MOLECULAR GENETIC DIVERSITY IN DWARF CATTLE OF KERALA

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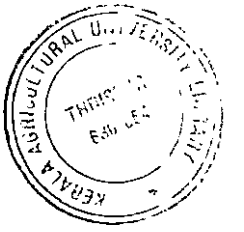

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DECLARATION

I hereby declare that this thesis entitled **"MOLECULAR GENETIC DIVERSITY IN DWARF CATTLE OF KERALA"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"MOLECULAR GENETIC DIVERSITY IN DWARF CATTLE OF KERALA"** is a record of research work done independently by **Dr. P. Suprabha**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Suprabha .P.

DEDICATED TO MY PARENTS

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Introduction

1. INTRODUCTION

India can be proud of possessing the richest domestic animal diversity in the world. Variation in agroclimatic conditions coupled with socio–economic factors of different regions resulted in development of various breeds and genetic groups of domestic animals. But the efforts taken to study, restore and multiply the resource is inadequate.

Kerala is not known outside for its cattle breeds except Vechur, which finds a place among the 30 recognised cattle breeds of India. The heavy monsoon and high humidity are the climatic peculiarities of Kerala. This hot humid tropical climate of the state is having profound influence in the physic of domestic animals. Intensive crossbreeding programme for milk production started in 1960's was instrumental in converting more than 85 per cent of the local animals as crossbreds of Jersey, Holstein Friesian and Brown Swiss. According to 1996 livestock census the local cattle accounts to 1.1 lakh out of 33.9 lakh cattle of Kerala. Four different groups of native animals (Vechur, Vatakara, Highrange dwarf and Kasargode) are described by different researchers based on physical characters.

The legendry smallest cattle breed of the world, Vechur, originated in Vechur village of Kottayam district are well known for its specific characters. The High range dwarf cattle are seen in high range areas of Idukki district. These are used as meat animal and are reared under zero input system. The Kasargode cattle is seen in northern parts of Kasargode district. The manure from these animals forms main source of organic fertilizer for areca plantations. The Vatakara animals are present in parts of Calicut district and are maintained as house cows.

In the era of globalisation, domestic animal diversity is recognized as one of the important wealths of the country. The characterization and documentation of domestic animal resources have great importance. Earlier studies on

characterization of domestic animals were based on phenotypic characters, blood typing, karyotyping, immunological assay, protein and biochemical polymorphisms. These approaches are time consuming, laborious and their power to detect genetic variation is low.

The recent advances in molecular genetics enabled the analysis of DNA directly providing a tool for interpretation of genetic variation that can be effectively measured within and between different genetic groups. This can be done by detecting DNA polymorphisms using suitable molecular marker systems.

In Kerala, no research work was undertaken to characterize the populations of dwarf cattle using DNA markers. So molecular genetic approach to characterize these animals was undertaken. Among the various molecular markers available, the Random Amplified Polymorphic DNA (RAPD) markers were selected for this study. It is the most simple and easy method of finding DNA polymorphisms. It is quick, efficient, and simple in experimental set up.

The present study on genetic characterization of dwarf cattle of Kerala was with the objectives to identify the RAPD polymorphism and to use the information to estimate the genetic distance between different groups of dwarf cattle of Kerala.

The results of the present study will be useful in opening up numerous vistas in the future research on this subject. Further more, prioritization of conservation efforts for these dwarf cattle would be made easy by this research.

Review of Literature

2. REVIEW OF LITERATURE

2.1 DESI/ DWARF CATTLE OF KERALA

According to Aiya (1906) there were no specialized breed of cattle in Travancore for any specified purpose. Generally the cattle of the state were best described as diminutive in size, small, wretched, low milk producing and poor workers. The colour of these animals were described as black, but brown animals were also found. He had mentioned about Vechur cattle, famous in its milking capacity, though extremely small in size.

Iyer (1937) reported that cattle of Kerala when compared to rest of India were very much smaller in built and were poorer milkers. These had been due to unfavourable rainfall and climatic conditions prevailing in the state. Premiscous mating and improper and insufficient feeding caused the deterioration of local animals which had gone to an extent that there was no specific breed of cattle in the state.

Velupillai (1940) emphasized the role of cattle as an important part in the Agricultural economy of the state. The number of cattle had been 382360 in 1816, 893403 in 1921, 1056943 in 1931 and 888154 in 1935. Though authorities labelled Travancore cattle as nondescript, they were divided into different groups according to the locality to which they belong. Of these, Vechur had noted position for its milking capacity.

Girija (1994) conducted studies on characterization and evaluation of dwarf cattle of Kerala, which include Vechur breed. Considering haemoglobin and transferrin polymorphism, it was summarized that the Vechur cattle has unique characteristics and suggested separate identity to this group. She stressed the necessity of conservation of dwarf cattle of Kerala, which is the smallest variety available in India and perhaps in the world.

Iype and Venkatachalapathy (2001) described Vechur breed, and its various phenotypic traits related to production and reproduction. The average height of male animal, was 89.43 ± 6.55 cm and that of female was 87.75 ± 0.77 cm. Horns were brown or black, curved forward, downward with pointing tips. They prepared a breed descriptor for Vechur breed.

Anilkumar (2002) outlined the physical profile of Highrange dwarf cattle. These small sized unique animals were identified from pockets in highlands of Idukki district. Coat colour of the animals was brown or shades of brown. Horn was black, straight, long with upward pointing tips.

The dwarf animals of Vatakara were with black coat, small hump and dewlap. They had black curved horns and upward pointing tips. The average body height at 1 year of age was 86.5 ± 1.5 cm (Anilkumar, 2003).

Anilkumar and Raghunandanan (2003) described the characters of four genetic groups of cattle in Kerala, namely Highrange dwarf, Vatakara, Vechur and Kasargode and prepared breed descriptor for all these different groups. The Kasargode animals are larger compared to Vechur but smaller than Highrange dwarf and Vatakara animals. Average height of adult animals was 91.2 ± 0.44 cm. Coat colour was black and its shades with large prominent hump. Horn was black, short and stumpy.

2.2 GENOMIC DNA ISOLATION

Blin and Stafford in 1976 introduced a new method of phenol extraction for isolation of high molecular weight DNA from eukaryotes.

The most widely accepted extraction procedure for genomic DNA from blood samples of cattle was that of Andersson *et al.*, 1986. Using phenol extraction procedure, the yield they obtained was in between 150 and 250 µg per 10 ml of blood.

Jeanpierre, 1987 followed the guanidine hydrochloride method for the isolation of genomic DNA from human blood and the yield of DNA was about 200 μg per 10 ml blood.

Montgomery and Sise (1990) evaluated phenol, high salt and guanidine hydrochloride methods for extraction of DNA in sheep. The extraction using guanidine hydrochloride method resulted in a gelatinous material that failed to resuspend in Tris-EDTA buffer. The mean yield of DNA from 20 ml whole blood by phenol and high salt method were 500 ± 0.19 μg and 640 ± 0.26 μg respectively. The quality of DNA extracted by high salt method was equivalent to that extracted by phenol method. They concluded that the high salt method can be used routinely for the extraction of DNA as it is less time consuming.

Apparao *et al.* (1994) described a rapid procedure to extract DNA from blood of cattle, buffaloe, goats, sheep and pigs. This modified phenol extraction procedure yielded 250-300 μg of DNA from 15 ml of whole blood.

Senthil *et al.* (1996) compared two methods of isolation of DNA from cattle blood samples namely phenol: chloroform method and high salt method. The high salt method was quick and produced good yield of pure high molecular weight DNA compared with phenol: chloroform method (615.55 ± 20.72 μg and 444.58 ± 25.54 μg respectively) from 15 ml blood.

Aravindakshan *et al.* (1998) compared high salt method, guanidine hydrochloride method and phenol: chloroform method for extraction of DNA from blood samples. The yield and quality of DNA extracted by high salt method was comparable to that of the commonly used phenol:chloroform method and was better than that produced by guanidine hydrochloride method. He reported the mean yields of DNA from 10 ml of blood by phenol:chloroform and highsalt method in cattle as 394.5 ± 34.26 μg and 446.16 ± 26.68 μg respectively.

2.3 RAPD-PCR TECHNIQUE

Gill *et al.* (1985) described forensic application of DNA fingerprints. They observed that DNA fingerprints were suitable for individual identification and had the capability of changing completely the emphasis of blood grouping in forensic science.

Mullis *et al.* (1986) first described Polymerase Chain Reaction as an *in vitro* method for synthesizing copies of a specific DNA fragment enzymatically. This method consists of repetitive cycles of denaturation, hybridization and polymerase extension.

Kuhnlein *et al.* (1989) reported DNA fingerprinting as a valuable tool for determining genetic distances between strains of poultry. DNA fingerprints of birds from five well defined populations of known genetic relationship were analysed and indices of genetic distances were computed.

Saiki *et al.* (1989) described that PCR involves merely combining DNA sample with oligonucleotide primers, deoxyribonucleoside triphosphates and the thermostable *Taq* DNA polymerase in a suitable buffer, followed by repeatedly heating and cooling the mixture for several hours until the desired amount of amplification was achieved.

The standard PCR buffer contains 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 1.5 mM $MgCl_2$ (Sambrook *et al.*, 1989). The reaction was extremely inefficient when concentration of primers were low. They designed primer pairs such that complementarity in their 3' ends were absent to reduce primer-dimer formation.

Lawyer *et al.* (1989) recommended the concentration of *Taq* DNA polymerase in most of the amplification reactions to be 1 to 2.5 units per 100 µl reaction mix, when other parameters are optimum.

Innis and Gelfand (1990) recommended that a primer concentration between 0.1 and 0.5 μM was optimum for polymerase chain reaction. Higher primer concentrations promote mispriming and accumulation of non-specific products and increase probability of formation of template independent artifact termed as primer-dimer. Furthermore, insufficient magnesium ions (Mg^{2+}) leads to low yield and excess Mg^{2+} result in the accumulation of non-specific products. They recommended a PCR buffer that contains 10-50 mM Tris HCl, with pH between 8.3 and 8.8 at 20°C. The four dNTPs should be used at equivalent concentration to minimize misincorporation errors. Theoretically 20 μM of each dNTP in a 100 µl reaction was sufficient to synthesize 2.5 µg of DNA.

Williams *et al.* (1990) first suggested the term RAPD markers after Random Amplified Polymorphic DNA, based on amplification of random DNA segments with single primers of arbitrary oligonucleotide sequence applied at low annealing temperature in PCR.

Eckert and Kunkel (1990) demonstrated that despite lacking 3'-5' proof reading exonuclease, the *Thermus aquaticus* (*Taq*) DNA polymease can catalyse highly accurate DNA synthesis *in vitro*. The unique capacity of *Taq* polymerase to withstand repeated exposures to temperatures at which DNA is denatured renders the enzyme an excellent tool for PCR. Under defined reaction conditions the error rate per nucleotide polymerized at 70°C can be as low as 10^{-5} for base substitution error and 10^{-6} for frame shift errors. The optimal concentration of dNTPs depends on several factors including $MgCl_2$ concentration, reaction stringency, primer concentration, length of amplified product, and number of cycles of PCR.

Lowe *et al.* (1990) designed a computer programme which rapidly scans nucleic acid sequences to select all possible pairs of oligonucleotides suitable for use as primers to direct efficient DNA amplification by PCR.

Dunnington *et al.* (1991) compared DNA fingerprinting between selected populations of chicken. Levels of band sharing between breeds were lowest, those between selected lines within breed were intermediate and those between the selected lines and their F_1 crosses were highest.

Appelhans (1991) delineated the potentials, methodology and applications of PCR in detail. Deoxyribonucleic acid fragments generated by PCR were comparable in yield and purity to fragments prepared from clonally isolated recombinants.

Erlich *et al.* (1991) and Haff (1993) reviewed some of the recent developments in PCR procedure that facilitated many tasks in molecular biology research like cloning and sequencing. The steps of denaturation, primer binding and DNA synthesis can be carried out at temperatures 94°C to 98°C, 37° to 65°C and 72°C respectively.

Bowditch *et al.* (1993) revealed that the number of cycles in PCR appeared more critical than the starting concentration of template DNA in RAPD-PCR analysis. When tested with 30 and 25 cycles, some bands were poorly reproducible and all bands were absent in 20 cycle reactions. It is imperative to keep reaction conditions constant in order to compare results from different reactions. They also found that 0.024 units/µl of *Taq* polymerase was sufficient for RAPD-PCR analysis using 0.2-0.6 ng/µl of template DNA with 0.24 p*M*/µl of primer DNA.

Liming and Shi (1993) reviewed general aspects of genetic diversity at the species, chromosome and molecular levels applicable to all living organisms. The state of genetic diversity in China, current threats to this diversity, the consequences of loss of genetic diversity and the means of preserving genetic diversity were outlined.

Penner *et al.* (1993) conducted research on reproducibility of RAPD analysis in seven laboratories in North America. The reproducibility of results

among laboratories was influenced by the overall temperature profiles especially, the annealing temperature.

Williams *et al.* (1993) detailed the experimental protocols for RAPD assays and applications emphasizing their use in genetic analysis. The technique required, only nanogram quantities of DNA, no knowledge of target DNA, was applicable to a broad range of species and was non-radioactive. They reported the use of 1.5 m*M* $MgCl_2$, 36°C annealing temperature, 0.1-2 μ*M* primer concentration and dNTP concentration of 100 μ*M* for each of the four bases, for reproducible amplification patterns.

Lear *et al.* (1994) evaluated the phylogenetic relationships of the Equidae using RAPD markers. Jaccard's similarity coefficients were found. Based on RAPD data, the equids are composed of two major groups and the phylogenetic tree thus constructed was different from those constructed based on other traits like protein systems, morphological tests or comparative karyotypes.

Newton and Graham (1994) described RAPD technique as ideally suited to fingerprinting applications because it is fast, requires less material and technically easy, but not the method of choice for paternity testing and pedigree analysis where absolutely unequivocal results are essential. They found that traces of phenol, SDS, and proteinase k used for DNA extraction were inhibitors of PCR and these could be removed by chloroform-isoamyl alcohol extraction and ethanol precipitation.

Bailey and Lear (1994) used 50 ng of template DNA for RAPD for 40 cycles in a study of comparison of Thoroughbred and Arabian horses using 11 primers.

In a study with 141 arbitrary oligonucleotide primers, Gwakisa *et al.*, 1994 used 10μl reaction mix comprising 20 ng template DNA, 6 p*M* primer, 200 μ*M* dNTPs and 0.5 unit *Taq* DNA polymerase. The temperature cycling comprised 40 cycles of 10 sec at 95°C, 5 sec at 35°C and 1 minute at 72°C.

Kemp and Teale (1994) also followed the same procedure with eighty 10 base oligonucleotide primers with GC content in the range of 60-80%. The temperature cycling consists of an initial denaturation of 30 sec at 95°C, followed by 40 cycles of 96°C for 10 sec, 35°C for 10 sec and 72°C for one minute.

Yu and Pauls (1994) reported the optimization of DNA extraction and PCR procedures for RAPD analysis in plants. They established that the efficiency of RAPD technique was determined by the procedures used for DNA extraction and PCR amplification. They optimized 35 cycle programme with denaturing 94°C for 5 sec, annealing 36°C for 30 sec and extension of 72°C for 60 sec.

