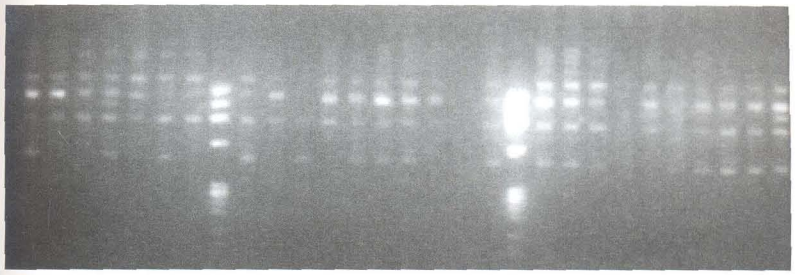

Fig.4.3. Fingerprints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 4
Line 1-7 , 8- 19 & 21-30 DNA samples
Line 8 & 20 PhiX 174 Hae III digest

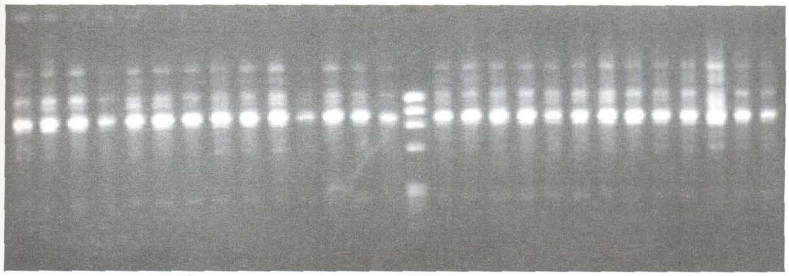

Fig.4.4. Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 7.
Line 1-14 & 16-30 DNA samples,
Line 15 PhiX 174 Hae III digest

Table 4.9. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA7 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA07a	2350	0.231	0.123	0.877
2	OPA07b	2130	0.577	0.350	0.650
3	OPA07c	1440	0.962	0.805	0.195
4	OPA07d	1010	1.000	1.000	0
5	OPA07e	690	0.327	0.180	0.820
6	OPA07f	600	0.212	0.112	0.888
7	OPA07g	450	0.096	0.049	0.951
8	OPA07h	290	0.789	0.540	0.459

Table 4.10 Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA8 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA08a	2310	0.175	0.092	0.908
2	OPA08b	2080	0.158	0.882	0.918
3	OPA08c	1770	0.877	0.649	0.351
4	OPA08d	1490	0.912	0.703	0.297
5	OPA08e	1250	0.474	0.274	0.725
6	OPA08f	1000	0.614	0.378	0.622
7	OPA08g	850	0.930	0.735	0.265
8	OPA08h	640	0.982	0.866	0.134
9	OPA08i	550	0.281	0.152	0.848
10	OPA08j	440	1.00	1.000	0
11	OPA08k	390	0.175	0.092	0.908
12	OPA08l	330	0.105	0.054	0.946

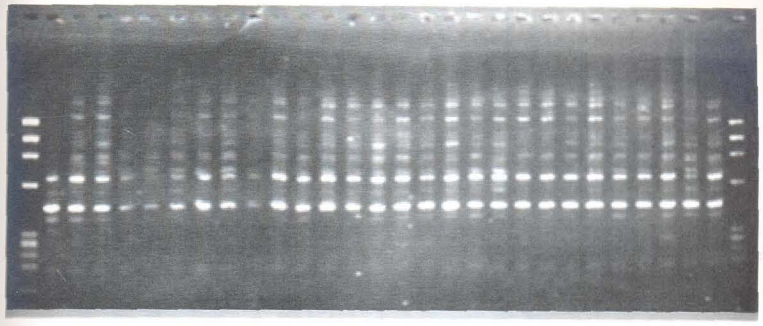

Fig.4.5. Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 8
Line 2-29 DNA samples.
Line 1 & 30 PhiX 174 Hae III digest

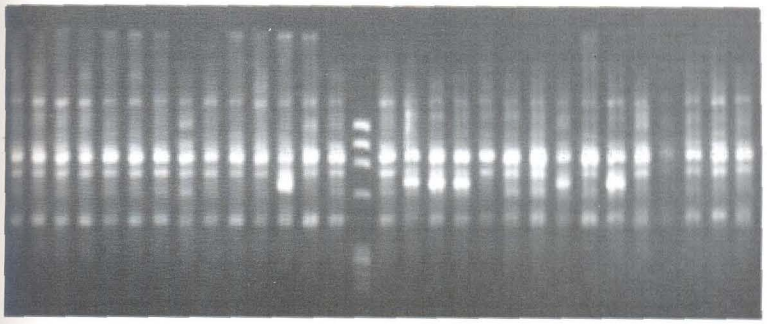

Fig.4.6. Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 9.
Line 1-14 & 16-30 DNA samples.
Line 15 PhiX 174 Hae III digest

Table 4.11. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA9 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA09a	2100	0.175	0.092	0.908
2	OPA09b	1750	0.856	0.621	0.379
3	OPA09c	1550	0.579	0.351	0.649
4	OPA09d	1190	0.105	0.054	0.946
5	OPA09e	940	1.000	1.000	0
6	OPA09f	770	0.983	0.870	0.130
7	OPA09g	700	0.281	0.152	0.848
8	OPA09h	630	0.246	0.132	0.868
9	OPA09i	490	0.983	0.870	0.130
10	OPA09j	400	0.035	0.018	0.982

Table 4.12. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA10 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA10a	2590	0.789	0.541	0.459
2	OPA10b	1930	0.895	0.675	0.324
3	OPA10c	1440	1.000	1.000	0
4	OPA10d	1050	0.842	0.603	0.397
5	OPA10e	980	0.702	0.454	0.546
6	OPA10f	780	0.965	0.813	0.187
7	OPA10g	510	0.351	0.194	0.806
8	OPA10h	340	0.211	0.112	0.888

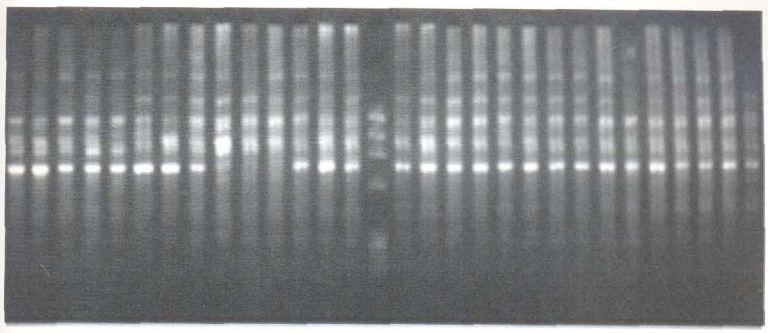

Fig 4.7 Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 10
Line 1-14 & 16-30 DNA samples,
Line 15 PhiX 174 Hae III digest

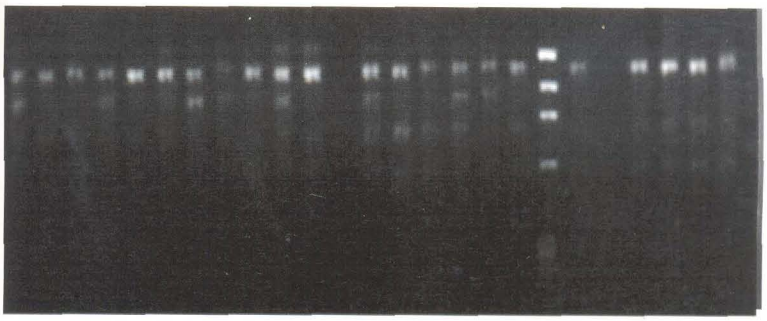

Fig 4.8 Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 12.
Line 1-18 & 20-26 DNA samples,
Line 19 PhiX 174 Hae III digest

Table 4.13. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA12 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA12a	1490	0.201	0.106	0.894
2	OPA12b	1250	0.940	0.755	0.245
3	OPA12c	1050	0.358	0.199	0.802
4	OPA12d	830	0.881	0.655	0.345
5	OPA12e	780	0.806	0.560	0.440
6	OPA12f	620	0.821	0.577	0.423
7	OPA12g	590	0.627	0.389	0.611
8	OPA12h	410	1.000	1.000	0
9	OPA12i	390	1.000	1.000	0

Table 4.14. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA14 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA14a	2400	0.406	0.229	0.771
2	OPA14b	1900	0.157	0.084	0.918
3	OPA14c	1640	0.672	0.427	0.573
4	OPA14d	1460	0.439	0.251	0.949
5	OPA14e	1300	0.812	0.566	0.434
6	OPA14f	1130	0.797	0.549	0.451
7	OPA14g	1080	0.125	0.065	0.935
8	OPA14h	830	1.000	1.000	0
9	OPA14i	650	0.875	0.646	0.354
10	OPA14j	560	0.172	0.090	0.910
11	OPA14k	410	0.344	0.190	0.810
12	OPA14l	300	0.281	0.152	0.848

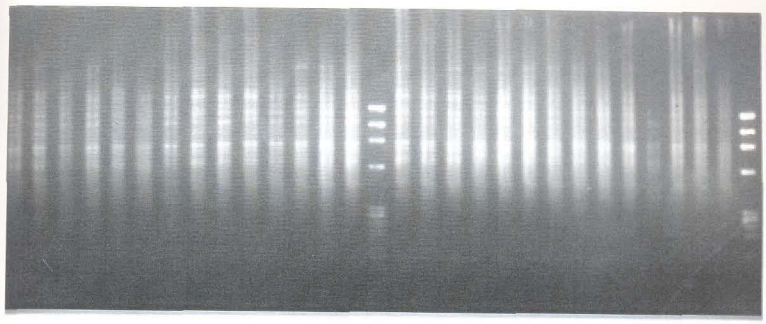

Fig.4.9 Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 14.
Line 1-14 & 16-29 DNA samples,
Line 15 & 30 PhiX 174 Hae III digest marker.

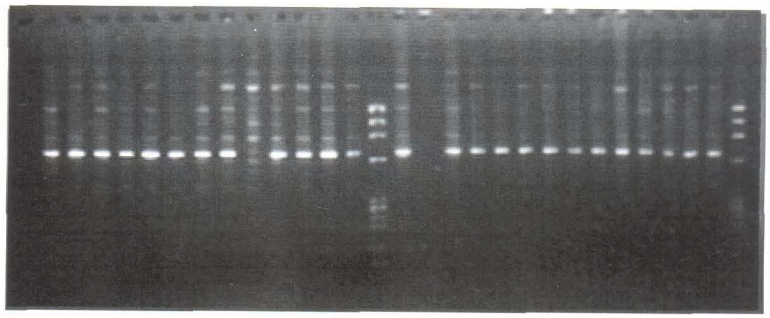

Fig.4.10 Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 15.
Line 1-14 & 16-29 DNA samples,
Line 15 & 30 PhiX 174 Hae III digest marker.

Table 4.15. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA15 on DNA samples of unrelated crossbred cattle of Kerala.