Rothuizen and Wolferen (1994) established the need for optimizing the protocol for generating RAPD markers with respect to the annealing temperature, concentration of $MgCl_2$, template DNA, primers and thermocycler. In optimized conditions, the reaction products were completely reproducible.

Antoniou and Skidmore (1995) tested 60 random primers for PCR and 26 primers yielded polymorphic fragments in cattle. They were able to identify a Y specific marker of 3100 bp length.

Teale *et al.* (1995) described a polymorphism in randomly amplified DNA that was generated in PCR with 10 base primer ILO 1065 that differentiated the Y chromosomes of *Bos indicus* and *Bos taurus*. The marker was generated in *Bos indicus* male templates but not from *B. taurus* male or female templates.

Rao *et al.* (1996) used 14 arbitrary primers to amplify DNA fragments in four species such as, Indian zebu cattle (*Bos indicus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*) and goat (*Capra hircus*). Clear and distinct RAPD patterns with a higher level of polymorphism were detected among species while fewer polymorphism were found within the species. Random amplified polymorphic DNA fragments were scored and Jaccard's similarity coefficients were calculated

to quantify genetic divergence among the species. Wagner Parsimony analysis revealed very low similarity among four species.

Carpio *et al.* (1996) used RAPD-PCR technique in cattle. Amplification patterns were analysed to identify polymorphic bands within and between groups. The average number of polymorphic bands per primer per bull and cow was found and the similarity index among animals were calculated based on bandsharing method.

Cushwa and Medrano (1996) discussed molecular genetic technologies that revolutionized genetic analysis of livestock species and suggested that the RAPD assay has the potential to play a useful role in genetic analysis of livestock species.

Balakrishna (1996) reported the importance of molecular markers in germplasm conservation programmes. Detection of molecular variation helped a lot to generate methods for efficient conservation of germplasms based on genetic distances between and within populations.

Ambady *et al.* (1996) conducted studies on optimization of RAPD-PCR conditions in cattle. They described the use of tricine buffer, the various reaction components and thermal cycling conditions using single primers and double primer combinations that enabled consistent reproducible amplification.

Parejo *et al.* (1997) described the optimum technique and environmental conditions required for success of RAPD technique in cattle.

Payne (1997) recommended highly polymorphic molecular markers for the measurement of genetic variability and genetic distances of closely related populations. Genetic distance, which is a measure of the allele frequency at a number of defined loci within a species indicate the probability that an allele chosen at random will be present in one but not the other breed.

Jones *et al.* (1997) described the advantages and disadvantages of molecular markers including RAPD. These are dominant markers due to their presence or absence at particular loci and they will segregate from a heterozygous diploid as Mendelian alleles. They are most often used as species specific markers for diversity and phylogenetic studies. The main disadvantages are poor reliability and reproducibility and their sensitivity to experimental conditions.

According to Joshi *et al.* (1998) the RAPD profile of DNA from blood samples of goat, sheep, buffaloe, cattle and dog was unique for each species in terms of the number and position of the bands, although some bands were common to cattle and buffaloes and to sheep and goats.

Mitra *et al.* (1999) reviewed the possible applications of molecular markers in livestock improvement. They suggested that the similarities between the DNA fingerprinting patterns, that are expressed by band sharing values, provide a reliable method for evaluating genetic distance amongst populations. Polymerase chain reaction based RAPD fingerprinting assays were used for characterization and detection of genetic variations in different species.

Kataria *et al.* (2000) had given an overview about Polymerase Chain Reaction. The components of PCR, modified PCR techniques, and the limitations of PCR were critically discussed. The concentration of magnesium was critical for getting specific product and needs to be titrated for each system.

Sodhi *et al.* (2001) depicted some general rules for PCR primer design, PCR optimization and the conditions favouring enhanced specificity. The efficiency, sensitivity and specificity of the PCR assay depends upon several parameters like primer concentration, initial substrate concentration, $MgCl_2$ concentration, annealing temperature etc. The optimization is essential to minimize production of incompletely amplified products, or extraneous primer dimer artifacts.

Eding *et al.* (2002) assessed the contribution of breed to genetic diversity in conservation schemes.

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

2.4 RAPD FINGERPRINTING IN DETECTING GENETIC VARIATION

Williams *et al.* (1990) described a new DNA polymorphism assay based on amplification of random DNA segments with single primers of arbitrary nucleotide sequence applied at low annealing temperature. They used short decamer primers and called these polymorphisms as Random Amplified Polymorphic DNA polymorphisms. The marker detects DNA polymorphism without prior sequence information of genomic DNA.

Welsh and McClelland (1990) established that simple and reproducible fingerprints of complex genomes can be generated using single arbitrarily chosen primers and polymerase chain reaction. This method known as Arbitrarily Primed PCR (AP-PCR) involved two cycles of low stringency amplification followed by PCR at highest stringency. The strains of bacteriae and rice were distinguished by comparing polymorphism in genomic fingerprints.

Welsh *et al.* (1991) applied arbitrarily primed PCR technique to distinguish between strains of mouse (*Mus musculus*). The method allowed rapid genetic mapping of DNA polymorphisms without southern blotting. The characteristic differences in the AP-PCR genomic fingerprints between strains were used in strain identification and verification.

Bardin *et al.* (1992) demonstrated that RAPDs are useful for measuring genetic variation in bovine populations. With an improved protocol, using three primers of 25 bp length, they obtained reproducible banding patterns.

Hetzel and Drinkwater (1992) stated that breed characterization requires the knowledge of genetic variation that can be effectively measured within and between populations.

The prospects for estimating nucleotide divergence with RAPD were described by Clark and Lanigan (1993). They summarized the restrictions and criteria that must be met when RAPD data are used for estimating population genetic parameters.

Bowditch *et al.* (1993) explained that the mutations that inhibit primer binding or otherwise interfere with amplification can be detected as the absence of the pertinent bands. Although the RAPD technique detects most of the classes of mutations (substitution, deletion and inversion) the basis of most polymorphism is presumably substitution.

Bailey and Lear (1994) compared pooled DNA samples of Thoroughbred and Arabian horses for the presence of RAPD markers. They found primer UBC-126 (5'CTTTCGTGCT3') detected a 1000 bp marker (designated as UBC 126^{C}) which was absent in Thoroughbred horses, but present in all Arabian horses sampled. This marker was effective for breed comparison and for detecting unwanted crossbreeding between two horse breeds.

Bardin *et al.* (1994) used bulked genomic DNA sample as templates in RAPD–PCR analysis. Several RAPD markers amplified and digested with restriction enzymes to study genetic variation and relationship in seven Italian cattle breeds.

The studies of Gwakisa *et al.* (1994) represent first application of RAPD-PCR technology for characterization of zebu cattle breeds in Tanzania. They employed 141 short sequence arbitrary primers singly in pools of DNA representing three zebu cattle breeds. Two primers (ILO 1127, ILO 1065) could differentiate breed specific DNA pools. They concluded that RAPD polymorphism was useful as genetic markers for cattle breed differentiation. The

band sharing and mean average percentage difference calculated within and between breeds showed a high degree of homogeneity within than across the breed and indicated measurable divergence between different breeds.

Kemp and Teale (1994) were able to identify RAPD markers which distinguish *Bos taurus* and *Bos indicus* species. Three RAPD markers were able to produce amplified products from *B. indicus* DNA pool but not from *B. taurus* DNA pool. The primer ILO 526 produced *B. indicus* specific product of 480 bp.

Chung *et al.* (1995) worked out DNA polymorphism and genetic characterization of Holstein dairy cattle using RAPD-PCR method. A total of ten primers produced 78 RAPD markers. They found that 74.4 per cent of bands were polymorphic. A combination of two of the primers produced Holstein specific RAPD markers.

Kantanen *et al.* (1995) investigated the use of RAPD method to detect genetic variation in five Finnish cattle breeds and in Finnsheep. They used 11 RAPD primers in cattle and 13 in sheep. Three polymorphic markers were identified for cattle and seven for sheep. They concluded that RAPD method was not efficient for finding new polymorphisms in either species.

Teale *et al.* (1995) described the characterization and cloning of a bovine RAPD, i.e., generated with the 10 base primer ILO 1065 from *Bos indicus* male templates. They concluded that ILO 1065 primed RAPD can be used in a single dot blot assay as a probe of RAPD-PCR products to provide reliable mean of detecting interogression of zebu genes in *B. taurus* cattle population.

Aravindakshan and Nainar (1998) evaluated the use of RAPD markers to analyse genetic variation in cattle and buffalo breeds using randomly designed ten base primers. Band sharing and mean average percentage difference calculated within and between different breeds using RAPD fingerprints showed a higher degree of homogeneity within than across the breed, indicated measurable divergence between breeds.

Jeon-Gi *et al.* (1998) used 30 random primers to study the DNA polymorphisms in Korean native, Holstein, Charolais, Aberdeen-Angus and Hereford cattle. The genetic distances were calculated and the lowest value was found between Korean native and Holstein cattle.

A dendrogram of relationship among Avilena, White Caceres, Charolais and Retinta was prepared by Parejo *et al.* (1998) using RAPD technique. The number of possible breed marker fragments of DNA found in the breeds were 68,34,68 and 42 in the four breeds respectively.

Joshi *et al.* (1999) opined that it was important to optimize and maintain constant reaction conditions of RAPD-PCR for reproducible DNA amplification. The application of RAPDs was less popular in variability analysis and individual specific genotyping because of poor reproducibility and faint or fizzy products.

Breed specific band patterns for Han Woo cattle [Korean native] were identified by Ahn *et al.* (1999) by comparison with those of Holstein and Hereford cattle. They found that the band patterns were not affected by PCR conditions.

Rincon *et al.* (2000) studied the genomic polymorphism in Uruguayan Creole cattle using RAPD and microsatellite markers. A total of 215 loci ranging between 300 and 2500 bp were amplified. Band sharing frequency among breeds were low between Creole and Hereford cattle and the highest similarity frequency corresponds to Holstein Friesian compared with Hereford.

Ganai *et al.* (2000) performed DNA amplification finger printing of cattle and buffaloes with short arbitrary 10 bp primers. Out of four primers tested, 5'GTGACGTAGG3' (G_1) and 5'TGCCGAGCTG3' (G_2) yielded amplification products in cattle and buffaloes.

Zubets *et al.* (2001) applied RAPD for investigation of molecular genetic polymorphism in three cattle breeds (Ukrainian red motley lactescent, Golshtine

and Simmental). A dendrogram reflecting genetic relations between analysed animals was drawn.

Ramesha *et al.* (2002) conducted RAPD-PCR assay to identify polymorphic markers in South Indian cattle breeds, Amrithmahal, Krishnavalley, Hallikar, Deoni, Khillari, Ongole and Malnad Gidda. The genetic distance between these breeds were estimated. Twenty six random primers were tested in breed specific pools and of these, seven primers were used to study different cattle breeds. No RAPD product specific to Indian zebu animals could be amplified. Malnad Gidda was found to be distinctly different from the other breeds studied.

Serrano *et al.* (2002) studied genetic diversity and population structures of five Brazilian native bovine breeds using RAPD markers. The genetic relationships were carried out by testing 120 primers, of which 22 were selected, generating 122 polymorphic bands. The results showed that the RAPD technique was capable of distinguishing the native breeds.

Yeo *et al.* (2002) used 60 random primers to identify specific DNA markers to distinguish Hanwoo cattle from five foreign cattle breeds. A specific DNA marker of 519 bp was identified and sequenced as the unique DNA marker for Hanwoo breed. They advocated the necessity to develop new probes of DNA primers from RAPD markers because of the extreme sensitivity of RAPD marker for amplification conditions.

Various authors worked on RAPD-PCR in different species to assess genetic diversity within and between breeds/lines

	Reference	Species
1.	Clouscard *et al.* (1994)	Sheep
2.	Semyenova *et al.* (1994)	Chicken
3.	Cushwa and Medrano (1996)	Chicken
4.	Semenova *et al.* (1996)	Chicken
5.	Giesel *et al.* (1997)	Ring necked pheasant
6.	Liu *et al.* (1997)	Pig
7.	Jiasheng and Kaiya (2000)	Goose
8.	Kumar *et al.* (2000)	Japanese quail
9.	Nath *et al.* (2000)	Chicken
10.	Nayal *et al.* (2000)	Chicken
11.	Mohd-azmi *et al.* (2000)	Chicken
12.	Jun *et al.* (2001)	Chicken
13.	Sharma *et al.* (2001)	Chicken
14.	Rao *et al.* (2001)	Chicken
15.	Yen *et al.* (2001)	Pig
16.	Akyuz *et al.* (2002)	Quail
17.	Zhang *et al.* (2002)	Goat
18.	Hong *et al.* (2002)	Pea fowl
19.	Guo *et al.* (2002)	Sheep
20.	Kulikova *et al.* (2002)	Pheasant
21.	Li *et al.* (2002)	Goat
22.	Mamuris *et al.* (2002)	Brown hare (*Lepus europaeus*)
23.	Semenova *et al.* (2002)	Swift hound dogs
24.	Yoon and Park (2002)	Crussian carp (*Carasssius carassius*)
25.	Zhang *et al.* (2002)	Chicken
26.	Geng *et al.* (2002)	Goat

2.5 ANALYTICAL TOOLS

Saitou and Nei (1987) proposed the Neighbor-Joining method for constructing phylogenetic trees from evolutionary distance data. They studied the efficiency of this method with unweighted pair group arithmetic mean method (UPGMA), Farris method, Sattath and Tversky's method, Li's method and Tateno Itali's modified Farris method and found that Neighbor-Joining method was generally better than other methods.

Lynch (1990) used DNA fingerprint similarity to make inferences about levels of genetic variation within and between natural populations. He determined pairwise similarity as fraction of shared band.

Gwakisa *et al.* (1994) described the application of pair-wise comparison in random amplified DNA fingerprinting data analysis. The genetic similarity was ascertained based on band sharing frequency and dissimilarity between genotypes expressed in the form of Mean Average Percentage Difference (MAPD). They got the MAPD value that varied for each primer and for each paired breed comparisons.

Genetic distance between breeds of chicken were estimated by Smith *et al.* (1996) using RAPD markers. He found that D value was lowest between Araucena (AR) and Rhode Island Red (RIR) and highest between Araucena (AR) and White Plymoth Rock.

Aravindakshan and Nainar (1998) investigated the genetic variation in cattle and buffalo breeds by RAPD markers. They calculated band sharing and mean average percentage difference by pairwise comparisons. A dendrogram was constructed based on Euclidean distance. Jersey crossbreds and Ongole cattle showed two distinct clusters whereas there were no distinct clusturing in Murrah and Surti animals.

Kumar *et al.* (2000) used band sharing frequency and calculate genetic distance in RAPD analysis for finding genetic relatedness in Japanese quail lines.

Mohd-Azmi *et al.* (2000) used pairwise comparison among different species of fowls and the estimate of band sharing was selected to calculate the similarities in genetic makeup populations in RAPD analysis.

Geng *et al.* (2002) studied the genetic relationships of three goat populations by genetic differentiation coefficient and genetic similarity coefficient in RAPD analysis.

Ramesha *et al.* (2002) used band sharing and mean average percentage difference as analytical tools for calculating genetic distance among seven South Indian breeds of zebu cattle using RAPD markers. They constructed a dendrogram of relationship among these breeds using genetic distances as estimated by the Distance Wagner procedure.

Materials and Methods

3. MATERIALS AND METHODS

3.1 STUDY MATERIALS

The study was conducted on four genetic groups of dwarf cattle available in Kerala, namely Vechur, Highrange dwarf, Vatakara and Kasargode. Animals of Vechur breed for the study were selected from the Vechur conservation unit, Kerala Agricultural University. (Plate 5). The Highrange dwarf animals were from Chinnar, Elappara, Pattumala, Fairfield and Glenmary estates of Idukki district in Highranges of Kerala. Vatakara animals were identified from Memunda and Kizhal areas of Vatakara and Kasargode animals were from Vorkadi and Meenja areas of Kasargode district of Northern Kerala.

3.2 COLLECTION OF SAMPLES

Blood samples (5 ml each) were collected from the jugular vein aseptically using sterile disposable needle and syringe and transferred immediately to sterile centrifuge tubes (Tarson polypropylene tube) containing Ethylene Diamine Tetra Acetic Acid (EDTA) (1 mg/ml of blood) as anticoagulant. The samples were brought under refrigerated conditions to the laboratory and stored at 4°C till processed.

3.3 EXTRACTION OF GENOMIC DNA

Genomic DNA from whole blood was extracted by using the standard phenol: chloroform extraction procedure (Chitra, 2002) with modifications. Though the minimum incubation period recommended for proteinase k/sodium dodecyl sulphate digestion was three hours, in this study the samples were kept overnight.

3.3.1 Phenol:chloroform Extraction Procedure

1. To five ml of blood, two volumes of ice cold red blood corpuscles (RBC) lysis solution were added, mixed and kept in ice with occasional mixing for 10 minutes to allow the complete lysis of erythrocytes.

2. The tubes were centrifuged at 4000 rpm for 10 minutes to pellet the contents. The dark coloured supernatant was discarded leaving the white pellet.

3. Step 1 and 2 were repeated till the pellet was without any dark or red colour. The pellet was then washed twice with Tris buffered saline (TBS) by centrifugation at 3000 rpm for 10 minutes.

4. Washed white cell pellet was resuspended completely by vortexing in 5 ml of saline EDTA (SE) buffer.

5. To this cell suspension, 25 µl of proteinase k (20 mg/ml in water) and 0.25 ml of 20% sodium dodecyl sulphate (SDS) were added and mixed gently.

6. The samples were then incubated at 50°C in a water bath with occasional mixing for overnight.

7. The digested samples were cooled to room temperature and 300 µl of 5 *M* NaCl was added to each sample and mixed by vortexing.

8. Equal volume of saturated phenol (pH 7.8) was added and mixed thoroughly by inversion for 10 minutes. The tubes were centrifuged at 4000 rpm for 15 minutes and the aqueous phase was collected in fresh tubes with the help of wide bore Pasteur pipettes.

9. Equal volume of saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added to the sample and mixed thoroughly by inversion for 10 minutes. The tubes were centrifuged at 4000 rpm for 15 minutes and the

supernatent aqueous phase was again collected in fresh tubes using wide bore Pasteur pipettes.

10. To the sample an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed for 10 minutes and centrifuged at 4000 rpm for 10 minutes. The upper aqueous phase was transferred into a fresh tube with the help of a wide bore Pasteur pipette and the step was repeated.

11. The sample was transferred to a sterile 50 ml beaker and one tenth volume of 3 *M* sodium acetate (pH 5.5) was added and mixed well.

12. An equal volume of chilled isopropyl alcohol was added and the precipitated DNA was spooled out on a clean micropipette tip. It was then washed in 70 per cent ethanol, air dried and resuspended in 500 µl TE buffer (pH 8.0) and stored at –20°C.

(The composition and methods of preparation of the reagents and buffers used with their sources are presented in the Annexure I and II).

3.3.2 Assessment of DNA Samples

From the DNA stock solution, 20 µl was diluted with 1980 µl of sterile triple distilled water. Optical densities (OD) were measured at 260 nm and 280 nm wavelengths using disposable cuvettes in an UV spectrophotometer (Jenway, UK) using sterile triple distilled water as blank. The yield and purity of DNA sample were estimated as follows.

3.3.2.1 Yield

An OD of 1 at 260 nm wavelength is equivalent to 50 µg/ml of double stranded DNA. Concentration of DNA stock solution per ml was found out by multiplying OD 260 with a factor of 5000. The total yield of DNA was calculated by multiplying the concentration and the volume of DNA stock solution.

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

3.3.2.2 Purity

Purity of DNA stock solution was estimated by finding the ratio between the optical density readings at 260 nm and 280 nm wavelengths (OD 260/OD 280).

3.3.3 Checking Quality of Genomic DNA

In order to determine the quality of isolated DNA, approximately 1-2 µg of DNA was run in 0.7% agarose gel in 1 X Tris Acetate EDTA (TAE) buffer in a horizontal submarine gel electrophoresis unit.

3.4 RAPD-PCR ANALYSIS

The RAPD-PCR analysis was done in four groups of dwarf cattle available in Kerala (Vechur, Highrange dwarf, Vatakara and Kasargode animals) to identify and establish RAPD marker polymorphism and genetic divergence in these animals.

3.4.1 Template DNA

Template DNA for PCR was prepared by diluting a sample of DNA stock solutions in sterile distilled water to get a final concentration of 25 ng/µl and was stored at-20°C.

Pooled breed specific DNA samples were prepared by mixing equal quantity of diluted DNA (25 ng/µl) from 10 unrelated animals, randomly selected from each group.

3.4.2 Primers

3.4.2.1 Primers Used

The random 10 base oligonucleotide primers used in this study have GC content of 60-90%. The Operon Kit A (Operon Technologies Inc., USA) containing 20 primers (OPA 01-20) and six primers (ILO 1127, ILO 876, ILO 526, OPAV15, G_1, G_2) selected from published reports (Custom synthesized from Life Technologies India Pvt. Ltd.) were used for this study.

3.4.2.2 Preparation of Primers

The primers obtained in the lyophillised form were spinned briefly and dissolved in sterile distilled water to get a final concentration of 200 p*M*/µl of stock solution.

The tubes were kept at room temperature, with occasional shaking for 1 hour. The tubes were centrifuged at 3500 rpm for 2 minutes to pellet down the insoluble particles if any. The working solution of primer for the PCR was prepared by diluting the stock solution to get a final concentration of 4-6 p*M*/µl. Both the stock solution and working solution were stored at –20°C.

3.4.2.3 Selection of Primers

A total of 26 arbitrary oligonucleotide primers were evaluated in this study for breed specific DNA pools using single primer method (Kemp and Teale, 1994).

The polymorphic patterns produced by the 26 primers were studied and 10 primers were selected for detecting polymorphism within and between group animals. The primers selected with their sequence and GC content are provided in Table 3.1.

Table 3.1 Sequence and GC content of primers used for the study

Primer	Sequence GC	GC content (%)	Reference
1. OPA 01	5'CAGGCCCTTC3'	70	
2. OPA 06	5'GGTCCCTGAC3'	70	
3. OPA 09	5'GGGTAACGCC3'	70	
4. OPA 10	5'GTGATCGCAG3'	60	
5. OPA 12	5'TCGGCGATAG3'	60	
6. OPA 18	5'AGGTGACCGT3'	60	
7. OPA 20	5'GTTGCGATCC3'	60	
8. ILO 1127	5'CCGCGCCGGT3'	90	Gwakisa *et al.*, 1994
9. ILO 876	5'GGGACGTCTC3'	70	Kemp and Teale, 1994
10. ILO 526	5'GCCGTCCGAG3'	80	Kemp and Teale, 1994

3.4.3 Standardization of PCR Conditions

It was essential to optimize the different PCR parameters like amount of template DNA, magnesium chloride ($MgCl_2$) concentration, annealing temperature and concentration of *Taq* DNA polymerase. The optimization minimizes the production of incompletely amplified products. This optimization was conducted by setting up a series of test PCR reactions. The reaction volume was kept constant as 20 µl and also the concentration of dNTP, primer and reaction buffer as 200 µ*M*, 5 p*M* and 1X respectively.

3.4.3.1 Annealing Temperature

The most important variable in RAPD is annealing temperature. Annealing temperature was optimized by conducting the reactions at 34-40°C with increments of 0.5°C, keeping annealing time constant at one minute. The number of cycles were also kept constant as 35 cycles.

3.4.3.2 Concentration of $MgCl_2$

Another variable is the concentration of $MgCl_2$ which affects the efficiency of annealing. Different reactions which uses $MgCl_2$ concentrations (1.5 m*M*, 2 m*M* and 2.5 m*M*) in 20 µl reaction were tried to find the optimal concentration for RAPD-PCR analysis.

3.4.3.3 Taq DNA Polymerase

Two different concentrations of *Taq* DNA polymerase (0.5 unit/20 µl reaction and 1 unit/20 µl reaction) were tried.

3.4.3.4 Preparation of Samples for PCR

To each reaction tube 1 µl of (25 ng) of template DNA and 19 µl of master mix were added. A master mix was prepared just before setting up of the PCR assay combining 10 X PCR buffer, $MgCl_2$, dNTP mix, 10 base primer, *Taq* DNA polymerase and sterile water to get a final concentration of 1 X PCR buffer, 1.5 m*M* $MgCl_2$, 200 µ*M* dNTPs, 5 p*M* primer and 0.5 unit *Taq* DNA polymerase in a total volume of 20 µl. The tubes were centrifuged briefly (3000 rpm 3 minutes) and placed in thermal cycler. One negative control without template DNA was also included to monitor contamination, if any.

3.4.4 RAPD-PCR Amplification

All amplification reactions were carried out in 0.2 ml thin wall reaction tubes using a programmable thermal cycler (MJ Research Inc., USA).

The temperature cycling comprised of an initial denaturation of 3 minutes at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 38°C and 1 min at 72°C. This was followed by a final extension of 10 minutes at 72°C. The samples were then cooled down to 4°C, until retrieved. The PCR amplified products were stored at –20°C till gel analysis was performed.

3.4.5 Analysis of Amplified Products

PCR amplified products were detected electrophoretically on 2% agarose in 1 X TAE buffer in a horizontal submarine electrophoresis unit.

3.4.5.1 Agarose Gel Preparation

The gel tray was placed on a level surface and the comb of thickness 0.7 mm and 30 wells was kept in proper position in the gel tray.

Agarose in 1 X TAE buffer (2%) was heated in a microwave oven until it was a clear solution and cooled to around 60°C and added 0.5 µg/ml of ethidium bromide. This molten agarose was poured carefully onto the gel tray avoiding air bubbles. The comb was removed after ensuring the setting of gel. Thus agarose gel of approximately 6 mm thickness was prepared.

A total of 60 wells were prepared in two rows each containing 30 wells having a depth of 4 mm.

3.4.5.2 Sample Loading

The gel tray was immersed in the buffer tank filled with 1 X TAE buffer (pH 8.3). Five µl of PCR products were mixed with one-sixth volume of 6 X gel loading buffer and the samples were loaded into the wells carefully. A total of sixty wells loaded each time in two rows. (seven samples each from four genetic groups and ϕ X 174 RF DNA digested with *Hae* III and 1 kb DNA ladder as markers in each row).

3.4.5.3 Electrophoresis

The electrophoresis was carried out in room temperature at 100 Volts and 300 mA current for 2 hrs (until the bromophenol blue dye reaches the two-third length of the gel) in moulded horizontal submarine electrophoresis unit (Amershom Pharmacia Biotech, USA).

3.4.5.4 Gel Analysis/scoring of Bands

RAPD fingerprints were visualized under a UV transilluminator and documented in a gel documentation system (Biorad Laboratories, USA). They were photographed subsequently.

The different bands were scored for all the 10 primers using the Quantity one software of Biorad Laboratories (User Guide Version 4.2, 2000).

3.5 DATA ANALYSIS

The polymorphic patterns produced by RAPD primers were identified as bands. The percentage of polymorphism was calculated as the proportion of bands polymorphic in at least one pair-wise comparison to the total number of bands.

3.5.1 Band Sharing

RAPD finger prints produced by 10 primers were used for band sharing calculation. For this, all possible 15400 pairwise combinations of ten primers were made both within as well as between breeds. The average band sharing for each primer and for 10 primers were calculated.

The band sharing was calculated as an expression of similarity of RAPD finger prints of animals from either the same or different breeds (Dunnington *et al.*, 1990; Gwakisa *et al.*, 1994; Aravindakshan and Nainar, 1998) using the formula,

$$BS = \frac{2\,B_{ab}}{B_a + B_b}$$

Where, B_{ab} is the number of bands shared by individuals a and b.

B_a is the total number of bands for individual a and B_b is the total number of bands for individual b.

3.5.2 Mean Average Percentage Difference (MAPD) between Breeds

Mean average percentage difference (MAPD) was calculated as an expression of interbreed dissimilarities. All interbreed pair-wise comparison of each animal were made. The value was calculated on RAPD fingerprints obtained with 10 primers using the formulae (Gwakisa *et al.*, 1994).

$$\text{Percentage difference, PD} = \frac{N_{ab}}{N_a + N_b} \times 100$$

Average percentage difference,

$$\text{APD} = \frac{I}{C} \Sigma^{c}_{i=1} PD_i$$

$$\text{MAPD} = \frac{1}{R} \Sigma_{i=1}^{R} APD_i$$

Where,

N_{ab} is the number of fragments that differed between two individuals for a single primer, N_a is the number of fragments resolved in individual a, N_b is the number of fragments resolved in individual b, C is the number of interbreed pair-wise comparison (C = 1, 2, …., 10720) and R is the number of random primers used (R = 1, 2, ….., 10).

3.5.3 Least Squares Analysis

The effect of genetic groups, GC content of primers and the different combinations of comparisons in gel on band sharing value and percentage differences were studied by least squares analysis using the technique described by Harvey (1986).

The mixed model least squares and maximum likelihood computer program PC-1 was used. The following mathematical model was constructed.

$$(BS)\ Y_{ijk} = \mu + GC_i + GEL_j + GG_k + e_{ijk}$$

$$(PD)\ Y_{ijk} = \mu + GC_i + GEL_j + GG_k + e_{ijk}$$

GC_i = Effect of GC content of primers

GEL_j = Effect of samples compared in upper rows, lower rows and both upper and lower rows of a gel.

GG_k = Effect of genetic groups

e_{ijk} = Error

μ = Overall mean

$(BS)\ Y_{ijk}$ = Band sharing value made by pairwise comparison of i^{th} GC content, j^{th} gel, k^{th} genetic group

$(PD)\ Y_{ijk}$ = Percentage difference value made by pairwise comparison of i^{th} GC content, j^{th} gel, k^{th} genetic group

3.5.4 Genetic Distance (D)

Measurement of genetic distance gives best description of genetic diversity. It is a measure of overall evolutionary divergence.

In the present study, the bands which were scored as clearly present or absent were considered for derivation of genetic distance, by taking mean band sharing frequency between breeds and the total number of bands in each breed. The genetic distance (D) among four cattle populations were calculated using Nei's method (Kumar *et al.*, 2000).

$$D = - \ln \frac{B_{xy}}{\sqrt{B_x B_y}}$$

Where,

B_{xy} = between breed band sharing value of breed x and breed y

B_x = within breed band sharing value of x

B_y = within breed band sharing value of y

3.5.5 Dendrogram

A dendrogram of relationship of 56 animals of four genetic groups was constructed using ten different RAPD fingerprinting patterns. The Unweighted Pair Group with Arithmetic mean (UPGMA) method was followed.