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA15a	2660	0.250	0.134	0.866
2	OPA15b	2120	0.673	0.428	0.572
3	OPA15c	1460	0.558	0.335	0.665
4	OPA15d	1120	0.212	0.112	0.888
5	OPA15e	940	0.519	0.306	0.694
6	OPA15f	700	0.019	0.009	0.990
7	OPA15g	650	1.000	1.000	0
8	OPA15h	610	0.058	0.029	0.971
9	OPA15i	440	0.269	0.145	0.855
10	OPA15j	400	0.153	0.079	0.920
11	OPA15k	370	0.173	0.090	0.909
12	OPA15l	340	0.058	0.029	0.971
13	OPA15m	230	0.096	0.049	0.951

Table 4.16. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA16 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA16a	3580	0.153	0.920	0.080
2	OPA16b	2980	0.128	0.934	0.066
3	OPA16c	2310	0.538	0.680	0.320
4	OPA16d	2130	0.795	0.547	0.453
5	OPA16e	1710	0.462	0.267	0.733
6	OPA16f	1350	1.000	1.000	0
7	OPA16g	1150	0.462	0.267	0.733
8	OPA16h	840	0.923	0.723	0.277
9	OPA16i	710	0.154	0.080	0.920
10	OPA16j	620	0.282	0.153	0.847
11	OPA16k	430	0.103	0.053	0.947

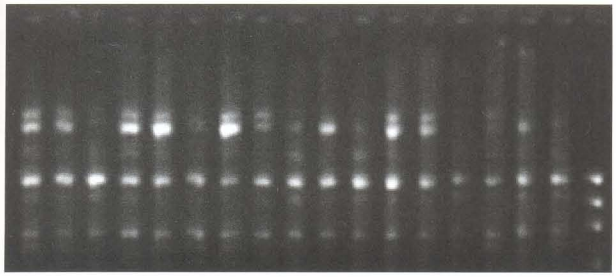

Fig 4.11. Fingerprints of unrelated DNA samplesof Crossbred cattle with the RAPD primer OPA 16
Line 1-17 DNA samples
Line 18 PhiX 174 Hae III digest.

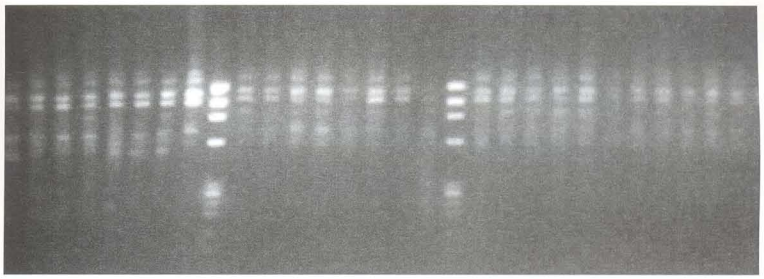

Fig.4.12. Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 17
Line 1-8, 10-17 & 19-29 DNA samples
Line 9 and 18 Phix 174 Hae III digest

Table 4.17. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA17 on DNA samples of unrelated crossbred cattle of Kerala.

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA17a	2740	0.259	0.139	0.861
2	OPA17b	2120	0.148	0.077	0.923
3	OPA17c	1660	0.852	0.615	0.385
4	OPA17d	1250	0.963	0.808	0.192
5	OPA17e	870	0.926	0.728	0.272
6	OPA17f	770	0.093	0.048	0.952
7	OPA17g	560	0.500	0.295	0.707
8	OPA17h	450	0.315	0.172	0.828
9	OPA17I	280	0.148	0.077	0.923

Table 4.18. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA18 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA18a	1840	0.136	0.070	0.930
2	OPA18b	1720	0.418	0.237	0.763
3	OPA18c	1350	0.942	0.759	0.241
4	OPA18d	1050	0.326	0.179	0.821
5	OPA18e	870	0.808	0.562	0.435
6	OPA18f	550	0.923	0.723	0.277
7	OPA18g	450	0.885	0.661	0.339

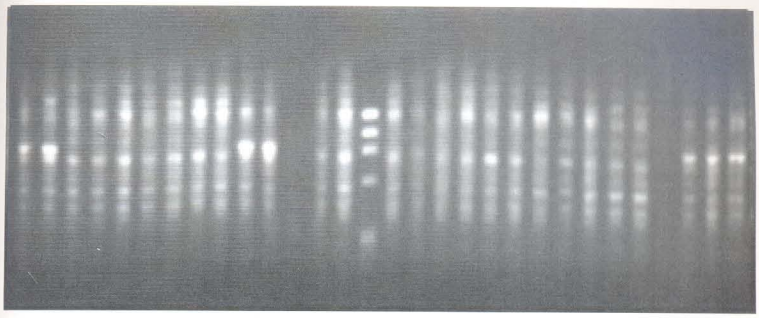

Fig.4.13. Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 18.
Line 1-14 & 16-29 DNA samples,
Line 15 PhiX 174 Hae III digest

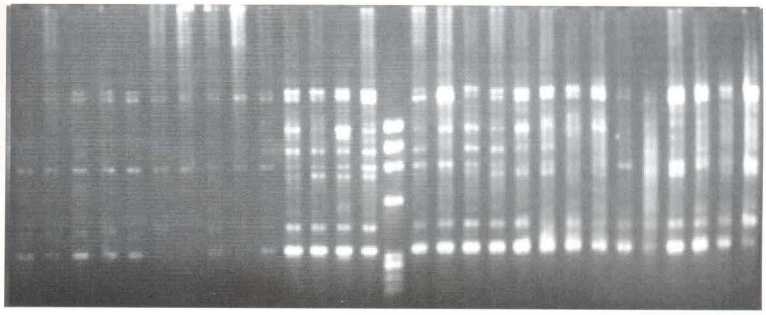

Fig.4.14 Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 19.
Line 1-14 & 16-30 DNA samples,
Line 15 PhiX 174 Hae III digest

Table 4.19. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA19 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in Kb	Frequency of the band	Allelic frequency of the band	Frequency of the allele of the band
1	OPA19a	2090	0.655	0.413	0.587
2	OPA19b	1820	0.862	0.629	0.371
3	OPA19c	1360	0.172	0.090	0.901
4	OPA19d	1300	0.655	0.413	0.587
5	OPA19e	1150	0.379	0.212	0.788
6	OPA19f	1010	0.586	0.357	0.643
7	OPA19g	860	1.000	1.000	0
8	OPA19h	780	0.241	0.129	0.871
9	OPA19i	660	0.172	0.090	0.910
10	OPA19j	610	0.172	0.090	0.910
11	OPA19k	540	0.207	0.009	0.891
12	OPA19l	420	0.818	0.573	0.427
13	OPA19m	310	0.724	0.475	0.525

Table 4.20. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer OPA20 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	OPA20a	2260	0.946	0.388	0.232
2	OPA20b	1650	0.625	0.767	0.612
3	OPA20c	1320	0.821	0.577	0.423
4	OPA20d	1210	0.982	0.866	0.134
5	OPA20e	1090	0.964	0.810	0.190
6	OPA20f	870	0.200	0.106	0.894
7	OPA20g	680	0.929	0.734	0.266
8	OPA20h	500	0.268	0.144	0.856

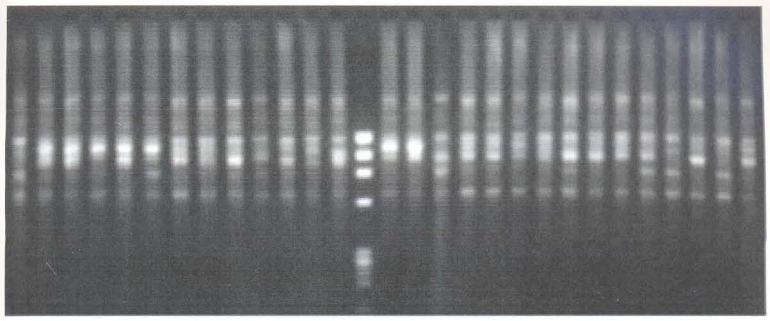

Fig.4.15 Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer OPA 20.
Line 1-14 & 16-29 DNA samples,
Line 15 PhiX 174 Hae III digest

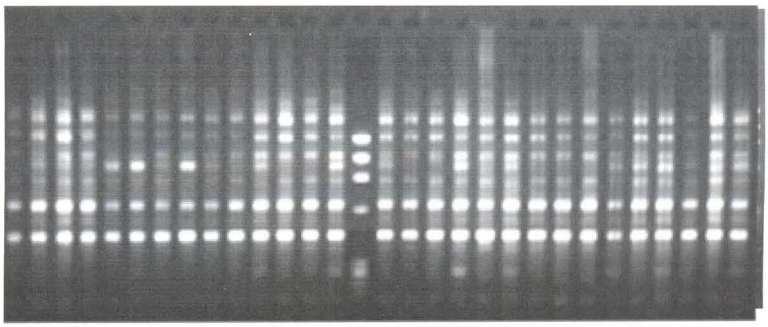

Fig.4.16 Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer G1.
Line 1-14 & 16-29 DNA samples,
Line 15 PhiX 174 Hae III digest

Table 4.21. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer G1 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	PRG01a	2270	0.351	0.194	0.806
2	PRG01b	1810	0.912	0.703	0.297
3	PRG01c	1450	0.860	0.626	0.374
4	PRG01d	1120	0.702	0.454	0.546
5	PRG01e	1000	0.731	0.481	0.519
6	PRG01f	850	0.912	0.703	0.297
7	PRG01g	640	0.981	0.862	0.138
8	PRG01h	460	1.000	1.000	0

Table 4.22. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer ILO 526 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	ILO526a	2250	0.830	0.412	0.588
2	ILO526b	1860	1.000	1.000	0
3	ILO526c	1210	1.000	1.000	0
4	ILO526d	1100	1.000	1.000	0
5	ILO526e	870	0.016	0.008	0.992
6	ILO526f	650	0.998	0.955	0.045
7	ILO526g	490	0.491	0.287	0.713
8	ILO526h	390	0.453	0.260	0.740
9	ILO526i	300	0.038	0.019	0.981

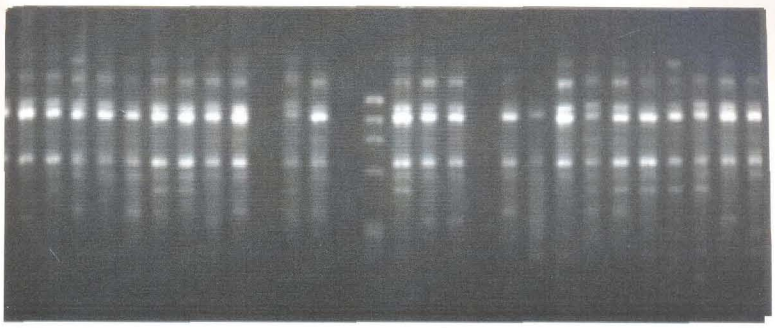

Fig 4.17 Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer ILO 526.
Line 1-14 & 16-30 DNA samples,
Line 15 PhiX 174 Hae III digest