Results

4. RESULTS

4.1 ISOLATION OF GENOMIC DNA

A total of 101 DNA samples were isolated from whole blood of four different groups of cattle comprising 18 Vechur, 25 Highrange dwarf, 23 Vatakara and 35 Kasargode animals using phenol: chloroform extraction procedure.

4.1.1 Yield and Quality of DNA

The average yield of DNA obtained per 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 1.6. (Table 4.1).

On agarose gel electrophoresis, the DNA samples appeared as single bands without shearing indicating good quality of DNA isolated.

4.2 RAPD-PCR CONDITIONS

4.2.1 Annealing Temperature

Annealing temperature was optimized to 38°C by conducting the annealing at 34-40°C with increments of 0.5°C keeping other conditions fixed.

4.2.2 Concentrations of $MgCl_2$

Different reactions which used $MgCl_2$ concentrations of 1.5 m*M*, 2 m*M*, 2.5 m*M* were tried. The differences between products obtained in the three concentrations of $MgCl_2$ were not significant. All three concentrations produced almost similar banding pattern. The optimal concentration of $MgCl_2$ was standardized as 1.5 m*M*.

4.2.3 *Taq* DNA Polymerase

Two different levels of *Taq* DNA polymerase in each reaction mixture, 0.5 unit and 1.0 unit were tried. 0.5 unit of *Taq* was found sufficient for the production of reproducible banding pattern.

4.2.4 Optimization of PCR Parameters

For efficient RAPD-PCR amplification and to minimize the production of incompletely amplified products, the different conditions were optimized in this study. The standardized reaction conditions are presented in Table 4.2.

4.3 RAPD-PCR ANALYSIS

4.3.1 Primers Used

Twenty six random oligonucleotide primers were used for the amplification of pooled DNA samples. Twenty four primers yielded products. RAPD profile of pooled DNA samples of four genetic groups with different primers differed (Plate 4).

Based on intensity, clarity and polymorphism of bands, ten primers (OPA 01, OPA 06, OPA 09, OPA 10, OPA 12, OPA 18, OPA 20, ILO 1127, ILO 876 and ILO 526) were selected for further studies. Random amplified polymorphic DNA (RAPD) analysis of individual DNA samples were carried out using these selected primers.

4.3.2 Number and Frequency of Bands

The number of bands produced by different primers ranged from four in OPA 10 and ILO 876 to 13 in OPA 18 (Table 4.3). The product size obtained ranged from 0.4 kb to 2.9 kb. The percentage of polymorphism represented by individual primer varied from zero to 100 per cent in different groups (Table 4.4).

Frequency of each band was determined from the ratio of the number of animals carrying fingerprints (n) to the total number of animals screened within a breed (N) (Gwakisa *et al.*, 1994).

4.3.3 Primer-wise Results

4.3.3.1 OPA 01 (5'CAGGCCCTTC3')

The number of bands produced in individual samples with OPA 01 ranged from four to seven. The frequency of different bands produced with their approximate size is provided in Table 4.5. The percentage of polymorphism of these primers in Vechur and Vatakara animals were 28.5 and 62.5 whereas that of Highrange dwarf and Kasargode animals were 42.8 each. The product, OPA 01 g was noticed only in four Vatakara animals but not in any other animals. The RAPD banding profile using OPA 01 primer in different genetic groups of dwarf cattle is presented in Plate 3B.

4.3.3.2 OPA 06 (5'GGTCCCTGAC3')

RAPD profile of animals amplified with OPA 06 is presented in Plate 3C. Individual samples gave to 8-9 bands when amplified with primer OPA 06. The primer had very low polymorphism in all the four groups. The different bands with their size and frequency are provided in Table 4.6. In Vehcur and Kasargode cattle, it was monomorphic whereas in the other two groups (Highrange dwarf and Vatakara cattle) it had 11.1% polymorphism. A prominent band, OPA 06 b was detected in some animals of Highrange dwarf cattle DNA samples. It needs further investigation to find the uniqueness of this band to the group. Eight bands (OPA 06 c, OPA 06 d, OPA 06 e, OPA 06 f, OPA 06 g, OPA 06 h, OPA 06 i and OPA 06 j) appeared in all the animals studied.

4.3.3.3 OPA 09 (5'GGGTAACGCC3')

Using OPA 09, the size and frequency of bands produced in different groups of animals are presented in Table 4.7. The number of bands obtained in

animals ranged from 2 to 6. The polymorphism obtained in Vechur and Vatakara animals were 85.7 per cent each. The polymorphism in Highrange dwarf and Kasargode animals were 57.1 per cent and 71.4 per cent respectively. OPA 09 produced only one common band (OPA 09 g) which appeared in all the individuals tested. The banding profile of OPA 09 is given in Plate 3A.

4.3.3.4 OPA 10 (5'GTVATCGCAG3')

RAPD fingerprints of primer OPA10 is presented in Plate 2D. The number of bands obtained by amplification with OPA 10 ranged from 1 to 4. The frequency and size of different bands produced are given in Table 4.8. The percentage of polymorphism was very high in Vechur and Kasargode (100) and it was 75 for Highrange dwarf. For Vatakara group, the polymorphism was comparatively lowest (33.3%). The band, OPA 10 d was absent in Vatakara animals.

4.3.3.5 OPA 12 (5'TCGGCGATAG3')

OPA 12 yielded 3 to 7 bands in individual samples tested. The size and frequency of each band is provided in Table 4.9. The level of polymorphism was comparatively higher for all the four groups. The percentage polymorphism observed for Highrange dwarf, Vatakara and Kasargode animals was same (85.7%) whereas it was 71.4 per cent in Vechur. A common band OPA 12 g well noticed in all the animals tested. The banding profile using OPA 12 is given in Plate 3D.

4.3.3.6 OPA 18 (5'AGGTGACCGT3')

The RAPD fingerprint of OPA 18 is presented in Plate 1B. It produced 6 to 11 bands in individual samples tested. The different bands with their size and frequency are given in Table 4.10. Fifty per cent of polymorphism was obtained in Vechur and Kasargode animals whereas it was 66.6 per cent in Highrange dwarf and 58.3 per cent in Vatakara animals. Four bands were common in all the

animals tested (OPA 18 c, OPA 18 d, OPA 18 l, OPA 18 m). OPA 18 f band was noticed only in two Vechur animals and the band OPA 18 h was absent in Vechur animals studied.

4.3.3.7 OPA 20 (5'GTTGCGATCC3')

Using OPA 20, the number of bands produced in different animals ranged from 2 to 8. The frequency of different bands produced with their approximate size is provided in Table 4.11. A polymorphism of 62.5 percent was noticed in Highrange dwarf and Kasargode animals and 87.5 percent and 66.7 percent respectively in Vatakara and Vechur. The RAPD banding profile is given in Plate 2C.

4.3.3.8 ILO 1127 (5'CCGCGCCGGT3')

ILO 1127 produced 3 to 9 bands in the individual samples tested. The frequency of bands produced with their approximate size is given in Table 4.12. The level of polymorphism was higher in all the four groups. It produced 100 per cent polymorphism in Kasargode, 90 per cent in Vechur, 77.8 per cent in Highrange dwarf and 75 per cent in Vatakara animals. ILO 1127 j band was noticed in half of Vechur animals and in one Kasargode animal whereas the band was absent in the other two groups. The fingerprinting pattern obtained using ILO 1127 is presented in Plate 2A.

4.3.3.9 ILO 526 (5'GCCGTCCGAG3')

The RAPD profile of animals with ILO 526 is presented in Plate 2B. The number of bands obtained by amplification with ILO 526 ranged from one to four. Their frequency and approximate band size is provided in Table 4.13. The level of polymoprhism was low in Kasargode (12.5%), Vatakara (28.6%) and Vechur (37.5%) and high in Highrange dwarf (75%).

4.3.3.10 ILO 876 (5'GGGACGTCTC3')

Primer ILO 876 yielded 4 to 8 bands in individual samples studied. The approximate size and frequency of bands produced are presented in Table 4.14. The level of polymorphism was high in all the four groups. It was 100 per cent polymorphic in Highrange dwarf, Vatakara and Kasargode whereas 75 per cent in Vechur. Two bands (ILO 876 d, ILO 876 e) were present invariably in all animals. The RAPD fingerprints of animals with ILO 876 is given in Plate 1A.

4.3.4 Band Sharing (BS)

4.3.4.1 Within Breed Band Sharing

RAPD fingerprints obtained with individual animal data were used for the calculation of band sharing values. All possible pair-wise combinations of different animals were made for each primer. The average within breed band sharing values were, 0.83 ± 0.03, 0.86 ± 0.04, 0.83 ± 0.03, 0.80 ± 0.04 for Vechur, Highrange dwarf, Vatakara and Kasargode animals (Table 4.15).

4.3.4.2 Intergroup Band Sharing

The interbreed band sharing calculated for different combinations were, 0.81 ± 0.03 between Vechur and Highrange dwarf, 0.82 ± 0.03 between Vechur and Vatakara, 0.80 ± 0.03 between Vechur and Kasargode, 0.82 ± 0.03 between Highrange dwarf and Vatakara, 0.80 ± 0.03 between Highrange dwarf and Kasargode and 0.80 ± 0.03 between Vatakara and Kasargode (Table 4.16).

4.3.4.3 Least Squares Analysis

The effects of genetic group, GC content of primer and the effect of gel were studied using Harvey's Least squares analysis, Model-1. The least squares means for these effects are given in Table 4.17 and the least squares analysis of variance is presented in Table 4.18. The effects of GC content of primer, genetic group and the gel on band sharing values were significant ($P<0.01$).

Ten combinations of genetic groups (VV, VH, VD, VK, HH, HD, HK, DD, DK and KK) with a total of 15400 observations on band sharing values were analysed. The analysis revealed that the band sharing values of HK was significantly different from that of Vechur, Vatakara and their combinations.

The variations in comparisons of upper gel to upper gel, lower gel to lower gel and upper gel to lower gel were analysed. A significant effect was observed for band sharing values. The upper gels had the highest band sharing values (0.84 ± 0.00) and then the lower gel (0.82 ± 0.00) and the combination had the least value (0.78 ± 0.00). From this, it was clear that between gel comparison had considerable effect on band sharing values.

Four different classes of primers based on GC content were formed (60%, 70%, 80% and 90%) and the effect of GC content of primer on band sharing was significant. Ninety percent of GC content yielded significantly lowest band sharing values followed by primers with 60%, 70% and 80%. Thus GC content of primer has a role on band sharing.

4.3.5 Percentage Difference

4.3.5.1 Average Percentage Difference

All possible interbreed pair-wise comparisons of different animals were made for the calculation of percentage difference. The average percentage difference values were calculated for all the ten primers.

4.3.5.2 Least Squares Analysis

The effect of GC content of primers, genetic group and gel were studied on percentage difference values. The least squares means for these effects are presented in Table 4.19 and the least squares analysis of variance in Table 4.20. Least square analysis of variance showed that GC content of primer, genetic group and gel had significant effect on percentage difference value ($P<0.01$).

The effect of genetic group on percentage difference value was analysed by taking ten combinations of genetic groups (VV, VH, VD, VK, HH, HD, HK, DD, DK and KK). The results show that VV (16.81 ± 0.56), VD (18.36 ± 0.39) and HD (18.16 ± 0.39) were uniform in percentage difference. They differed significantly from VH (18.36 ± 0.39), HH (19.06 ± 0.56), DK (19.07 ± 0.39), VK (19.37 ± 0.40) and KK (19.66 ± 0.56).

Gel variations on percentage difference values were analysed by taking upper, lower and their combinations. The percentage difference value was lowest in upper gel (16.25 ± 0.29) and highest in the combination (21.70 ± 0.22).

Four classes of primers with varying GC content were analysed (60%, 70%, 80%, 90%). The highest percentage difference value was observed with primers having GC content 90% (28.66 ± 0.43) followed by those with 60% (21.12 ± 0.22), 70% (14.99 ± 0.22) and 80% (9.30 ± 0.43).

4.3.5.3 Mean Average Percentage Difference (MAPD)

The interbreed dissimilarities were calculated on RAPD fingerprints obtained with10 primers. All interbreed pair-wise comparisons of individual animals in each group were made. The value for Vechur x Highrange dwarf was 19.21, Vechur x Vatakara 17.93, Vechur x Kasargode 19.04, Highrange dwarf x Vatakara 18.68, Highrange dwarf x Kasargode 20.92 and Vatakara x Kasargode 19.64 (Table 4.21).

4.3.6 Genetic Distance (D)

The genetic distance among four cattle groups in Kerala were computed using Nei's method as given by Kumar, *et al.*, 2000. It revealed values 0.006 between Vechur and Vatakara, 0.012 between Vatakara and Kasargode, 0.014 between Vechur and Kasargode, 0.031 between Vatakara and Highrange dwarf, 0.039 between Vechur and Highrange dwarf and the highest value 0.046 for Highrange dwarf and Kasargode. The results are given in Table 4.22.

It showed the same results as given by mean average percentage difference with lowest genetic distance for Vechur and Vatakara and highest for Highrange dwarf and Kasargode.

4.3.7 Dendrogram

A dendrogram of relationship of animals in four genetic groups were constructed using RAPD fingerprints by unweighted pair Group Arithmetic Mean (UPGMA) method (Plate 6).

Table 4.1. The yield and purity of DNA extracted from four genetic groups of dwarf cattle of Kerala

	Vechur	Highrange dwarf	Vatakara	Kasargode	Overall average
Number of samples	18	25	23	35	
Average yield (µg) (Mean ± SE)	80.2 ± 4.3	90.5 ± 5.8	208 ± 31.3	57.6 ± 3.1	110.1 ± 9.4
Average OD 260/ OD 280	1.6	1.6	1.7	1.6	1.6

Table 4.2. Optimum PCR conditions applied for RAPD-PCR analysis

Parameter/variable	Level
$MgCl_2$	1.5 m*M*
Taq DNA polymerase	0.5 unit
Primer concentration	5 picomoles
Template DNA	25 ng
dNTPs	200 µ*M*
Reaction volume	20 µl
Denaturation	94°C, 1 minute
Primer annealing	38°C, 1 minute
Primer extension	72°C, 1 minute
Number of cycles	35

Table 4.3. The number of bands detected in four groups of cattle for different primers

Primer	Genetic group							
	Vechur		Highrange dwarf		Vatakara		Kasargode	
	Range	Average ± SE	Range	Average ± SE	Range	Average ± SE	Range	Average ± SE
OPA 01	5 – 7	6.4±0.2	5 – 7	5.7±0.2	5 – 8	5.9±0.3	4 – 7	5.1±0.3
OPA 06	8	8.0±0	8 – 9	8.3±0.1	8 – 9	8.1±0.1	8	8.0±0.1
OPA 09	3 – 6	4.4±0.3	4 – 6	4.7±0.2	3 – 6	4.6±0.3	3 – 6	4.6±0.3
OPA 10	1 – 4	2.1±0.2	2 – 3	2.1±0.1	2 – 3	2.1±0.1	1 – 3	2.1±0.1
OPA 12	4 –6	5.0±0.2	3 – 5	4.4±0.2	3 – 7	4.6±0.3	3 – 6	4.1±0.2
OPA 18	8 – 11	8.9±0.3	7 – 11	8.5±0.3	6 – 10	8.1±0.3	7 – 9	8.7±0.2
OPA 20	5 – 8	6.6±0.3	2 – 7	5.9±0.3	4 – 7	5.1±0.2	5 – 7	5.4±0.2
ILO 1127	3 – 9	5.6±0.5	3 – 8	5.2±0.4	4 – 7	5.3±0.3	4 – 8	6.1±0.3
ILO 526	2 – 4	2.8±0.2	1 – 4	2.9±0.2	2 – 4	2.9±0.3	1 – 4	2.4±0.3
ILO 876	4 – 7	6.5±0.2	4 - 7	6.2±0.2	6 - 7	6.6±0.1	6 - 8	7.2±0.2

Table 4.4. Percentage of polymorphism of RAPD bands for different primers

Primer	Genetic group			
	Vechur	Highrange dwarf	Vatakara	Kasargode
OPA 01	28.5	42.8	62.5	42.8
OPA 06	0	11.1	11.1	0
OPA 09	85.7	57.1	85.7	71.4
OPA 10	100	75	33.3	100
OPA 12	71.4	85.7	85.7	85.7
OPA 18	50	66.6	58.3	50
OPA 20	66.7	62.5	87.5	62.5
ILO 1127	90	77.8	75	100
ILO 526	37.5	75	28.6	12.5
ILO 876	75	100	100	100

Table 4.5. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer OPA 01

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	OPA 01 a	1.9	1	1	0.79	1
OPA 01	OPA 01 b	1.6	1	1	0.93	1
	OPA 01 c	1.2	0.93	0.64	0.28	0.5
	OPA 01 d	1.0	1	1	1	1
	OPA 01 e	0.9	0.57	0.35	0.71	0.14
	OPA 01 f	0.7	0.86	0.79	0.93	0.35
	OPA 01 g	0.6	0	0	0.28	0
	OPA 01 h	0.5	1	1	1	1

kb – kilo base pair

Table 4.6. Frequency and approximate size of bands in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer OPA 06

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	OPA 06 a	2.6	0	0	0.14	0
OPA 06	OPA 06 b	2.5	0	0.28	0	0
	OPA 06 c	1.9	1	1	1	1
	OPA 06 d	1.7	1	1	1	1
	OPA 06 e	1.4	1	1	1	1
	OPA 06 f	1.2	1	1	1	1
	OPA 06 g	0.9	1	1	1	1
	OPA 06 h	0.7	1	1	1	1
	OPA 06 i	0.7	1	1	1	1
	OPA 06 j	0.5	1	1	1	1

kb – kilo base pair

Table 4.7. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer OPA 09

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	OPA 09 a	1.4	0.57	0.35	0.50	0.428
OPA 09	OPA 09 b	1.2	0.71	0.64	0.64	0.64
	OPA 09 c	1.0	0.93	1	1	1
	OPA 09 d	0.9	0.64	1	0.79	0.64
	OPA 09 e	0.7	0.35	0.21	0.21	0.64
	OPA 09 f	0.7	0.43	0.50	0.50	0.28
	OPA 09 g	0.5	1	1	1	1

kb – kilo base pair

Table 4.8. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer OPA 10

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	OPA 10 a	1.0	0.86	1	1	0.86
OPA 10	OPA 10 b	0.9	0.21	0.21	0.14	0.21
	OPA 10 c	0.8	0.86	0.71	1	0.86
	OPA 10 d	0.4	0.14	0.21	0	0.28

kb – kilo base pair

Table 4.9. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer OPA 12

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	OPA 12 a	1.5	0.43	0.21	0.28	0.14
OPA 12	OPA 12 b	1.3	0.43	0.57	0.71	0.71
	OPA 12 c	1.3	0.50	0.57	0.50	0.50
	OPA 12 d	1.0	0.93	0.71	0.71	0.71
	OPA 12 e	0.8	1	0.79	0.79	0.79
	OPA 12 f	0.6	0.71	0.57	0.64	0.43
	OPA 12 g	0.3	1	1	1	1

kb – kilo base pair

Table 4.10. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer OPA 18

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	OPA 18 a	2.3	1	0.85	0.85	1
OPA 18	OPA 18 b	1.8	1	0.92	1	1
	OPA 18 c	1.5	1	1	1	1
	OPA 18 d	1.4	1	1	1	1
	OPA 18 e	1.2	0.21	0.12	0.21	0.28
	OPA 18 f	1.1	0.14	0	0	0
	OPA 18 g	0.9	0.90	0.64	0.64	0.71
	OPA 18 h	0.9	0	0.35	0.14	0.07
	OPA 18 i	0.8	0.85	0.50	0.35	0.42
	OPA 18 j	0.7	0.14	0.42	0.50	0.50
	OPA 18 k	0.6	0.64	0.71	0.40	0.14
	OPA 18 l	0.5	1	1	1	1
	OPA 18m	0.4	1	1	1	1

kb – kilo base pair

Table 4.11. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer OPA 20

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	OPA 20 a	2.3	1	1	0.85	1
OPA 20	OPA 20 b	1.7	0.85	0.79	0.57	0.71
	OPA 20 c	1.6	0.07	0	0	0
	OPA 20 d	1.5	1	1	1	1
	OPA 20 e	1.3	0.28	0.57	0.07	0.07
	OPA 20 f	1.2	0.93	0.79	0.71	0.57
	OPA 20 g	1.1	0.64	0.43	0.43	0.50
	OPA 20 h	1.0	0.86	0.43	0.43	0.50
	OPA 20 i	0.7	1	1	1	1

kb – kilo base pair

Table 4.12. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer ILO 1127

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	ILO 1127 a	2.9	0.35	0.35	0.14	0.42
ILO 1127	ILO 1127 b	2.5	0.42	0.64	0.71	0.85
	ILO 1127 c	2.2	0.07	0.07	0.78	0.85
	ILO 1127 d	1.8	0.85	1	1	0.92
	ILO 1127 e	1.8	0.14	0.21	0	0.14
	ILO 1127 f	1.4	1	1	1	0.93
	ILO 1127 g	1.4	0.43	0.28	0.28	0.50
	ILO 1127 h	0.9	0.14	0.50	0.57	0.21
	ILO 1127 i	0.9	0.78	0.42	0.78	0.85
	ILO 1127 j	0.5	0.50	0	0	0.07

kb – kilo base pair

Table 4.13. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer ILO 526

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	ILO 526 a	0.8	1	0.86	0.86	0.86
ILO 526	ILO 526 b	0.7	0.79	0.79	0.71	0.35
	ILO 526 c	0.5	0.71	0.86	0.86	0.86
	ILO 526 d	0.4	0.35	0.35	0.43	0.35

kb – kilo base pair

Table 4.14. Frequency and approximate size of bands produced in Vechur, Highrange dwarf, Vatakara and Kasargode cattle with primer ILO 876

Primer	Bands	Approximate size (kb)	Vechur	Highrange dwarf	Vatakara	Kasargode
	ILO 876 a	2.1	1	0.93	1	1
ILO 876	ILO 876 b	1.7	1	0.93	1	1
	ILO 876 c	1.3	0.64	0.93	0.64	0.64
	ILO 876 d	1.2	1	1	1	1
	ILO 876 e	0.6	1	1	1	1
	ILO 876 f	0.5	1	0.85	1	1
	ILO 876 g	0.4	0.35	0.14	0	0.50
	ILO 876 h	0.4	0.50	0.50	0.93	1

kb – kilo base pair

Table 4.15. Band sharing values within Vechur, Highrange dwarf, Vatakara and Kasargode cattle for different primers and their average

Primers	Genetic group			
	Vechur	Highrange dwarf	Vatakara	Kasargode
OPA 01	0.95	1.00	0.88	0.93
OPA 06	1.00	1.00	0.98	0.98
OPA 09	0.71	0.96	0.75	0.73
OPA 10	0.72	0.64	0.95	0.69
OPA 12	0.78	0.88	0.71	0.7
OPA 18	0.90	0.80	0.82	0.86
OPA 20	0.85	0.77	0.73	0.80
ILO 1127	0.69	0.79	0.77	0.69
ILO 876	0.76	0.84	0.74	0.67
ILO 526	0.88	0.90	0.95	0.93
Average ± SE	0.83 ± 0.03	0.86 ± 0.04	0.83 ± 0.03	0.80 ± 0.04

Table 4.16. Interbreed band sharing values for different combinations of dwarf cattle of Kerala for different primers and their average

Primers	Genetic group					
	Vechur x Highrange dwarf	Vechur x Vatakara	Vechur x Kasargode	Highrange dwarf x Vatakara	Highrange dwarf x Kasargode	Vatakara x Kasargode
OPA 01	0.94	0.91	0.90	0.89	0.93	0.85
OPA 06	0.98	0.99	1.00	0.99	1.00	0.99
OPA 09	0.76	0.74	0.73	0.80	0.75	0.73
OPA 10	0.74	0.83	0.72	0.76	0.69	0.80
OPA 12	0.73	0.75	0.73	0.69	0.67	0.71
OPA 18	0.83	0.85	0.85	0.81	0.80	0.84
OPA 20	0.81	0.79	0.82	0.77	0.80	0.78
ILO 1127	0.68	0.70	0.68	0.79	0.70	0.72
ILO 876	0.76	0.76	0.69	0.81	0.70	0.69
ILO 526	0.87	0.90	0.90	0.88	0.86	0.93
Average ± SE	0.81 ± 0.03	0.82 ± 0.03	0.80 ± 0.03	0.82 ± 0.03	0.79 ± 0.03	0.80 ± 0.03

Table 4.17. Least squares means of band sharing values for different genetic groups, G C content of primer and gel

Factors		BS value
Variable	Level	Least square mean ± SE
GC content of primer	60%	0.79 ± 0.00^{a}
	70%	0.85 ± 0.00^{b}
	80%	0.91 ± 0.00^{c}
	90%	0.71 ± 0.00^{d}
Genetic group	VV	0.83 ± 0.00^{b}
	VH	0.82 ± 0.01^{c}
	VD	0.83 ± 0.00^{b}
	VK	0.81 ± 0.00^{c}
	HH	0.81 ± 0.00^{c}
	HD	0.82 ± 0.01^{c}
	HK	0.80 ± 0.00^{a}
	DD	0.83 ± 0.00^{b}
	DK	0.81 ± 0.01^{c}
	KK	0.80 ± 0.00^{c}
Gel	Upper	0.84 ± 0.00^{a}
	Lower	0.82 ± 0.00^{b}
	Combination	0.78 ± 0.00^{c}

The least square means with same superscript within the same variable does not vary significantly ($P \leq 0.01$)

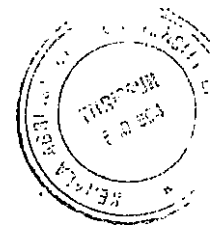


Table 4.18. Least squares analysis of variance for the effects of GC content of primer, genetic group and gel on band sharing

Source	df	Mean sum of squares	F value	P
GC content of primer	3	13.54	499.06**	0.00
Genetic group	9	0.20	7.44**	0.00
Gel	2	4.53	166.88**	0.00
Error	15385	0.03		
Total	15400			

** $P \leq 0.01$

Table 4.19. Least squares mean of percentage difference values for genetic groups, GC content of primer and gel

Factors		PD value
Variable	Level	Least squares mean ± SE
GC content of primer	60%	21.12 ± 0.22[a]
	70%	14.99 ± 0.22[b]
	80%	9.30 ± 0.43[c]
	90%	28.66 ± 0.43[d]
Genetic group	VV	16.81 ± 0.56[a]
	VH	18.36 ± 0.39[b]
	VD	17.44 ± 0.38[d]
	VK	19.37 ± 0.40[b]
	HH	19.06 ± 0.5b[b]
	HD	18.16 ± 0.39[a]
	HK	20.41 ± 0.39[b]
	DD	16.83 ± 0.56[d]
	DK	19.07 ± 0.39[b]
	KK	19.66 ± 0.56[b]
Gel	Upper	16.25 ± 0.29[a]
	Lower	17.61 ± 0.29[b]
	Combination	21.70 ± 0.22[c]

The least square means with same superscript within the same variable does not vary significantly ($P \leq 0.01$)

Table 4.20. Least squares analysis of variance for the effects of GC content of primer, genetic group and gel on percentage difference

Source	df	Mean squares	F value	P
GC content of primer	3	135383.3	499.10**	0.00
Genetic group	9	2017.71	7.44**	0.00
Gel	2	4526.6	166.88**	0.00
Error	15385	271.25		
Total	15400			

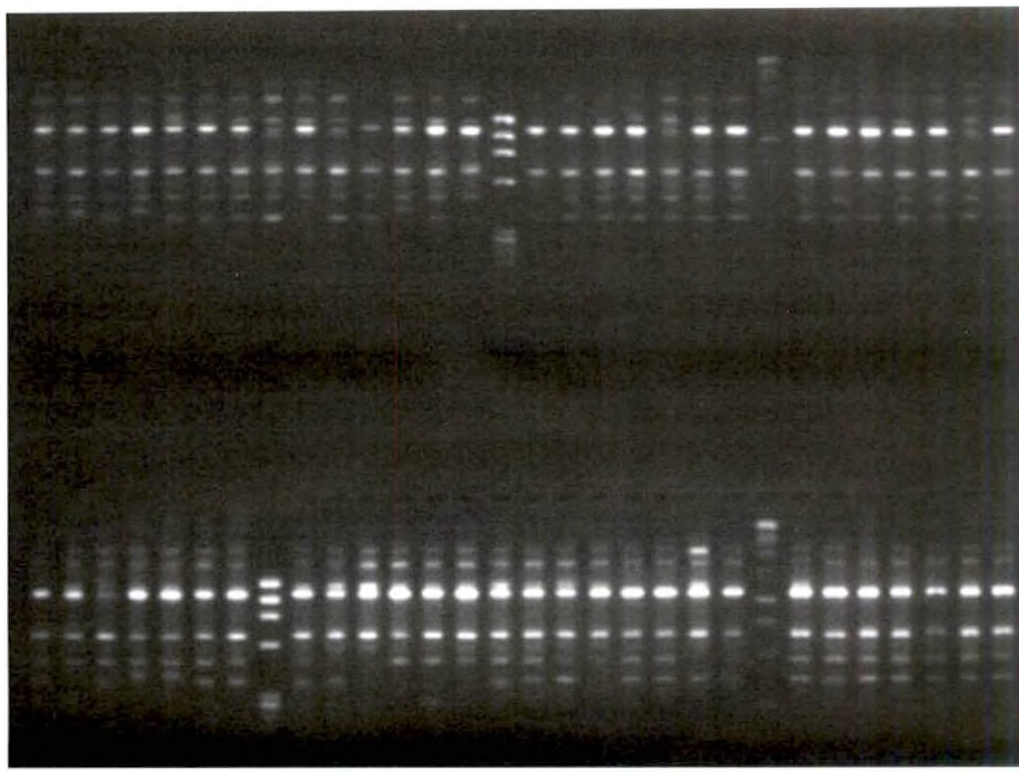
** $P \leq 0.01$

Table 4.21. The Mean average percentage difference (MAPD) of 4 different groups of cattle for 10 primers