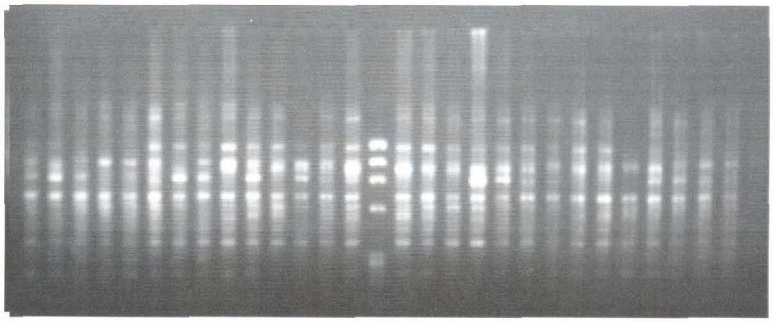

Fig 4.18. Finger prints of unrelated DNA samples of crossbred cattle with the RAPD primer ILO 876.
Line 1-14 & 16-30 DNA samples,
Line 15 PhiX 174 Hae III digest

Table 4.23. Name. approximate size of product. frequency and allelic frequencies of the bands produced by the primer ILO 876 on DNA samples of unrelated crossbred cattle of Kerala

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	ILO876a	2020	0.103	0.053	0.947
2	ILO876b	1700	0.705	0.457	0.543
3	ILO876c	1510	0.754	0.504	0.496
4	ILO876d	1320	0.718	0.469	0.531
5	ILO876e	1100	0.934	0.743	0.257
6	ILO876f	910	0.515	0.304	0.696
7	ILO876g	710	0.949	03774	0.225
8	ILO876h	620	0.282	03152	0.847
9	ILO876i	560	0.091	03046	0.953
10	ILO876j	460	0.091	03046	0.953
11	ILO876k	350	0.975	0.842	0.158
12	ILO876l	230	0.846	0.608	0.392

Table 4.24. Name, approximate size of product, frequency and allelic frequencies of the bands produced by the primer ILO 1127 on DNA samples of unrelated crossbred cattle of Kerala.

Sl No	Name of the band	Approximate size in base pairs	Frequency of the band	Frequency of the band allele	Frequency of the non band allele
1	ILO 1127a	3030	0.273	0.147	0.852
2	ILO 1127b	2620	0.491	0.287	0.713
3	ILO 1127c	2340	0.200	0.106	0.894
4	ILO 1127d	2130	0.546	0.326	0.674
5	ILO1127e	1950	0.873	0.644	0.356
6	ILO1127f	1850	0.055	0.028	0.972
7	ILO1127g	1480	0.891	0.670	0.330
8	ILO1127h	1220	0.400	0.225	0.775
9	ILO1127I	1040	0.273	0.147	0.853
10	ILO1127j	980	0.491	0.287	0.713
11	ILO1127k	940	0.220	0.117	0.883
12	ILO1127l	810	0.927	0.730	0.270
13	ILO1127 m	580	0.802	0.557	0.445
14	ILO1127n	510	0.236	0.126	0.874
15	ILO1127o	470	0.618	0.382	0.618
16	ILO1127p	340	0.836	0.595	0.401

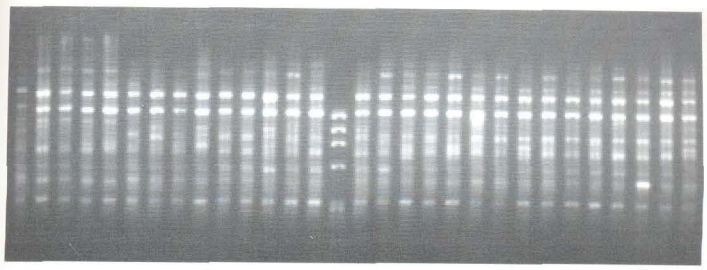

Fig 4.19. Finger prints of unrelated DNA samplesof crossbred cattle with the RAPD primer ILO 1127
Line 1-14 & 16-30 DNA samples,
Line 15 PhiX 174 Hae III digest

Fig 4.20. Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 1
Line 1-9, 11-19 & 21-27 DNA samples
Line 10 & 20 1 kb ladder

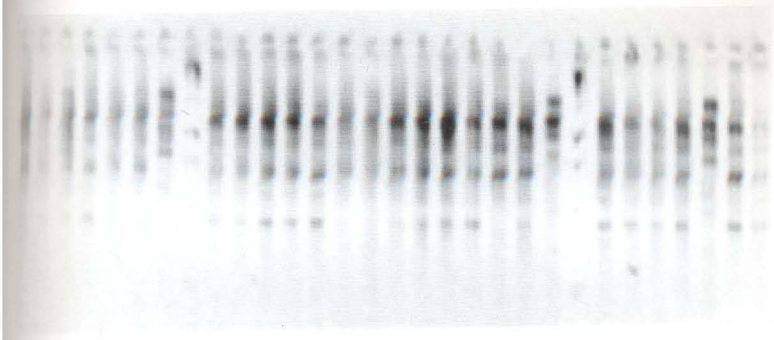

Fig.4.21 Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 4
Line 1-10, 12-20 & 22-30 DNA samples
Line 8& 23 1PhiX 174 Hae III digest marker.

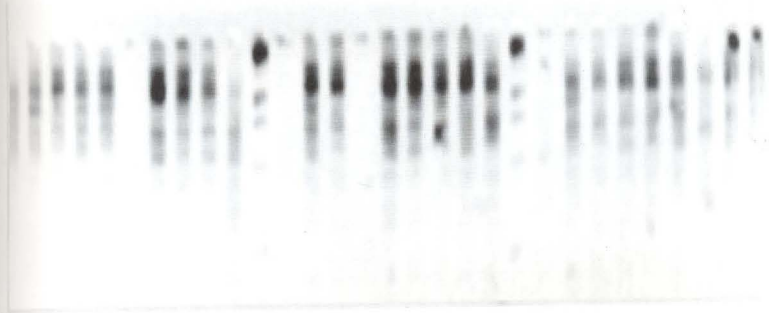

Fig.4.22 Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 4
Line 1-10, 12-20 & 22-30 DNA samples
Line 11& 21 1 kb ladder

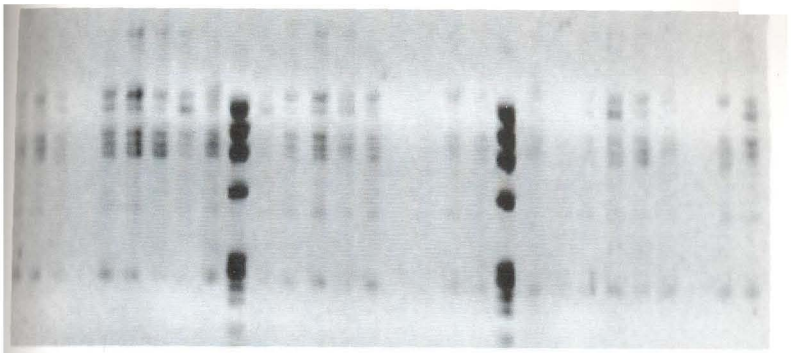

Fig 4.23 Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 7
Line 1-9, 11-19 & 21-27 DNA samples
Line 10 & 20 PhiX 174 Hae III digest

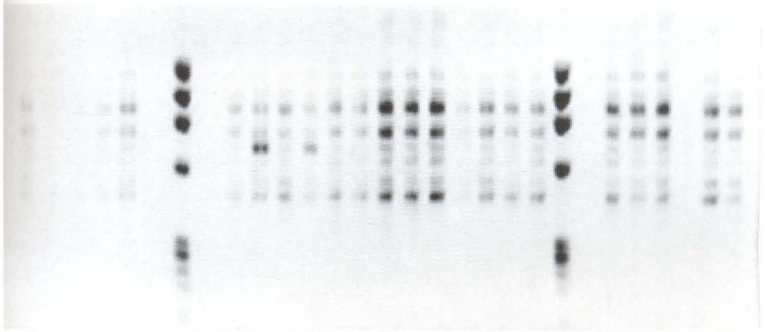

Fig 4.24 Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 9
Line 1-8, 10-24 & 25-30 DNA samples
Line 9 & 25 1 kb ladder

Fig 4.25. Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 14
Line 1-8, 10-18 & 20-30 DNA samples
Line 9 PhiX 174 Hae III digest & 19 1 kb ladder

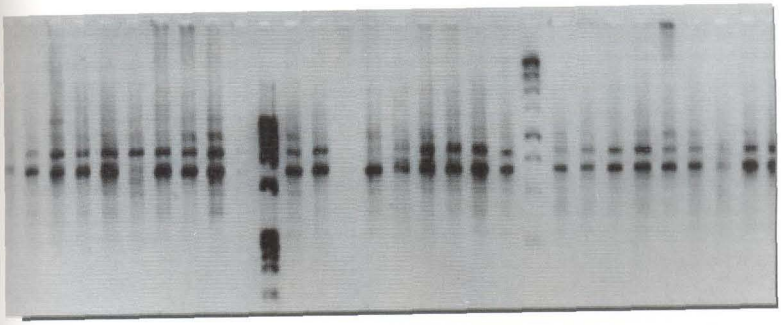

Fig 4.26. Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 15
Line 1-10, 12-20 & 22-30 DNA samples
Line 12 PhiX 174 Hae III digest & 11 1 kb ladder

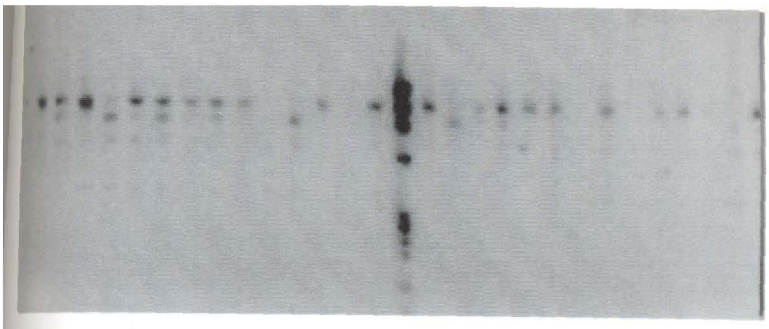

Fig.4.27. Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 18
Line 1-15 & 17-30 DNA samples
Line 16 PhiX 174 Hae III digest marker.

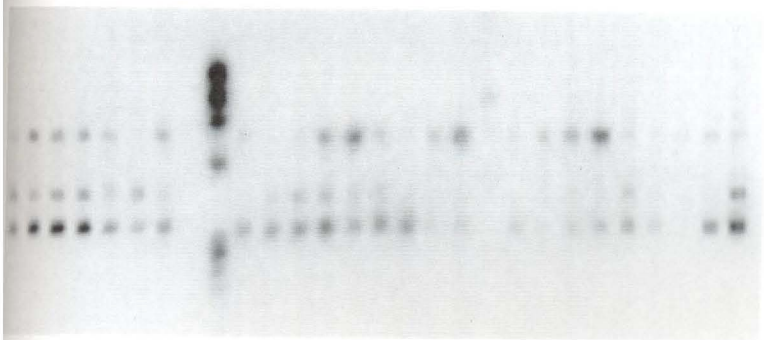

Fig.4.28. Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 19
Line 1-8, 10-24 & 25-30 DNA samples
Line 9 PhiX 174 Hae III digest marker.