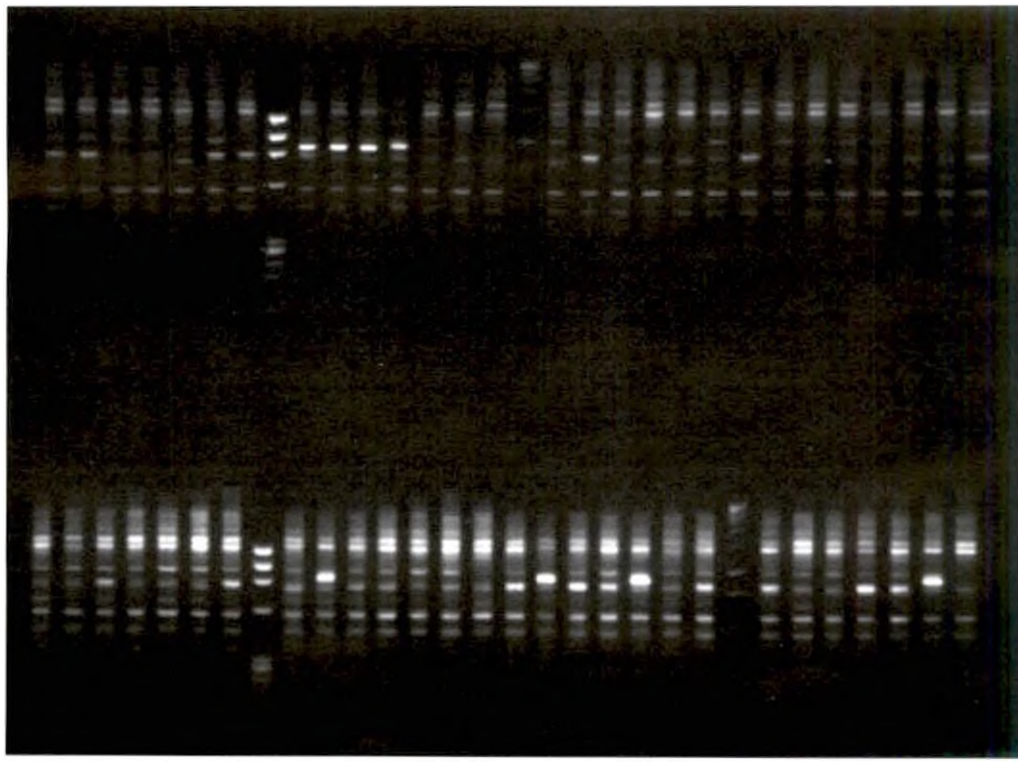
	Highrange dwarf	Vatakara	Kasargode
Vechur	19.21	17.93	19.9
Highrange dwarf		18.68	20.92
Vatakara			19.64

Table 4.22. The Genetic distance (D) among four cattle groups in Kerala

	Vechur	Highrange dwarf	Vatakara
Highrange dwarf	0.039		
Vatakara	0.006	0.031	
Kasargode	0.014	0.046	0.012



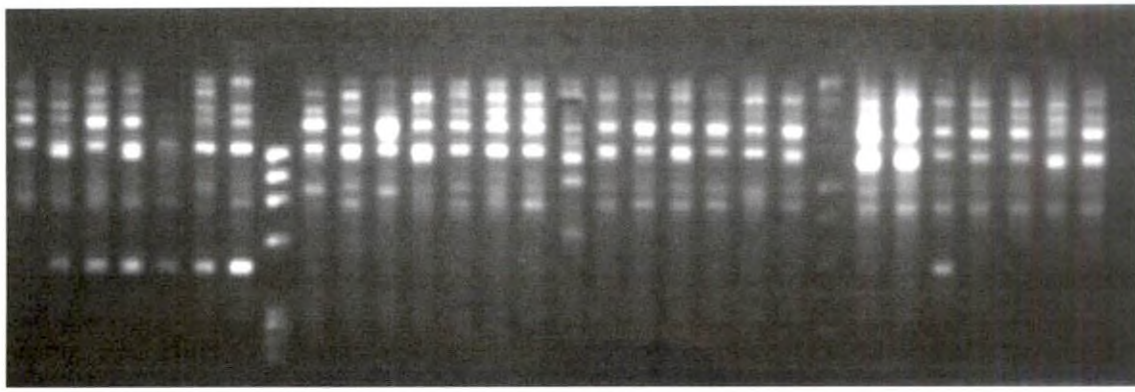
A



B

PLATE 1: RAPD profile of indivdual samples with primer A: ILO 876 B: OPA 18

A: 1-7 and 31-37 V, 8-14 and 39-45 H, 16-22 and 46-52 D, 24-30 and 54-60 K, 8 and 38 M1,15 and 53 M2
B: 1-7 and 31-37 V, 9-15 and 39-45 H, 16-22 and 46-52 D, 24-30 and 54-60 K, 8 and 38 M1,23 and 53 M2
V- Vechur, H- Highrange dwarf, D- Vatakara, K- kasargode, M1- phiX 174 Hae III digest, M2- 1 kb ladder



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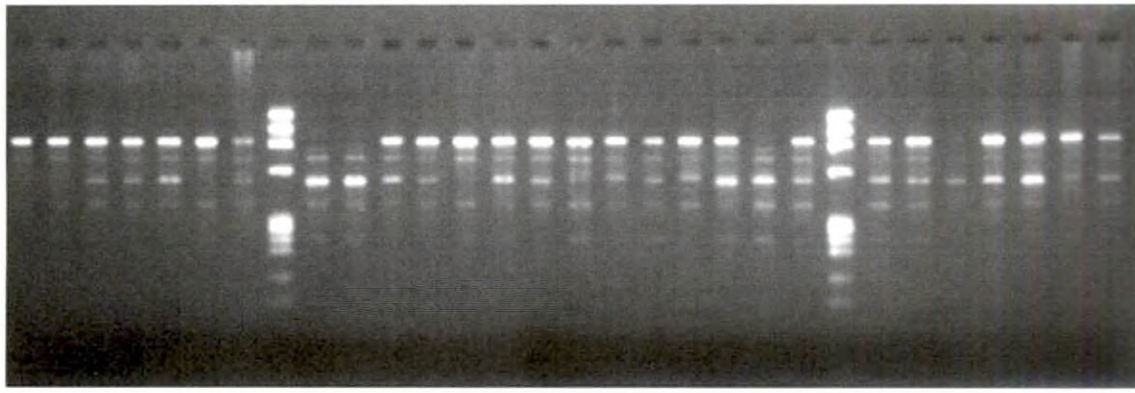

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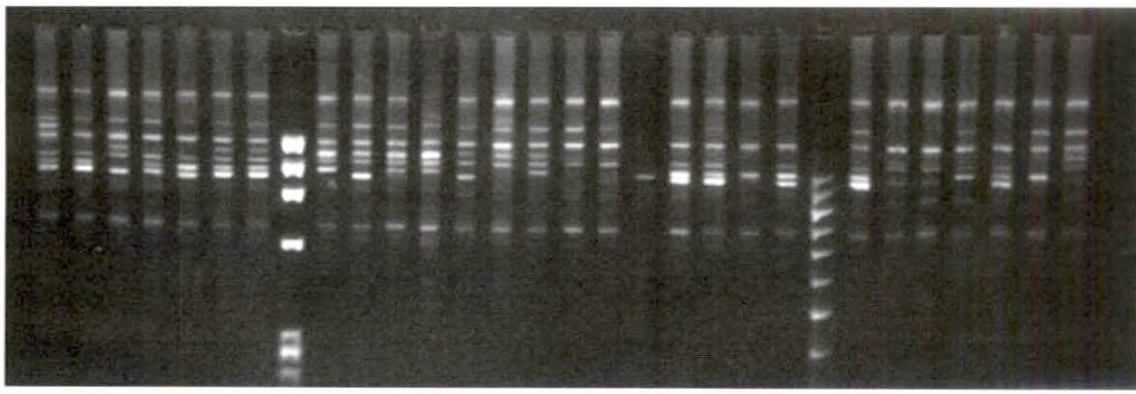

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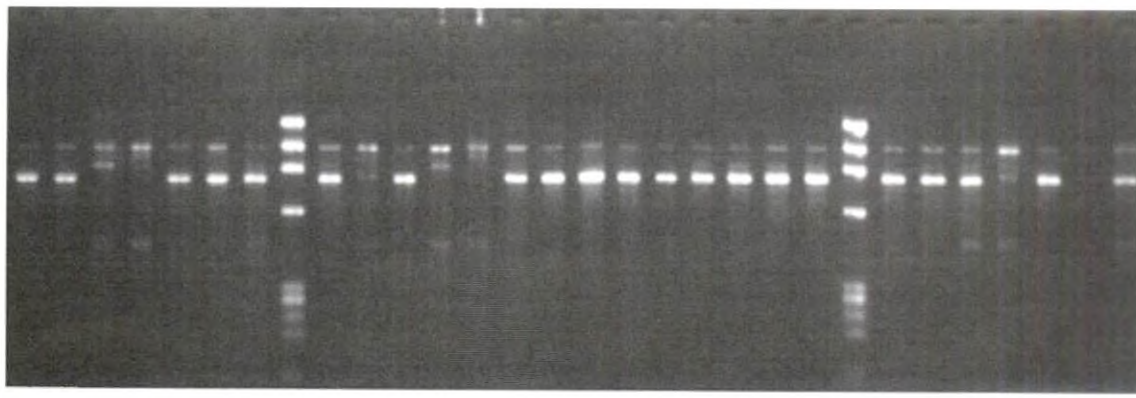
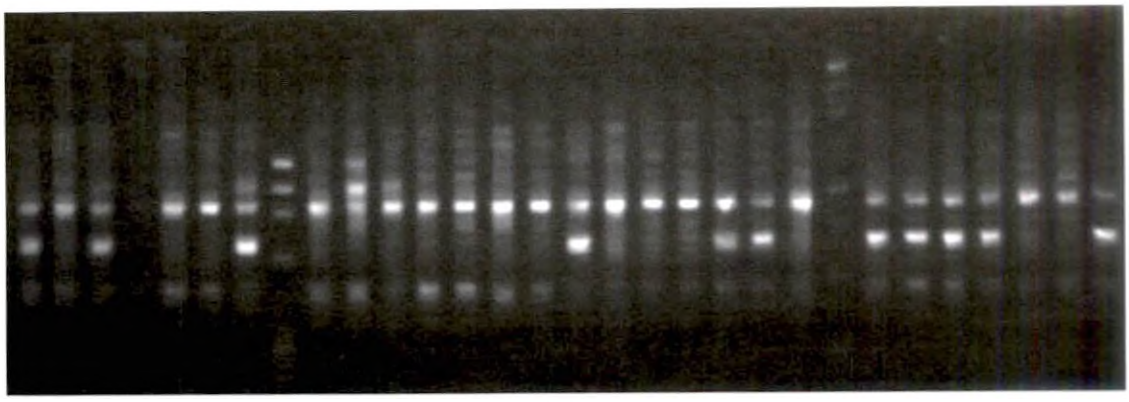


D

PLATE 2: A. ILO 1127 B. ILO 526 C. OPA 20 D. OPA 10
1-7 V, 9-15 H, 16-22 D, 24-30 K, 8- phiX174 Hae III digest, 23- 1kb ladder
V- Vechur, H- Highrange dwarf, D- Vatakara, K- Kasargode



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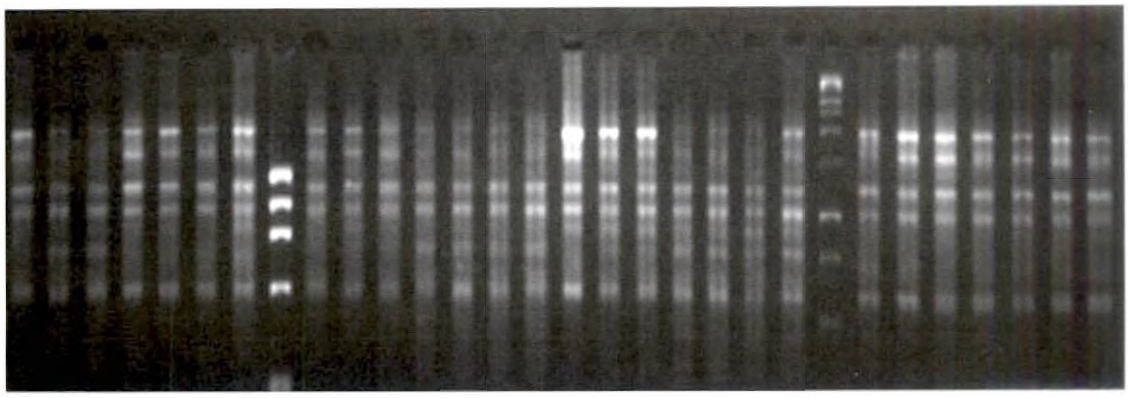
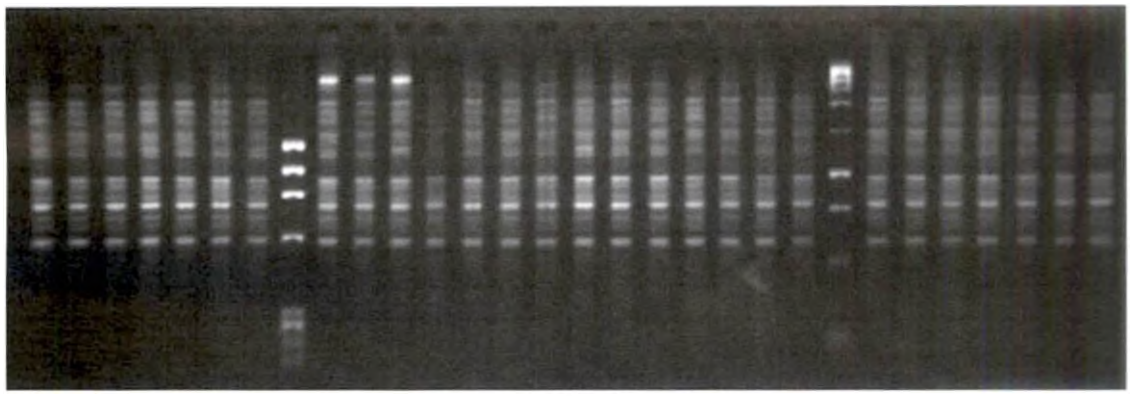
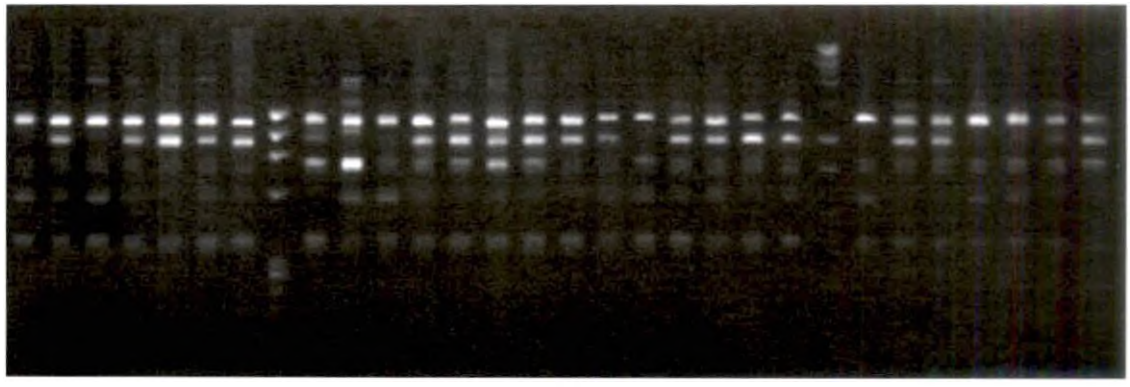


B



C



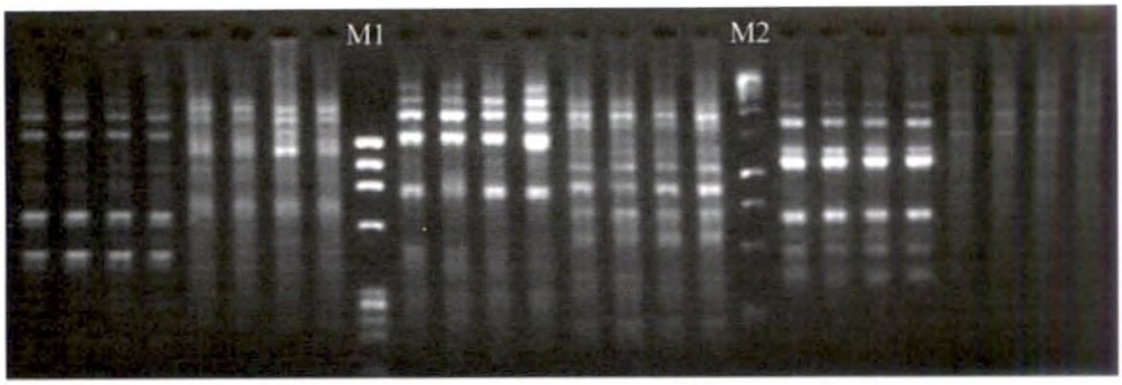
D

PLATE 3: RAPD fingerprints of individdual DNA samples.