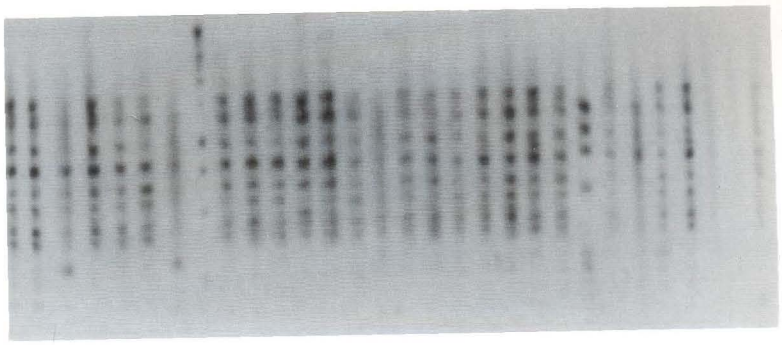

Fig.4.29. Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer OPA 20
Line 1-7 & 9-30 DNA samples
Line 8 PhiX 174 Hae III digest marker.

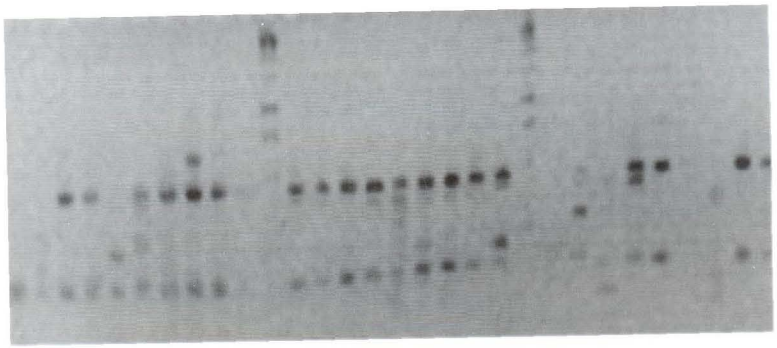

Fig.4.30.. Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer G 1
Line 1-11, 13-20 & 22-30 DNA samples
Line 12 & 22 1 Kb marker

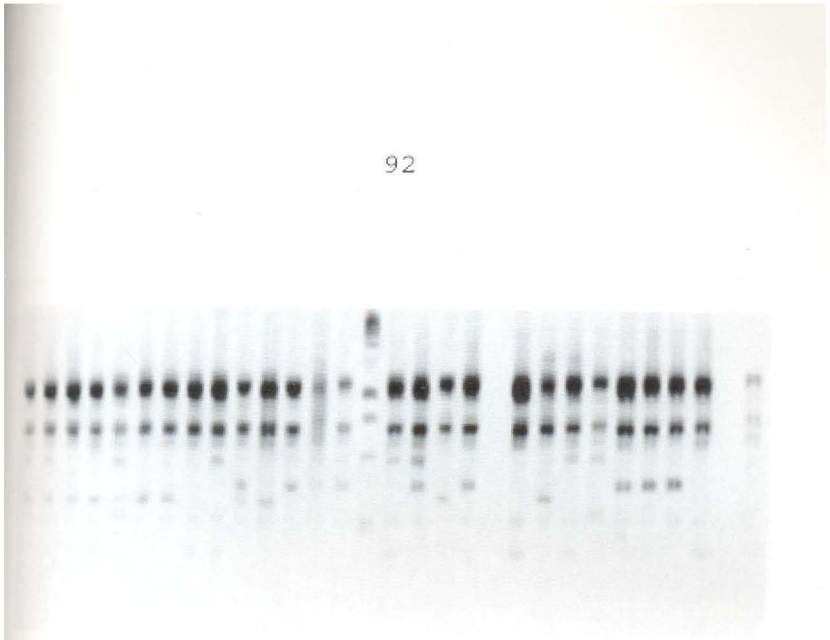

Fig.4.31. .Fingerprints of offspring, dam and sire DNA samples of crossbred cattle with the RAPD primer ILO 1127
Line 1-14 & 16-29 DNA samples
Line 15 1kb marker & 30 PhiX 174 Hae III digest marker.

Table 4.25. Primers selected for Offspring. Dam. Sire analysis with their sequence and average number of bands and percentage of non parental bands produced

Sl No	Name of the Primer	Primer sequence	Average number of bands produced	Percentage of Non parent bands
1	OPA 1	5'CAGGCCCTTC3'	5.89 ± 0.31	4.86
2	OPA 2	5'TGCCGAGCTG3'	5.72 ± 0.11	6.21
3	OPA 4	5'AATCGGGTTG3'	3.53 ± 0.12	0.00
4	OPA 7	5'GAACGGGCTG3'	4.65 ± 0.10	2.43
5	OPA 9	5'GGGTAACGCC3'	5.13 ± 0.11	11.03
6	OPA 14	5'TCTGTGCTGG3'	6.12 ± 0.15	0.00
7	OPA 15	5'TTCCGAACCC3'	4.18 ± 0.13	14.6
8	OPA 18	5'AGGTGACCGT3'	5.68 ± 0.31	2.00
9	OPA 19	5'CAAACGTCGG3'	6.03 ± 0.26	13.6
10	OPA 20	5'GTTGCGATCC3'	5.68 ± 0.21	4.89
11	PRG 1	5'GTGACGTAGG3'	6.62 ± 0.19	0.00
12	ILO 1127	5'CCGCGCCGGT3'	8.46 ± 0.17	6.20

Table 4.26. The PCR conditions fixed for Microsatellite analysis for DRB3 locus.

	Parameter / variable	Level
REACTION MIX		
1	Template DNA	25 ng
2	Forward primer concentration	5 p*M*
3	Reverse primer concentration	5 p*M*
4	dNTP concentration	200 μ*M*
5	Concentration of $MgCl_2$	1.5 m*M*
6	*Taq* DNA polymerase	0.5 unit
7	Reaction volume	10 μl
CYCLE PARAMETERS		
8	Denatuaration for PCR cycles	94°C for 1 minute
9	Primer annealing	60°C for 1 minute
10	Primer extension	72°C for 1 minute
11	Number of cycles	35
12	Final extension	72°C for 10 minutes

Table 4.27. The PCR conditions fixed for Microsatellite analysis for ETH 131 locus.

	Parameter / variable	Level
REACTION MIX		
1	Template DNA	25 ng
2	Forward primer concentration	5 p*M*
3	Reverse primer concentration	5 p*M*
4	dNTP concentration	200 µ*M*
5	Concentration of $MgCl_2$	1.5 m*M*
6	*Taq* DNA polymerase	0.5 unit
7	Reaction volume	10 µl
CYCLE PARAMETERS		
8	Denatuaration for PCR cycles	94°C for 1 minute
9	Primer annealing	55°C for 1 minute
10	Primer extension	72°C for 1 minute
11	Number of cycles	35
12	Final extension	72°C for 10 minutes

Table 4.28. The PCR conditions fixed for Microsatellite analysis for HEL-6 locus.

	Parameter / variable	Level
REACTION MIX		
1	Template DNA	25 ng
2	Forward primer concentration	5 p*M*
3	Reverse primer concentration	5 p*M*
4	dNTP concentration	200 µ*M*
5	Concentration of $MgCl_2$	1.5 m*M*
6	*Taq* DNA polymerase	0.5 unit
7	Reaction volume	10 µl
CYCLE PARAMETERS		
8	Denatuaration for PCR cycles	94°C for 1 minute
9	Primer annealing	60°C for 1 minute
10	Primer extension	72°C for 1 minute
11	Number of cycles	35
12	Final extension	72°C for 10 minutes

Table 4.29. The PCR conditions fixed for Microsatellite analysis for FSHβ locus.

	Parameter / variable	Level
REACTION MIX		
1	Template DNA	25 ng
2	Forward primer concentration	5 p*M* / sample
3	Reverse primer concentration	5 p*M* / sample
4	dNTP concentration	200 µ*M*/ sample
5	Concentration of $MgCl_2$	1.25 m*M* / sample
6	*Taq* DNA polymerase	0.5 unit / sample
7	Reaction volume	10 µl
CYCLE PARAMETERS		
8	Denatuaration for PCR cycles	94°C for 1 minute
9	Primer annealing	60°C for 1 minute
10	Primer extension	72°C for 1 minute
11	Number of cycles	35
12	Final extension	72°C for 10 minutes

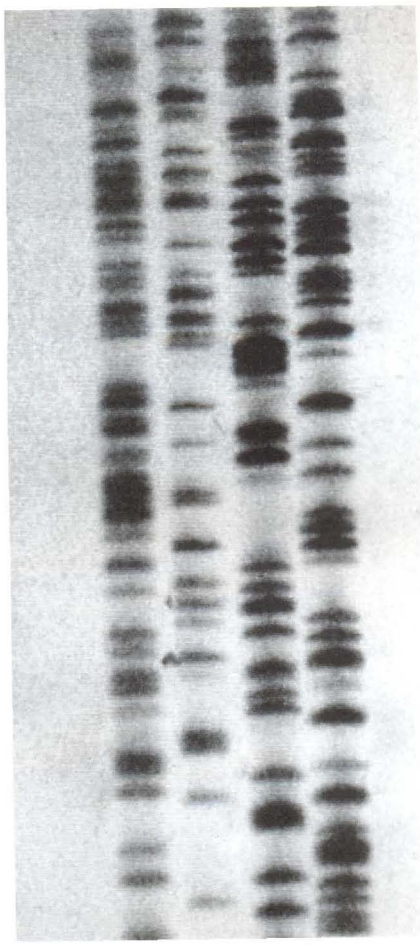

Fig 4.32. Autoradiograph of gel shoowing sequence of M13 phage using sequenase version 2.0,DNA sequencing kit.

Table 4.30. The size and frequencies of alleles of DRB3 in dwarf cattle of Kerala.