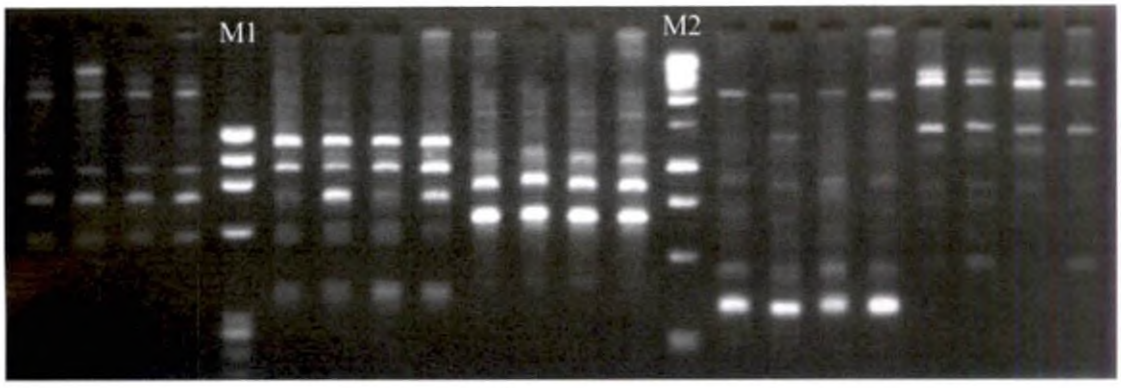
A. OPA 09 B. OPA 01 C. OPA 06 D. OPA 12

1-7 V, 9-15 H, 16-22 D, 24-33 K, 8-phiX174 Hae III digest, 23- 1 kb ladder

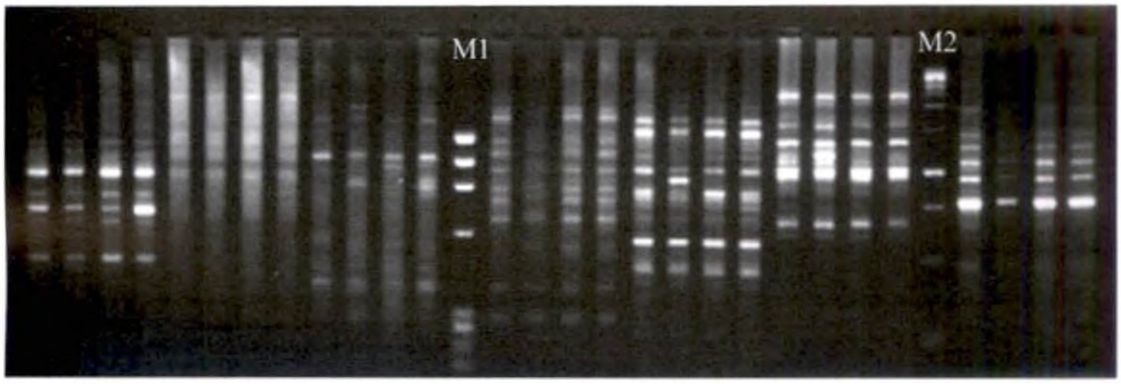
V- Vechur, H- Highrange dwarf, D- Vadakara, K- Kasargod



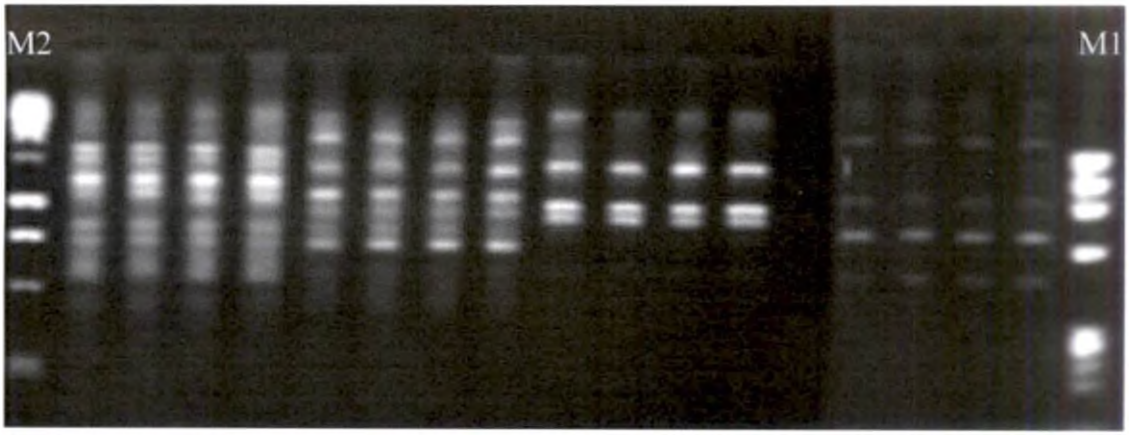
A



B



C



D

PLATE 4: RAPD fingerprints of pooled DNA samples of Vechur, Highrange dwarf, Vatakara and Kasargode respectively with different primers

A. Primer G1, G2, ILO 1127, ILO 876, ILO 526, OPAV 15
B. Primer OPA 06, OPA 12, OPA 15, OPA 19, OPA
C. Primer OPA 09, OPA 13, OPA11, OPA14, OPA 18, OPA 20, OPA10
D. Primer OPA O4, OPA05, OPA 07, OPA 08

M1 - phiX174 HaeIII digest
M2- 1kb DNA ladder

VECHUR

VATAKARA

HIGHRANGE DWARF

KASARGODE

PLATE: 5. The dwarf cattle of Kerala

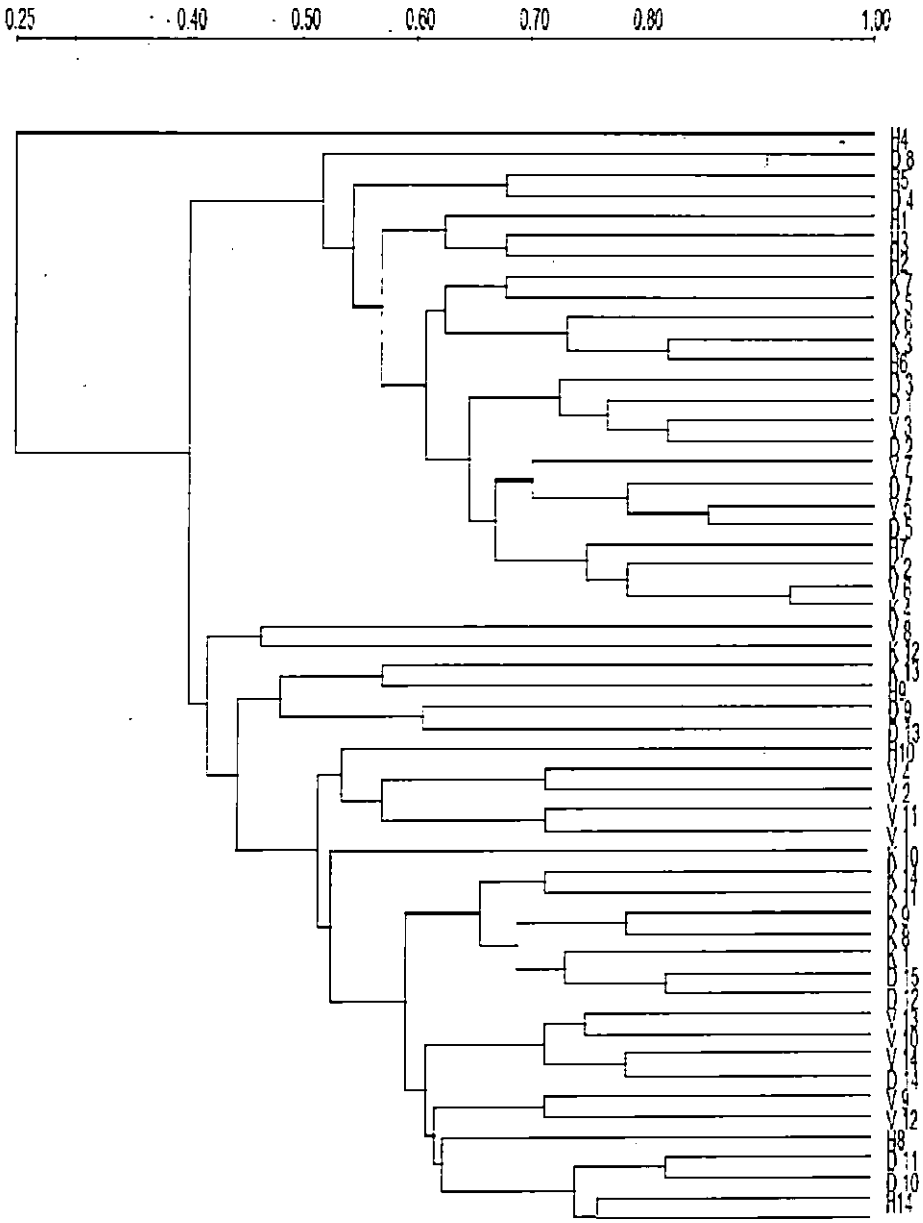


Plate 6. Dendrogram of relationship between 52 animals of four genetic groups using UPGMA method

V1-V14 Vechur, D1-D14 Vatakara, K1-K14 Kasargode and H1-H10 and H 14 Highrange dwarf cattle

Discussion

5. DISCUSSION

Random amplified polymorphic DNA, RAPD uses a single short primer (10 bases) of arbitrary sequence and a lower annealing temperature than the average PCR. These modifications lower the specificity of the reaction so that more number of fragments get amplified from most complex genomes (Williams *et al.*, 1993). The greatest advantage of this technique is the ability to obtain DNA polymorphisms without prior knowledge of genomic sequence. Universal set of primers can be used for genomic analysis in a wide variety of species. The technique is simple, fast and relatively cheaper permitting rapid generation of almost unlimited number of polymorphism. An attempt was made using RAPD-PCR technique to characterize the dwarf cattle of Kerala and to find out the genetic relationship among these groups.

5.1 ISOLATION OF GENOMIC DNA

The mean yield of DNA obtained in this study from five ml of whole blood was 110.07 ± 9.4 µg using phenol: chloroform extraction procedure. Overnight incubation with sodium dodecyl sulphate (SDS) and proteinase k was the modification made in this study, to make sure the complete digestion of proteins. Andersson *et al.* (1986) reported the yield of 150-250 µg DNA from 10 ml of whole blood in cattle whereas Aravindakshan *et al.* (1998) obtained a higher yield of 394.5 ± 34.26 µg. The yield from 20 ml of blood reported by Montgomery and Sise (1990) was 500 ± 0.19 µg in sheep.

5.2 RAPD-PCR CONDITIONS

The RAPD-PCR conditions to get reproducible banding pattern varies from laboratories to laboratories (Penner *et al.*, 1993; Rothuizen and Wolferen, 1994; Ambady *et al.*, 1996; Parejo *et al.*, 1997). Standardization of PCR

conditions for the laboratory was done under the study. The various levels of variables which were best suited were identified after conducting a series of tests.

One of the important variables in RAPD procedure is the annealing temperature. Annealing temperature of 35°C or 36°C are routinely used by researchers. Gwakisa *et al.* (1994); Kemp and Teale (1994); Aravindakshan and Nainar (1998) and Ramesha *et al.* (2002) applied the annealing temperature of 35°C in RAPD-PCR works. Yeo *et al.* (2002) and Yoon and Park (2002) standardized the annealing temperature at 36°C in their studies. Erlich *et al.* (1991) and Haff (1993) recommended the annealing temperature of 37°C. Annealing temperature above 40°C prevented amplification by many of the 10 base primers (Williams *et al.*, 1990). In the present study, the annealing temperature was fixed at 38° which was significantly higher than those selected by many other researchers. Because of the increased annealing temperature, the number of products produced was slightly lower than those reported. But the reproducibility of the bands produced was satisfactory.

Another important variable is the concentration of magnesium, which was reported to affect polymerase activity and efficiency of annealing. Since insufficient magnesium ions leads to reduced number of products and excess magnesium ions results in nonspecific products, the level fixed is very critical for optimum RAPD-PCR conditions. In this study, no significant differences in the product pattern were observed with 1.5 m*M*, 2 m*M* and 2.5 m*M* $MgCl_2$ in 20 µl reaction. Hence, the lowest concentration tested namely 1.5 m*M* was fixed as the optimal level of magnesium chloride. Williams *et al.* (1990), Kemp and Teale (1994) and Gwakisa *et al.* (1994) recommended 1.5 m*M* $MgCl_2$ in 25 µl, 10 µl, 10 µl reaction respectively and Rothuizen and Wolferen (1994) recommended 2.5 m*M* $MgCl_2$ in 35 µl reaction as the optimum concentration. But Welsh and McClelland (1990) used a higher level, 4 m*M* in RAPD-PCR assay in 10 µl reaction.

Of the two quantities (0.5 unit and 1 unit) of *Thermophilus aquaticus* (*Taq*) DNA polymerase, tested, the lower level of 0.5 units per 20 µl reaction was adjudged as sufficient for RAPD-PCR. This is half of that reported by Aravindakshan and Nainar in 1998 and Ramesha in 2002. Williams *et al.* (1990) used 0.5 units *Taq* DNA polymerase per 25 µl reaction. Bowditch *et al.* (1993) suggested that, 0.02 units per µl of *Taq* polymerase is sufficient for RAPD reactions using 0.2-0.6 ng/µl of template DNA with 0.24 p*M*/µl of primer DNA. Excess *Taq* polymerase can cause the products to appear as a smear rather than discrete bands. Rothuizen and Wolferen (1994) used 1.25 µl of *Taq* DNA polymerase in 35 µl reactions.