Sl No	Size in Basepairs	Allelic frequencies				Average
		Vechur	Highrange Dwarf	Vatakara	Kasargode	
1	176	0.167				0.031
2	178	0.083	0.040	0.050		0.039
3	180		0.040		0.028	0.023
4	184		0.060			0.023
5	186		0.040	0.100		0.031
6	188			0.180		0.023
7	190	0.167	0.140	0.100		0.100
8	192	0.292	0.160	0.050	0.028	0.131
9	194	0.042	0.120	0.100		0.069
10	196	0.125	0.060	0.200	0.083	0.100
11	198		0.060		0.011	0.054
12	200				0.011	0.031
13	202		0.080	0.050	0.056	0.054
14	204		0.040		0.278	0.092
15	206	0.083	0.040	0.050	0.139	0.077
16	208	0.042	0.080			0.038
17	210				0.056	0.015
18	212				0.056	0.015
19	218		0.040	0.100		0.031
20	224			0.050		0.015
21	236				0.056	0.015

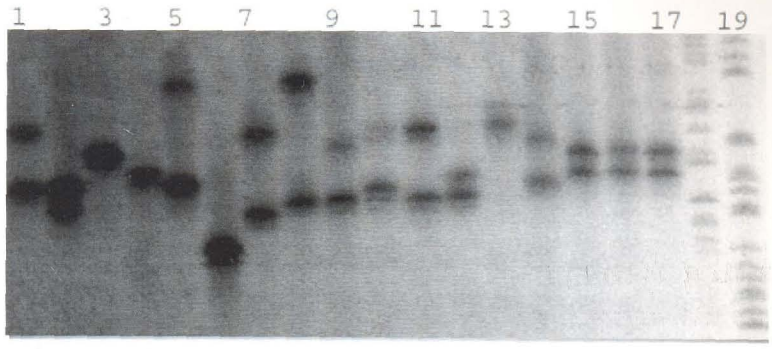

Fig.4.33. Autoradiograph of gel showing polymorphism of DRB3 locus in dwarf cattle of Kerala
Line 1-17 DNA samples
Line 18 & 19 G & A markers of M13 phage.

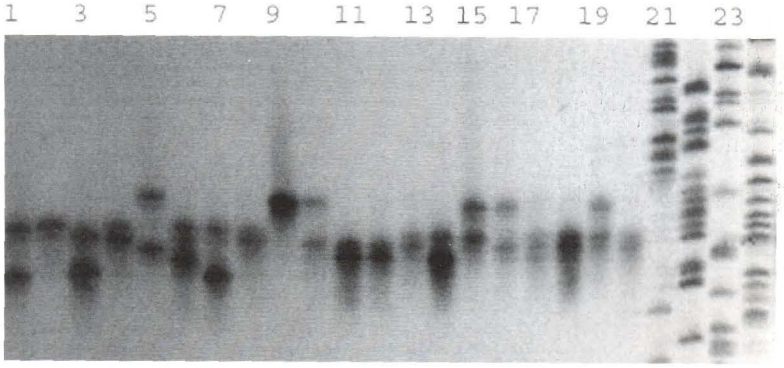

Fig 4.34. Autoradiograph of gel showing polymorphism of ETH-131 locus in dwarf cattle of Kerala
Line 1-20 DNA samples
Line 21-24 G , A,T & C markers of M13 phage.

Table 4.31. The size and frequencies of alleles of ETH 131 in dwarf cattle of Kerala.

Sl No	Size of the allele	Allelic frequencies				
		Vechur	Highrange Dwarf	Vatakara	Kasargode	Average
1	160			0.196		0.044
2	162			0.083		0.019
3	164				0.059	0.025
4	166		0.132		0.015	0.038
5	168		0.053	0.083	0.103	0.075
6	170	0.500	0.026	0.167	0.324	0.238
7	172		0.211	0.083	0.059	0.094
8	174	0.167	0.263	0.222	0.206	0.219
9	176	0.222			0.103	0.069
10	178		0.158	0.139		0.069
11	180	0.056	0.132		0.103	0.081
12	182		0.026			0.006
13	184	0.056	0.026	0.028	0.015	0.025

Table 4.32. The size and frequencies of alleles of HEL-6 in dwarf cattle of Kerala

Sl No	Size of the allele	Allelic frequencies			
		Vechur	Vatakara	Kasargode	Average
1	263		0.125		0.027
2	265	0.056			0.027
3	267	0.0139	0.1875		0.108
4	271	0.056		0.250	0.095
5	273		0.063	0.050	0.027
6	275	0.028	0.125		0.041
7	277	0.028	0.250		0.068
8	279	0.083	0.063		0.041
9	281	0.056	0.063	0.050	0.054
10	283	0.056			0.027
11	285	0.167	0.063	0.300	0.176
12	289	0.083	0.063		0.054
13	293	0.025		0.350	0.216
14	295	0.056			0.027

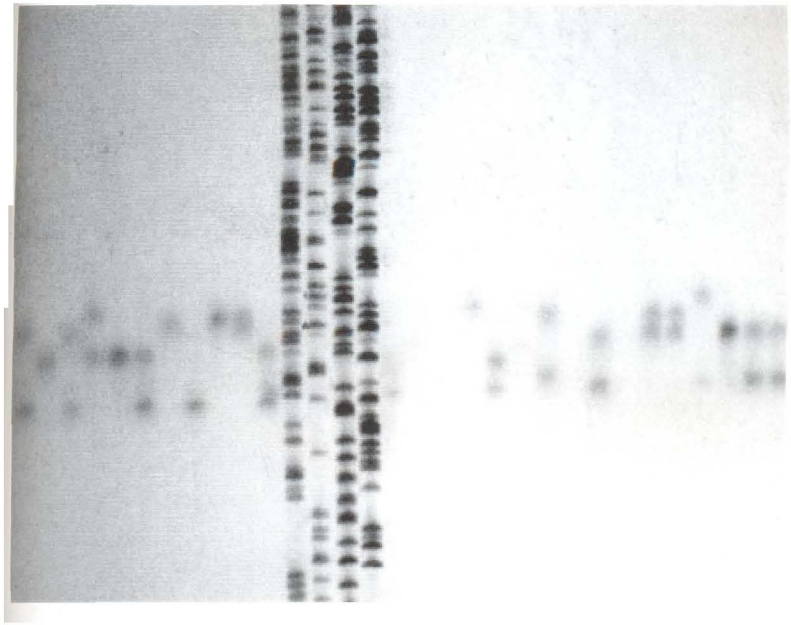

Fig.4.34. Autoradiograph of gel showing polymorphism of HEL-6 locus in dwarf cattle of Kerala
Line 1-11 & 16-30 DNA samples
Line 12-15 G,A,T & C markers of M13 phage.

Table.4.33. Depicting number of observations, alleles, unbiased heterozygosity, direct count heterozygosity and PIC values in Highrange dwarf cattle.

Sl No		DRB3 locus	ETH 131 Locus
1	Number of observations	50	38
2	Number of alleles	14	9
3	Size range of alleles (bp)	178-218	166-184
3	Unbiased heterozygosity	0.9257	0.8439
4	Direct count heterozygosity	0.9072	0.8219
5	PIC value	0.9071	0.8110

Table.4.34. Depicting number of observations, alleles, unbiased heterozygosity, direct count heterozygosity and PIC values in Vechur cattle.

Sl No		DRB3 locus	ETH 131 Locus	HEL 6 Locus
1	Number of observations	24	18	36
2	Number of alleles	8	5	12
3	Size range of alleles (bp)	176 - 208	170-184	265 - 295
3	Unbiased heterozygosity	0.8619	0.8217	0.8237
4	Direct count heterozygosity	0.8260	0.7058	0.7825
5	PIC value	0.8260	0.6496	0.9235

Table.4.35. Depicting number of observations, alleles, unbiased heterozygosity, direct count heterozygosity and PIC values in Vatakara cattle.

Sl No		DRB3 locus	ETH 131 Locus	HEL 6 Locus
1	Number of observations	20	36	16
2	Number of alleles	11	8	9
3	Size range of alleles (bp)	178 - 224	160-184	263 - 289
3	Unbiased heterozygosity	0.9212	0.8677	0.9247
4	Direct count heterozygosity	0.8751	0.8436	0.8669
5	PIC value	0.8750	0.8396	0.8640

Table.4.36. Depicting number of observations, alleles, unbiased heterozygosity, direct count heterozygosity and PIC values in Kasargode cattle.

Sl No		DRB3 locus	ETH 131 Locus	HEL 6 Locus
1	Number of observations	36	68	20
2	Number of alleles	11	9	5
3	Size range of alleles (bp)	186 - 236	164-184	271 - 293
3	Unbiased heterozygosity	0.9074	0.8439	0.8237
4	Direct count heterozygosity	0.8822	0.8217	0.7825
5	PIC value	0.8822	0.8089	0.7600

Table.4.37. Depicting number of observations, alleles, unbiased heterozygosity, direct count heterozygosity and PIC values in dwarf cattle of Kerala

Sl No		DRB3 locus	ETH 131 Locus	HEL 6 Locus
1	Number of observations	130	160	72
2	Number of alleles	21	13	14
3	Size range of alleles (bp)	176 - 136	160-184	263 - 295
3	Unbiased heterozygosity	0.9358	0.8652	0.8974
4	Direct count heterozygosity	0.9286	0.8599	0.8849
5	PIC value	0.9282	0.8568	0.8840

Discussion

5. DISCUSSION

The use of short arbitrary primers of eight or ten base length and a lower annealing temperature are the two most important features of Random Amplified Polymorphic DNA (RAPD) technique. The results of the attempt to delineate the RAPD polymorphic patterns are discussed.

5.1. RAPD-PCR

5.1.1. Yield and quality of DNA

The yield of DNA obtained from whole blood was more as compared to those obtained from frozen semen and fresh semen. An average yield of 256 µg ± 2.12 µg / 5 ml of blood was higher than those reported by Andersson *et al.* (1986) in cattle, Jeanpierrie (1987) in humans, Montgomery and Sise (1990) in sheep, Apparao *et al.* (1994) from different domestic animals, Senthil (1995) from cattle, Annapoorni (1996) from buffalo, Aravindakshan (1997) from buffalo, Aravindakshan *et al.* (1998a) from cattle, Nagaraja (1998) from cattle, Nagarajan (1998) from sheep, Saravanan (1999) from sheep and Appannavar (2001) from Deoni breed of cattle.

Isolation of DNA from semen samples was carried out after washing with PBS to remove the seminal plasma. Average yield of DNA obtained from semen was 91.32 ± 6.01 µg/ 0.25 ml frozen straw and 241.23 ± 8.42 µg/ 4 X 10^5 sperms of chilled semen is comparable with that obtained by Bahnak *et al.*, 1988 (200 µg / 10^8 sperm cells), Lien *et al.*, 1990 (300-500µg / 2.5 X 10^8 sperm cells) and Aravindakshan *et al.*, 1998 (441.85µg/ 3 X 10^8 sperm cells).

The OD ratio which is given by OD_{260} / OD_{280} was taken as the indicator of quality of DNA. The ratio obtained in this study was between 1.61 ± 0.34 and 1.81

± 0.26 for frozen semen and fresh semen respectively. This is in agreement with the values obtained by Montgomery and Sise (1990), Aravindakshan (1997), Miller *et al.* (1998), Aravindakshan *et al.* (1998b), Nagarajan (1998) and Appannavar (2001).

5.3 UNRELATED CROSSBRED CATTLE

5.3.1 Primers

The Primers chosen for RAPD-PCR were of 10 base length in this study. Because of the short size of the primers, the specificity was also low. Slight alterations in the PCR conditions can affect the products of the reaction as they depend on the sequence and length of oligonucleotide primers and reaction conditions.

In the present study, 26 random oligonucleotide primers of 10 bp length were used for a preliminary test and out of that 22 primers yielded different sized products in RAPD-PCR. Among these 22 primers, 20 primers were chosen for depiction of RAPD polymorphisms of unrelated crossbred cattle. According to Bowditch *et al.* (1993), mutations or other factors influencing primer annealing can be detected by the absence of amplified products or presence of new products.

Kemp and Teale (1994) also used 26 random 10 base oligonucleotide primers in pooled samples for identification of *Bos indicus* cattle. Aravindakshan and Nainar (1998) used three primers in individual analysis of cattle and buffaloes and Ganai *et al.* (2000) used 4 RAPD primers to fingerprint buffaloes. Ramesha *et al.* (2002) evaluated seven primers in individual samples for detecting genetic variation in cattle breeds. Yeo *et al.* (2002) used 60 primers in their study on native identification of Hanwoo cattle of Korea.

5.3.1.1. Primer OPA 1

The primer OPA 1 had four bands with frequency less than 0.25. This is sufficient to use it extensively for studies on genetic polymorphisms. Three bands produced by the primer were having frequency more than 0.5 and hence are of limited use in genetic studies. There were three bands with a frequency of 1 which were of no use in the studies of genetic polymorphism.

5.3.1.2. Primer OPA 2

The primer OPA 2 did not produce any common band. The presence of five bands with frequency less than 0.25 indicates its usefulness in the analysis of genetic polymorphism. The other six bands were having frequency more than 0.5 and are of limited use.

5.3.1.3. Primer OPA 4

Of the twelve bands produced by the primer OPA 4 identified four bands had frequency less than 0.25, and six bands were with frequency between 0.25 and 0.5. This makes OPA 4 worthy in the assessment of genetic polymorphism.

5.3.1.4. Primer OPA 7

The number of bands produced by the primer OPA 7 is less compared to many other primers. It also produced three bands with frequency between 0.5 and 1 and one band with frequency 1. The usefulness of the primer for polymorphic studies is less compared to many other primers.

5.3.1.5. Primer OPA8

Four bands of the primer OPA 8 were having frequency less than 0.25, qualifying the primer for genetic studies. Two other bands were with frequency between 0.25 and 0.5, which were also useful for the purpose. Of the other six bands one was common for all the animals, which is of no use in such studies. The other five bands had frequencies between 0.5 and 1 making them less useful in polymorphic studies.

5.3.1.6. Primer OPA 9

The primer OPA 9 had four bands with less than 0.25 frequency and one band with frequency between 0.25 and 0.5. These make the primer useful in genetic polymorphism studies. Other five bands were with frequency more than 0.5 and are of little use in genetic studies.

5.3.1.7. Primer OPA 10

Primer OPA 10 produced only one band with frequency less than 0.25 hence, the primer is of limited use in genetic polymorphism studies.

5.3.1.8. Primer OPA 12

The primer OPA12 produced a total number of 9 bands of which only one band had a frequency less than 0.25 and another band with frequency between 0.25 and 0.5. The other seven bands produced were with frequency more than 0.5. Since the primer generated a very low number of rare bands, it is of limited use in genetic polymorphism studies.

5.3.1.9. Primer OPA 14

OPA 14 produced three bands with frequency less than 0.25 and another four with frequency between 0.25 and 0.5 which make it useful in genetic polymorphism studies. Bands with frequency between 0.5 and 1 is of limited use and band with frequency 1 is of no use in such studies.

5.3.1.10. Primer OPA 15

Maximum number of bands (seven) with frequency less than 0.25 was observed with the primer OPA 15. Another two bands were present with frequency between 0.25 and 0.5. Hence it is suggested that the primer OPA 15 is one of the best suited RAPD primer for genetic polymorphism studies in crossbred cattle of Kerala.

5.3.1.11. Primer OPA 16

The primer OPA 16 was also highly polymorphic with four bands of frequency less than 0.25 and three bands of frequency between 0.25 and 0.5. Which qualify for genetic polymorphism studies in crossbred cattle.

5.3.1.12. Primer OPA 17

OPA 17 was also fairly polymorphic with three bands of frequencies less than 0.25 and two bands of frequencies between 0.25 and 0.5. The other four bands with frequencies between 0.5 and 1 are of limited used in genetic studies.

5.3.1.13. Primer OPA 18

The primer OPA 18 produced the lowest number of bands. One band was with frequency less than 0.25 and two bands were with frequency between 0.25 and 0.5. Hence the use of the primer for analysis of genetic polymorphism is limited in crossbred cattle of the state.

5.3.1.14. Primer OPA 19

Of the 13 bands produced by the primer OPA 19 five were with frequency less than 0.25 and one with frequency between 0.25 and 0.5. This makes the primer suitable for genetic polymorphism studies.

5.3.1.15. Primer OPA 20

The primer OPA 20 was having one band with frequency less than 0.25 and another band with frequency between 0.25 and 0.5. Hence its usefulness for genetic analysis is limited.

5.3.1.16. Primer G1

Of the eight bands generated by the primer G1, only one band was found with a frequency less than 0.5. No rare bands (< 0.25 frequency) could be detected. Overall assessment suggests that this primer is of no use in genetic polymorphism analysis.

5.3.1.17. Primer ILO 1127.

The number of bands produced in this study with the primer ILO 1127 was three to nine against those reported by Aravindakshan and Nainar in 1998 (3 to 10)

and Ramesha *et al.* in 2002 (7 to 9). Two bands with frequencies less than 0.25 and two bands with frequencies between 0.25 and 0.5 were identified with this primer. Three common bands and two bands with frequencies between 0.5 and 1 were also detected with the primer. Overall observations suggest that this primer is of limited use in genetic studies.

Gwakisa *et al* (1994) found that two RAPD primers ILO 1127 and ILO 526 could differentiate three zebu cattle breeds of Tanzania. With ILO 1127 Ramesha *et al* (2002) observed breed specific bands for Malanad Gidda and Amritmahal breed. The genetic make up of the crossbred may be the reason for not finding such specific bands in this study.

5.3.1.18. Primer ILO 876

Of the twelve bands identified with the primer eight were having frequency between 0.5 and 1 making them less suitable for genetic studies. The other three bands with frequency less than 0.25 and one with frequency between 0.25 and 0.5 are useful for genetic analysis of crossbred cattle. Ramesha *et al.* (2002) was able to obtain a 0.95 kb amplified product in South Indian cattle breeds (*Bos indicus*) with the primer ILO 876 which was similar to one reported in African zebu breeds. Similar findings were also reported by Nagaraja (1998). But such a band could not be identified in the crossbred cattle of the state and it may be due to the genetic make up of the crossbred cattle of the state.

5.3.1.19 Primer ILO 526.

The primr ILO 526 produced 16 bands, which was found to be the maximum number of bands generated in this study. Four bands with frequency less than 0.25 and another five with frequency between 0.25 and 0.5 make it suitable for genetic analysis of crossbred cattle of the state.

Presence of genetic group specific bands with the primer ILO 527 was not noticed in this study. But Ramesha *et al.* (2002) observed moderate to high levels of polymorphism with ILO 526 in seven South Indian Cattle breeds. A specific product observed in Krishna Vally and Ongole breeds but absent in Malanad Gidda, Amritmahal, Hallikar, Deoni and Khillari was identified. Gwakisa *et al.* (1994) were unable to identify breed specific bands with ILO 526 in zebu cattle breeds of Tanzania.

5.4.OFFSPRING, DAM AND SIRE COMBINATIONS

Twelve different RAPD primers were selected based on polymorphism and intensity of bands to analyse the offspring, dam and sire combinations. Presence of non parental bands were observed in 9 of these primers. The primers OPA 4, OPA 14 and G1 did not produce any non parental bands. The % of non parental bands ranged between 2 and 14.6.

Studies on application of RAPD- PCR technique in parentage determination are limited. Riedy *et al.* (1992) reported high frequency of non parental bands in primates. They could identify on an average percentage frequency of non parental bands as 4.4 in baboons and 2.7 in humans.

Thus the presence of non parent bands in offspring of the present limit the use of RAPD-PCR technique for parentage verification.

5.5.MICROSATELLITE ANALYSIS

The microsatellite markers are highly polymorphic, relatively abundant and are amenable to PCR assay and hence have a significant advantage over other molecular markers. The increasingly important role played by microsatellite

markers such as $(dC\text{-}dA)_n$, $(dG\text{-}dT)_n$ are stressed by different researchers (Weber and May, 1989; Love *et al.* 1990; Moore *et al.* 1991.).

Moore *et al* (1992) listed a number of primer sequences and annealing temperatures of different microsatellites. The location of these loci was also described by Fries *et al.* (1995).

5.5.1. Primers

5.5.1.1. DRB3

DRB3 locus in the present study was found to have 21 alleles of size ranging from 176 to 236 base pairs. Ellegren *et al.* (1993) found that DRB3 locus had alleles between the sizes of 150 to 200 base pairs while Usha (1995) reported the presence of 23 different alleles varying in size from 144 to 220 base pairs. The size of the alleles in the present study differed from that of earlier reports. Presence of new alleles in *Bos indicus* cattle can be the reason for this.

Polymorphic Information Content (PIC) value of the locus has been found to be 0.928 ,which was in agreement with the findings of Usha (1995). Among the various microsat4ellite loci analysed, highest PIC value (0.928) was also observed for DRB3 locus which agrees with the observations (PIC value 0.93 for DRB3 locus) reported by Usha (1995).

5.5.1.2. ETH 131

The alleleic size of the ETH 131 locus in this study has been found to lie between 160 and 184 with 13 alleles. Mullis and Fries (1992) could obtain 8 alleles in ETH 131 locus with the size range of 141 to 163 base pairs in *Bos taurus* breeds. Stephen *et al.* (1993) depicted the loci of ETH 131 as D21S4. He could obtain 8

different amplified products for the locus in *Bos taurus* cattle. The allelic size ranged from 150 to 200 base pairs. Usha (1995) found 25 alleles with a size range of 137-171 base pairs. The increase in size range and number of alleles could be due to the fact that dwarf cattle of Kerala are *Bos indicus* type and where the possibility of new alleles was more.

5.5.1.3. HEL 6

Hel 6 locus was found to have 14 alleles of the size range 263-295 base pairs. Kaukinen and Varvio (1993) reported the allelic size between 260 and 290 base pairs. Usha (1995) obtained 19 alleles in the *Bos taurus* population with a size range of 252 to 276 base pairs.

5.6.STASTISTICAL ANALYSIS

Direct count heterozygosity, unbiased heterozygosity and PIC values were found out in this study to measure the genetic polymorphism of different genetic groups of dwarf cattle of Kerala.The values obtained for different genetic groups of the dwarf cattle would be helpful for the future application of the technique in genetic studies. Analysis of microsaterllite loci of the dwarf cattle can be applied in different ways.

The allelic frequencies at four different loci depicted in this study could be used for the phylogeny analysis of these genetic groups of animals. The contribution of different indigenous breeds in the development of Highrange dwarf, Vatakara, Kasargode and Vechur cattle can be studied with the help of this information.

Though other markers and techniques are available for genetic characterization of the dwarf cattle of Kerala the use of microsatellite markers are

advantageous since it is more sensitive and highly polymorphic. Relatively small allele size, reproducibility and the possibility of automation make them suitable for the work compared with other molecular markers.

The microsatellites are accepted as the most suitable molecular marker for parentage verification. From the data on allele frequencies, heterozygosity and PIC values the probabilities of exclusion of random sire could be calculated (Usha *et al.* 1995). The technique can be made use in establishing a parentage verification facility. Hagelberg *et al* (1991) opined that because of detection of hypervariable sequences, the PCR based microsatellite typing can be used as a powerful tool for identification or paternity testing. Selected microsatellite loci, the multiplex PCR system and electrophoresis form extremely powerful tools for parentage testing (Heyen *et al.* 1997, Peelman *et al.* 1998; Luikat *et al.* 1999).

Summary

6.SUMMARY

Three panels of DNA samples were prepared for the study. The first panel contained samples from 84 unrelated crossbred cattle, the second panel included 52 combinations of Offspring-dam- sire and the third panel contained samples from 108 dwarf cattle of Kerala belonging to four genotypes namely Vechur, Highrange dwarf, Vatakara and Kasargode.

The average yields of DNA obtained from whole blood was 256 µg / 5 ml, from fresh semen was 241.23 µg/ 400 million sperms and from frozen semen was 91.32 µg/ 0.25 ml semen straw. The ratio of optical density at 260 nm and 280 nm were 1.72 ± 0.14, 1.81 ± 0.26 and 1.61 ± 0.34 for the DNA obtained from whole blood, frozen semen and fresh semen respectively.

6.1.RAPD PCR STUDIES

The conditions for RAPD-PCR amplification were standardized. A total volume of 10 µl reaction contained 25 ng template DNA, 1 µl 10X PCR buffer, 0.5 unit of Taq DNA polymerase and 200 µ*M* each of the four dNTP.

Nineteen random oligonucleotide primers were selected from among 26 primers based on intensity, clarity and polymorphism of bands for amplification of DNA samples. Twelve primers were used for offspring, dam and sire analysis.

Number of bands obtained for different primers ranged from 2 to 16. The average number of bands produced by different primers ranged from 3.78 ± 0.15 in OPA 4 to 8.15 ± 0.25 in ILO1127. The size of amplification products ranged from ~230 base pairs to ~3580 base pairs. Percentage of polymorphism represented by individual primers varied from 66.66 to 100 in different groups. Seven primers namely OPA2, OPA 4, OPA 17, OPA18, OPA 20, ILO 1127 and ILO 876 yielded 100% polymorphic bands. Frequencies of the bands and their allelic frequencies were worked out.

The primer ILO 1127 yielded on an average 8.15 ± 0.25 bands which was followed by ILO 876 with 6.81 ± 0.16 bands. The lowest number of bands was observed for the primer OPA 4 (3.78 ± 0.15).

Three common bands obtained for primers were OPA1 and ILO 526. Two common bands were observed for OPA 12 and one common band was seen in the primers G1,OPA 7, OPA 8, OPA 9, OPA 10, OPA14, OPA 15, OPA 16 and OPA 19.

The bands having frequency less than 0.25 were classified as rare bands. One rare band each was observed with the primers OPA 10, OPA 12, OPA 18 and OPA 20, two rare bands were observed in ILO 526, three rare bands were observed with primers OPA7, OPA 14, OPA 17 and ILO 876, four rare bands for OPA1, OPA4, OPA 8, OPA 9,OPA 16 and ILO 1127 and five rare bands for the primer OPA 19 and seven rare bands for the primer OPA 15.

Amplification of DNA with the primer OPA 1 revealed 10 products. The approximate size of products ranged from ~290 to ~2240 base pairs. The primer OPA 2 yielded 11 products with a size range of ~270 to ~2340 base pairs.

Twelve products of the size range from ~ 280 base pairs to ~ 2240 base pairs were observed for OPA 4. In OPA 7, eight polymorphic bands of size from ~290 base pairs to 2350 base pairs were observed. Twelve bands of size from ~330 base pairs to ~2310 base pairs were observed for the primer OPA 8.

The primer OPA 9 yielded nine polymorphic bands where as there were eight bands for the primer OPA 10. The sizes of these bands were from ~400 base pairs to 2100 base pairs and ~340 base pairs to 2590 base pairs for the primers Opa 9 and OPA 10 respectively.

OPA 12 produced 12 bands with a narrow size range (~390 to 1490 base pairs). Of the twelve bands identified for OPA 14, the smallest product had the size of ~300 base pairs and the largest had the size of ~2400 base pairs. Thirteen bands with a size range of ~ 230 base pairs to ~2660 base pairs were observed for the primer OPA 15.

The largest band of the size ~3580 was observed for the primer OPA 16. Of the eleven bands, four were having size more than 2000 base pairs. Nine products of the primer OPA 17 showed a size range from ~280 base pairs to 2740 base pairs.

The primer OPA 18 had the smallest number of products (7) from ~450 to 1840 base pairs. Thirteen bands (~310 to ~2090 base pairs) were observed by amplification with OPA 19 and eight bands (~500 base pairs to 2260 base pairs) were observed for the primer OPA 20.

Products size ranged from ~460 base pairs to 2270 base pairs for the primer G1 and nine bands of the size from ~300 base pairs to 2250 base pairs were observed for the primer ILO 526.

The primer ILO 876 produced 12 bands with a size range from ~230 base pairs to ~2020 base pairs. Primer ILO 1127 produced maximum number of bands (16) in the present study. The product size ranged from ~340 base pairs to ~3030 base pairs.

Twelve RAPD primers were used to study the offspring, dam and sire combinations. Three of these primers namely OPA 14, G1 and ILO 526 showed only parental bands in offspring. Other primers produced non parental bands and the percentage of non parental bands in offspring ranged from 2 to 13.6.

It was concluded that RAPD-PCR technique cannot be the method of choice for parentage verification because of the presence of non parental bands in offspring.

6.2.MICROSATELLITE ANALYSIS

DNA samples were isolated from whole blood of 24 Vechur, 25 Highrange dwarf, 24 Vatakara and 35 Kasargode animals using phenol: chloroform extraction procedure. The average yield of DNA obtained per 5 ml of blood samples was 210.1 ± 9.4 µg with the ratio of optical density 1.86 ± 0.23.

The procedure for end labelling of forward primers with $\gamma^{32}P$ ATP was standardized. . The reaction was carried out in a total volume of 10µl, containing 1µl of 10 X PNK buffer, 1 µl, 200 p*M* primer, 0.5µl of PNK, 1µl 10 mCi of $\gamma^{32}P$ dATP and 6.5µl water.

PCR conditions for the four microsatellite loci namely DRB3, ETH 131, HEL 6 and FSHβ were standardised. For the microsatellite locus DRB3, template DNA used was 25 ng, with 1.5 m*M* $MgCl_2$ and 5 p*M* each of both forward and reverse primers in 1X buffer. 35 PCR cycles of denaturation at 94°C for one minute, annealing at 60oC for one minute, extension at 72°C for one minute with a final extension of 72°C for ten minutes were used in the study.

The concentration of $MgCl_2$ was fixed at 1.25 mM and the primer annealing of the PCR cycles was done at 55°C for one minute for the locus ETH 131. Other conditions were same as that of the locus DRB3.

For HEL6 and FSH*β*, the PCR conditions used were same as that of DRB3 locus except for the concentration of $MgCl_2$. which was fixed at 1.0 m*M* for HEL6 and 1.25 m*M* for FSHβ.

Sequencing of M 13 phage was done using sequenase version 2.0 sequencing kit as per manufacturer's instructions. The sequenced phage was used as molecular size marker for microsatellite studies.

Twenty one different alleles having size range from 176bp to 236 bp were identified for DRB3 locus. The PIC value of the primer was highest among the four loci compared.

Number of alleles identified for ETH 131 locus was 13 with a size range of 160 bp to 184 bp. The direct count heterozygosity and PIC value of the primer was calculated as 0.8599 and 0.8568 respectively.

Fourteen alleles with a size range of 263 to 295 base pairs were identified in HEL-6 locus. The PIC value of the primer was 0.884 and unbiased heterozygosity and direct count heterozygosity were 0.8974 and 0.8849 respectively.

Number of alleles of the DRB3 locus and ETH 131 locus in Highrange dwarf cattle were 14 and 9 respectively. The values of unbiased heterozygosity, direct count heterozygosity and Polymorphic Information Content were 0.9257, 0.9052 and 0.9071 for DRB3 locus and 0.8439, 0.8219 and 0.8110 respectively for ETH 131 locus.

Eight alleles were found in DRB3 locus of Vechur cattle with size ranging from 176 bp to 208 bp. Number of alleles for ETH locus observed in the breed was five with the size range 170 to 184 base pairs and that observed in HEL 6 locus was 12 of the size range from 265 to 295. PIC values for DRB3, HEL 6 and ETH 131 loci were 0.826, 0.9235 and 0.6496 respectively. The direct count heterozygosity and unbiased heterozygosity were 0.8260 and 0.8619 for DRB3 locus, 0.7058 and 0.8217 for ETH 131 locus and 0.7825 and 0.8237 for HEL 6 locus respectively.

Number of alleles identified and the size range of the three microsatellite loci of Vatakara cattle namely DRB3, ETH 131 and HEL 6 were eleven (178 bp-224 bp), eight (160 bp-184 bp) and nine (263 bp-289 bp) respectively. The values of direct count heterozygosity, unbiased heterozygosity and PIC value were worked out for the different loci.

The number of alleles for DRB3 locus in Kasargode cattle was 11. The size of the alleles ranged from 186 to 236 bp. For ETH 131 locus nine alleles of size from 164 to 184 base pairs and for Hel 6 loci five alleles with a size range of 271 to 293 base pairs were identified. The PIC values of the DRB3, ETH 131 and HEL 6 in Kasargode cattle were 0.8822, 0.8099 and 0.7600. The direct count heterozygosity worked out for the three loci were 0.8822, 0.8217 and 0.7825 where as the unbiased heterozygosity was 0.9074, 0.8439 and 0.8237 respectively.

Microsatellite analysis can be used for genetic characterization of dwarf cattle of the state and phylogenetic studies. Parentage verification facility can be established under the university where this technique can be employed in disputed cases.

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*Originals not seen

Appendix

APPENDIX

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

Acrylamide solution (40%)

Acrylamide	380 g
Bis Acrylamide	2 g

Dissolved the above ingredients in 1 litre of distilled water. Since the chemicals are neurotoxic and harmful gloves were used with utmost care in preparation of the solution

Agarose 2%

3g of agarose powder was weighed and put in a conical flask. To this was added 200 ml of 1X TAE buffer. The solution was then put in microwave oven for 3 minutes and the agarose 2% solution is ready.

EDTA (0.5 M , pH 8.3)

Dissolved 18.61 g of EDTA (disodium di hydrate) in 80 ml of distilled water. The pH was then adjusted to 8.3 using sodium hydroxide solution. The volume was made up to 100 ml and stored at room temperature after filtration and autoclaving.

Ethidium Bromide (10 mg/ml)

100 mg of ethidium bromide power was dissolved in 10ml of distilled water. The solution is stored at 4 °C in dark coloured bottle.

Gel Loading Buffer (6X)

Brom phenol blue	0.25 %	50 mg
Xylene cyanol	0.25%	50mg
Sucrose	40%	8 g

Stirred the components well in 20 ml of distilled water and stored at 4 °C.

Formamide dye

Formamide 95%

1X TBE

Xylene cyanole, 0.01%

Bromphenol Blue 0.01%

Phenol (Satuarated, pH 7.8)

Phenol crystal are first melted at 68 °C. To 250 ml the melted phenol 0.25 g hydroxy quinoline was added and then 25 ml of Tris hydrochloride (pH 8.0) was also added. The mixture was stirred on a magnetic stirrer for 30 minutes. The contents were then transferred to a separating funnel. When the two phases had separated, the lower phenolic phase was collected. Equal volume of 0.1*M* tris (ph 8.0) was added to the phenol and using magnetic stirrer the mixture was mixed for 30 minutes. Phenolic phase was again collected using a separating funnel and pH was checked. If it is less than 7.8 the extraction with tris hydrochloride was continued. Once the pH is above 7.8 the phenol was transferred to a dark coloured bottle and 10% volume of 0.01 *M* tris hydrochloride was added and stored at 4 °C.

Phosphate Buffered Saline (PBS, pH 7.4)

Sodium Chloride (NaCl)	138 m*M*	8.0647 g
Sodium Dihydrogen phosphate(NaH_2PO_4 2 H_2O)	4 m*M*	0.6240 g
Di sodium hydrogen Phosphate (Na_2H PO_4 12 H_2O)	6 m*M*	2.1488 g

All the chemicals listed above were accurately weighed and added to the beaker containing 800 ml distilled water. The solution was stirred using magnetic stirrer and the pH was adjusted to 7.4 using diluted hydrochloric acid. The volume was then made up to 1000ml.

RBC lysis solution

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

All the chemicals listed above were accurately weighed and added to the beaker containing 1000 ml distilled water. The solution was stirred, filtered, autoclaved and stored at 4 °C.

Sodium acetate (3*M*, pH 5.5)

40.824 g of sodium acetate was dissolved in 70 ml of distilled water and the pH was adjusted to 5.5 using glacial acetic acid. The volume was made upto 100 ml, autoclaved and stored at 4 °C.

Sodium Chloride (5*M*)

29.22 g odium chloride was dissolved in 80 ml distilled water. The volume of the solution was made up to 100ml. The solution was filtered and stored at room temperature.

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

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

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

Sodium dodecyl sulphate (SDS) 20%

20g Sodium dodecyl sulphate was taken on a beaker, and volume was made up to 100 ml with distilled water. The solution was stirred in a magnetic stirrer, filtered and stored at room temperature.

Sperm lysis buffer

	Stock solution	For making 100 ml	Final concentration
0.5% SDS	10%	5 ml	0.5 5
Tris (pH 8.0)	1M	1 ml	10m*M*
2mercaptoethanol	100%	2 ml	2%
EDTA (pH 8.0)	0.5M	2 ml	10m*M*
Sodium chloride	5M	2 ml	10m*M*

The volume of the solution was made up to 98 ml with distilled water. Just before use heated up to 65 °C and mercaptoethanol was added.

TAE buffer (50 X)

Tris base	48.4 g
Glacial Acetic acid	11.42 ml
0.5 *M* EDTA	20 ml

All the chemicals were dissolved in 800 ml of distilled wataer. The volume was made up to 1000 ml with distilled water. Autoclaved and stored at room temperature.

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

Tris base	108 g
Boric acid	55 g
EDTA	9.3g

The chemicals were dissolved in 700 ml of distilled water and the volume was made up to 1000 ml with distilled water after adjusting the pH to 8.3. Autoclaved and stored at room temperature.

TBE Gelmix (0.5X)

40% Acrylamide solution	150 ml
10 X TBE buffer	50 ml
Urea	450 g

The urea was weighed out in a beaker. Acrylamide solution and TBE buffer were added in to it. The volume of the solution was made up to 1000 ml. The beaker was then kept in magnetic stirrer for complete dissolving of urea. It was then stored in the refrigerator.

Tris Buffered Saline (TBS) pH 7.4

Sodium Chloride	140m*M*	8.18 g
Potassium Chloride	0.50m*M*	0.0373 g
Tris base	0.25m*M*	0.0303 g

Dissolved the chemicals in 900 ml distilled water. The pH was then adjusted to 7.4. Volume made up to 100ml, autoclaved and stored at room temperature.

Tris EDTA (TE) buffer pH 8.0

Tris base	10m*M*	0.3029 g
EDTA	0.1m*M*	0.0073 g

Dissolved the chemicals in 200 ml of distilled water. The volume was made up to 250 ml after adjusting the pH to 8.0. Filtered, autoclaved and stored at 4 °C.

Tris 1*M*

Dissolved 121.14 g tris base in one liter of distilled water. Filtered and stored in bottles at room temperature.

ANNEXURE - II

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

A. CHEMICALS

Agarose (Low EED)	-	Bangalore Genei Pvt. Ltd.
6 X gel loading buffer	-	Bangalore Genei Pvt. Ltd
Ammonium chloride	-	Sisco Research Laboratories Pvt.Ltd. (SRL), Bombay
Crystalline phenol	-	Merk
EDTA	-	SRL, Bombay
Ethanol	-	Merk
Ethidum bromide	-	BDH lab, Engaland
Glacial acetic acid	-	BDH-E, Merk (India) Ltd.
Hydroxy quinolone	-	Qualigens Chemicals Mumbai
Methanol	-	SRL Bombay
Potassium chloride	-	SRL Bombay
Sodium acetate	-	SRL Bombay
Sodium chloride	-	SRL Bombay
Sodium S	-	SRL Bombay
Tris base	-	SRL Bombay

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B. PRIMERS

Life Technologies India Pvt. Ltd.

Operon Technologies Inc., USA

C. Molecular Markers

ϕ X 174 RF DNA/*Hae III* digest	-	Bangalore Genei Pvt. Ltd.
(11 fragments of 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 base pairs)		
1 kb ladder DNA	-	BDH Lab, England
(0.25, 0.5, 0.75,1.0, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 kilo base pairs)		

D. Enzyme

Taq DNA polymerase	-	Bangalore Genei Pvt. Ltd.
Proteinase k	-	Bangalore Genei Pvt. Ltd.
dNTPs	-	Bangalore Genei Pvt. Ltd.

DELINEATION OF RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS IN CROSSBRED CATTLE

By
ANILKUMAR K.

ABSTRACT OF A THESIS

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ABSTRACT

A study was conducted to delineate the Random Amplified Polymorphic DNA markers of crossbred cattle of Kerala. DNA was isolated from whole blood, fresh semen or frozen semen samples for this study. Three panels of DNA samples were used in this study. They were DNA samples of 84 unrelated crossbred cattle, which was used for assessing the RAPD polymrophic patterns, DNA samples from 52 offspring, dam and sire combinations for assessing the possibility of using the technique in parentage identification and DNA samples of 108 dwarf cattle for microsatellite analysis.

The average yields of DNA obtained from whole blood was 256 ± 2.12µg/ 5 ml, fresh semen was 241.23 ± 8.42µg/ 400 million sperms and from frozen semen was 91.32 ± 6.01 µg/ 0.25 ml straw. The optical density ratio calculated as an indicator of purity of the sample were 1.72 ± 0.14, 1.81 ± 0.26 and 1.61 ± 0.34 for the DNA obtained from whole blood, frozen semen and fresh semen respectively.

Twenty random oligonucleotide primers from Operon kit A and six custom synthesised primers were used for the study. Based on intensity, clarity and polymorphism of bands, 19 primers were selected for amplification of DNA samples of crossbred cattle.

Number of bands obtained from different primers ranged from 2 to 16. The average number of bands produced by different primers ranged from 3.78 ± 0.15 in OPA 4 to 8.15 ± 0.25 in ILO1127. The size of amplified products ranged from ~230 base pairs to ~3580 base pairs. Percentage of polymorphism represented by individual primer varied from 66.66 to 100 in different groups. Seven primers namely OPA2, OPA 4, OPA 17, OPA18, OPA 20, ILO 1127 and ILO 876 yielded 100% polymorphic bands. Frequencies of the bands and their allelic frequencies were worked out.

The largest average number of bands was produced by the primer ILO 1127 (8.15 ± 0.25) and the lowest average number of bands was observed for the primer OPA 4 (3.78 ± 0.15).

The bands having frequency less than 0.25 were classified as rare bands. The primer OPA 15 produced 7 rare bands, the primer OPA 2 and OPA 19 five bands each, the primers for OPA1, OPA4, OPA 8, OPA 9,OPA 16 and ILO 1127 four each and primers OPA7, OPA 14, OPA 17 and ILO 876 three each.ILO 526oduced two rare bands, four primers namely OPA10, OPA 12, OPA 18 and OPA 20 produced one rare band each , and no rare bands were observed for the primer G1.

Twelve RAPD primers were selected to study 52 offspring, dam and sire combinations. Non parent bands were observed in offspring to the extent of 2 to 13.6%. Three of these primers namely OPA 14, OPA 4 and G1 did not produce any non parent bands in offspring. It was concluded that due to presence of the non parent bands in offspring, RAPD-PCR technique cannot be the method of choice for parentage verification.

Phenol chloroform procedure was used to isolate DNA from whole blood of 24 Vechur, 25 Highrange dwarf, 24 Vatakara and 35 Kasargode animals for microsatellite studies. The average yield of DNA obtained per 5 ml of blood samples was 210.1 ± 9.4 μg with the ratio of optical density at 260 nm and 280 nm as 1.86 ± 0.23.

PCR conditions for the four microsatellite loci namely DRB3, ETH 131, HEL 6 and FSHβ were standardised. The size of the alleles ranged from 176 to 236 bp for DRB3 locus, 160 to 184 bp in ETH 131 locus and 263 to 295 base pairs in HEL-6 locus. The number of alleles identified in different loci were 21, 14 and 13 respectively for DRB3, ETH131 and HEL 6 loci. The PIC value of the primers, direct count heterozygosity and unbiased heterozygosity were worked

out. The identification of new alleles in this study was attributed to the fact that dwarf cattle of Kerala are *Bos indicus* .

The possibility of using the microsatellite marker analysis for genetic characterization of dwarf cattle of Kerala and their phylogenic studies are indicated in this work. Parentage verification facilities can be established, where this technique can be employed in disputed cases.