A primer concentration of 5 p*M* in 20 µl reaction was used in this study, much lower than that suggested by Gwakisa *et al.* (1994) and Kemp and Teale (1994). They recommended 6 p*M* in 10 µl reactions. Ramesha *et al.* (2002) used much higher level of 40 p*M* in 30 µl reaction. 0.2 µ*M* was the recommended level of primer by Williams *et al.* (1990, 1993). Aravindakshan and Nainar (1998) employed 15 ng primer in 20 µl reaction.

5.3 RAPD-PCR ANALYSIS

5.3.1 Primers

The products of reaction depends on the sequence and length of oligonucleotide primers as well as reaction conditions. At appropriate annealing temperature, the primer binds to the complementary sequence on both strands of genomic DNA. A discrete DNA segment is produced when the primer binding sites are within an amplifiable distance of each other.

In the present study, 26 random oligonucleotide primers of 10 bp length were used. Of these, 24 primers yielded amplified products after RAPD-PCR and 10 primers were selected for individual sample study. Kemp and Teale (1994) also used 26 random 10 base oligonucleotide primers in pooled samples. Aravindakshan and Nainar (1998) used three primers in individual analysis and

Ramesha *et al.* (2002) evaluated seven primers in individual samples for detecting genetic variation in cattle breeds. Different primers produce different RAPD bands, the variations are due to differences in primer binding sites. Mutations that inhibit primer binding or otherwise interfere with amplification can be detected as the absence of bands or presence of new bands in those individuals (Bowditch *et al.*, 1993).

In this study, the amplification with ILO 1127 produced a distinct band (ILO 1127j) which was present in half of Vechur animals and in one Kasargode cattle only. Ramesha *et al.* (2002) observed two breed specific bands, one for Malnad Gidda and other in Amritmahal breed with the same primer. Gwakisa *et al.* (1994) used ILO 1127 to differentiate *B. taurus* and *B. indicus* cattle. 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).

With ILO 526, 75 per cent polymorphism was observed in Highrange dwarf cattle whereas it was only 12.5 per cent in Kasargode cattle. The other two genetic groups had the polymorphic patterns in between these two. This is an indication of uniformity in genetic content of Kasargode cattle and diversity in Highrange dwarf cattle. Ramesha *et al.* (2002) observed moderate to high levels of polymorphism with this primer in South Indian cattle breeds. They identified a specific fragment which was found in Krishna Valley and Ongole breeds, but was absent in Malnad Gidda, Amritmahal, Hallikar, Deoni and Khillari. Gwakisa *et al.* (1994) were unable to identify breed specific bands with ILO 526 in zebu cattle breeds of Tanzania.

Primer ILO 876 produced one to four bands in various genetic groups of dwarf cattle of Kerala. The polymorphism was high in all the four groups. Two bands ILO 876 d and ILO 876 e were common to all animals tested. Ramesha *et al.* (2002) was able to obtain 0.95 kb product similar to one reported in African zebu breeds. Similar findings were also reported by Nagaraja (1998).

A prominent population specific band of 2.5 kb (OPA 06 b) was identified in Highrange dwarf cattle with the primer OPA 06, may be specific to that genetic group since it was absent in other animals screened. A detailed investigation is needed to ascertain the hypothesis. OPA 06 produced monomorphic patterns in Vechur and Kasargode and eight common bands were detected. Antoniou and Skidmore (1995) was able to identify a 3100 bp male specific fragment in bovines with the primer OPA 06. All the female samples tested failed to produce the samples.

A product OPA 01 g was identified only in four Vatakara cattle with frequency of 0.28. The band OPA 01 h was common to animals in all the four groups. It produced moderate degree of polymorphism in all the groups studied.

The polymorphicity of OPA 09 was significantly higher as evidenced by the fact that only one common band OPA 09 g appeared in all animals studied.

Primer OPA 10 was also highly polymorphic. One of the products of its amplification, OPA 10 d was absent in all Vatakara animals. This information can be made used in verifying the purity of Vatakara animals.

The polymorphism was very high with OPA 12 in different genetic groups. The frequency of OPA 12 e band produced by OPA 12 amplification was 0.79 for all groups except Vechur in which it was one. One common band OPA 12 g found in all the animals sampled.

With the primer OPA 18, two significant findings were observed in Vechur breed. A distinct band, OPA 18 h was absent in all the Vechur animals and the band OPA 18 f was present in only few of Vechur animals studied. It produced four common bands.

By the use of primer OPA 20, two common bands namely OPA 20 d and OPA 20 i were identified. The band OPA 20 c was absent in all the animals except a couple of Vechur animals.

The possibility of use of RAPD markers in genetic characterization of various animals was established by many scientists. The works of Bardin *et al.* (1994) in cattle, Bailey and Lear (1994) in Thoroughbred and Arabian horses, Gwakisa *et al.* (1994) in zebu cattle breeds of Tanzania, Kemp and Teale (1994) in *B. taurus* and *B. indicus* species, Kantanen *et al.* (1995) in Finnish cattle and Finsheep, Aravandakshan and Nainar (1998) in cattle and buffaloes, Parejo *et al.* (1998) in cattle, Natarajan (1998) in indigenous sheep breeds, Ramesha *et al.* (2002) in South Indian cattle breeds and Yeo *et al.* (2002) in Korean cattle using RAPD markers as a tool for genetic characterization is confirmed by this study. The presence of genetic group specific products with different primers paves a way for the use of these information in genetic group identification. Absence of some amplification products in different genetic groups of dwarf cattle of Kerala will also be useful as a confirmation tool for the genetic group identification.

5.3.2 Band Sharing

The band sharing values within and between the four genetic groups were calculated under this study as an indicator of genetic similarities. Pairwise comparison and band sharing value estimates were used by Gwakisa *et al.* (1994), Mohd-Azmi *et al.* (2000); Ramesha *et al.* (2002) and Yoon and Park (2002). The various variables affecting the band sharing values were taken into account on least square analysis of variance. The effect of genetic group, gel and GC content of primer were found significant.

The within group bandsharing values of Vechur and Vatakara animals are comparatively higher than Kasargode and Highrange dwarf animals, indicating more within group homogeneity in Vechur and Vatakara animals. The between group bandsharing values not differ significantly.

Of the ten different combinations of genetic groups of animals compared the band sharing values of HK (Highrange dwarf x Kasargode) was significantly different from that of Vechur, Vatakara and their combination. The significantly

low value of band sharing for HK compared with VD and VV genetic group suggests that HK is heterogenous compared to the other two.

Gwakisa *et al.* (1994) made 15 pairwise comparisons in each RAPD primers and concluded that interbreed band sharing was lower than intrabreed band sharing in three zebu cattle breeds of Tanzania. Aravindakshan and Nainar (1998) got similar results with band sharing values with breeds of cattle and buffaloes studied. Smith *et al.* (1996) established that band sharing value between turkies and chicken was lower than those between populations of same species. But Kantanen *et al.*(1995) in their study on sheep population found that genetic variation between the populations were smaller than genetic variation within grey sheep.

In RAPD analysis, minute variations in different factors can affect the product profile. In a comparison between three types of gels namely upper gel, lower gel and the combination, a significant effect was observed for band sharing values. The upper gels had the highest band sharing values followed by the lower gel and the combination respectively. This indicates that the comparison of between gel band sharing values may not yield exactly the same results as that of a single gel. Gwakisa *et al.* (1994) and Aravindakshan and Nainar (1998) used samples run on same gel for comparison.

The GC content of the primer also plays a significant role in band sharing values. Of the four different classes of primers based on the GC content, the one with 90 per cent GC content yielded significantly lowest band sharing values followed by primers with 60 per cent, 70 per cent and 80 per cent GC content respectively. This information emphasizes the role of primers and their GC content in band sharing values. Williams *et al.* (1990) reported that 10-base random primers with GC content of 60-90 per cent was suitable for amplification of genomic DNA segments to study the polymorphisms between amplified products of different individuals.

5.3.4 Percentage Difference

The percentage difference, average percentage difference and mean average percentage difference were used as measures to identify the genetic divergence between different groups of individuals. The values of percentage difference was found to be significantly affected by genetic groups of animals, GC content of primer and the gel used.

The percentage difference values between samples was lowest in upper gel and highest in the combination. The highest percentage difference in the combination indicates erratic product differentiation in gels. The most probable reason for this can be the quality of agarose gel used, since maximum care was taken to provide uniform electrophoresis condition for running all the gels.

The highest percentage difference was observed with primer GC content 90% followed by those with 60%, 70% and 80% respectively.

The percentage difference between ten combinations of four different genetic groups of dwarf cattle of Kerala showed interesting results. VV (Vechur x Vechur) DD (Vatakara x Vatakara), VD (Vechur x Vatakara) and HD (Highrange dwarf x Vatakara) were not significantly different in percentage difference. They differed significantly from VH (Vechur x Highrange dwarf), HH (Highrange dwarf x Highrange dwarf), DK (Vatakara x Kasargode), VK (Vechur x Kasargode), HK (Kasargode and Highrange dwarf) and KK (Kasargode x Kasargode) comparisons.

The three combinations namely VD, VH and HD were showing uniform percentage difference. This indicates that Vechur, Vatakara and Highrange dwarf cattle are more uniform compared with Kasargode cattle. The significantly higher percentage difference levels for Kasargode cattle and its crosses confirms this finding.

According to Aravindakshan and Nainar (1998) genetic divergence was narrower between Murrah and Surti breeds of buffalo and the genetic divergence was higher for Jersey crossbred and Ongole. Gwakisa *et al.* (1994) found interbreed dissimilarities of true zebu breeds of Tanzania which varied with each of four primer used.

5.3.5. Mean Average Percentage Difference

The mean average percentage difference value is the measure of interbred dissimilarities. The value was lowest for Vechur x Vatakara combination and highest for Highrange dwarf Kasargode combination.

Gwakisa *et al.* (1994) calculated MAPD in RAPD analysis by pairwise comparison method as a measure of interbreed dissimilarities. They found that the interbreed divergence was narrower between Mpwapwa and Tangaryika shorthorned zebu cattle breeds in Tanzania. Aravindakshan and Nainar (1998) calculated MAPD between Murrah and Surti buffaloes and the low value they found is an indicative of narrow genetic divergence between them when compared with Jersey crossbred and Ongole. Ramesha *et al.* (2002) found the MAPD between Surti Indian zebu cattle breeds. The value for MAPD between different breeds using RAPD fingerprints ranged from 16.7 ± 4.06 (between Krishna Valley and Ongole) to 36.9 ± 0.05 (between Deoni and Malnad gidda).

5.3.6. Genetic Distance (D)

The genetic distances among the four genetic groups were calculated using Nei's method. Vatakara and Vechur animals were found to have the least genetic dissimilarity between them as evidenced by the lowest value of genetic distance. The genetic distance between Kasargode and Highrange dwarf was the highest indicating huge dissimilarity between them. Almost similar values obtained for VK and KD suggests similar genetic base for Vatakara and Vechur animals. The geographical isolation of the Highrange dwarf cattle and the high

geographic distance between Kasargode and highranges of Idukki may be the reason for high genetic distance between these groups.

Smith *et al.* (1996) used D value obtained from RAPD bands to analyse the genetic distance between breeds of chicken. Kumar *et al.* (2000) used the informationof amplified fragements scored from RAPD patterns of three primers and presented the results of genetic distance estimates among five Japanese quail lines.

Based on the results obtained in band sharing value, percentage difference, MAPD and genetic distance, the genetic relationship among the four groups of dwarf cattle in Kerala can be summarized. From these information, it is evident that Vechur and Vatakara animals are more similar than Kasargode and Highrange dwarf cattle, and comparatively high genetic divergence was noticed between Highrange dwarf and Kasargode. The identification of different groups of animals, in this study was based on physical characters. Breed record or even pedigree of most of them was not available. Hence there are chances for mixing of animals, between these groups.

A dendrogram of relationship among four genetic groups of dwarf cattle was prepared. Mixing of animals irrespective of genetic groups was observed. The absence of distinct clustering in the dendrogram suggest a common gene pool for all the dwarf cattle of Kerala.

Summary

6. SUMMARY

Genetic erosion and extinction threaten an increasing number of plant and animal species, the major consequence being a loss of global genetic diversity. Development of an efficient strategy for preservation requires as set of genetic markers which can efficiently characterise different populations. The development of molecular genetics had helped the research to find the polymorphisms at DNA level.

The genetic diversity in dwarf cattle of Kerala using RAPD-PCR technique was evaluated in the present study. The important findings are summarized as follows.

1. Genomic DNA was isolated from 101 animals belonging to four cattle populations namely Vechur, Highrange dwarf, Vatakara and Kasargode using phenol:chloroform method. The mean yield of DNA and the ratio of optical densities at 260 nm and 280 nm were calculated.

2. The reaction conditions were standardized with respect to annealing temperature, magnesium chloride concentration and the amount of *Taq* DNA polymerase.

3. RAPD-PCR analysis was performed in pooled DNA samples using 26 random 10-base oligonucleotides to achieve rapid screening of large numbers of primers for their capability to reveal population specific polymorphisms. Twenty four primers produced good amplification pattern.

4. Individual sample analysis was done by selecting ten primers which gave good scorable amplification and polymorphism in pooled samples. The products obtained were reproducible.

5. Different populations of cattle showed varying degrees of genetic polymorphism in their RAPD profiles from zero to 100%. The number and frequencies of each band were scored.

6. The primer ILO 1127 produced a distinct band which was present in half of Vechur cattle and in one Kasargode cattle.

7. Primer ILO 526 gave a low degree of polymorphism in Kasargode where as moderate levels in other groups.

8. The polymorphism of primers ILO 876, OPA 20, ILO 1127, OPA 12, OPA 09 are higher for all groups tested where as ILO 526, OPA 10 and OPA 01 gave moderate levels of polymorphism and the primer OPA 06 gave very low level of polymorphism and it was monomorphic in Vechur and Kasargode groups.

9. A prominent population specific band of 2.5 kb was identified in Highrange dwarf cattle with primer OPA 06.

10. OPA 10 d fragment was absent in all Vatakara animals when tested with primer OPA 06.

11. A distinct band OPA 18 h was absent in all Vechur animals.

12. The band OPA 20 C was absent in all animals except two Vechur animals when amplified with primer OPA 20.

13. OPA 01 h, OPA 09 g and OPA 12 g are the common bands for OPA 01, OPA 09 and OPA 12 primers. Primer OPA 20 produced two common bands, primer OPA 06 produced eight common bands and the primer OPA 18 produced four common bands.

14. The band sharing values within and between four genetic groups were calculated by pair wise comparison as an interpretation of genetic similarity within group.

15. The various variables like genetic group, gel and GC content of primer have got significant effect on band sharing values. The HK (Highrange dwarf x Kasargode) combination is significantly different from Vechur, Vatakara and their combinations.

16. Percentage difference as a measure to identify the genetic divergence between groups was calculated.

17. The effect of genetic group, gel and GC content of primer on percentage difference were found significant.

18. The combinations VV (Vechur x Vechur), VD (Vechur x Vatakara) and HD (Highrange dwarf Vatakara) were not significantly different but they differed from VH, HH, DK, VK, HK and KK combinations.

19. Mean average percentage difference as a measure of dissimilarities between groups were found. It showed lowest value for VD combination to a highest value for HK combination.

20. Genetic distances among the four genetic groups were calculated. Vechur and Vatakara animals were found to have the least genetic distance between them and the Highrange dwarf and Kasargode animals have the highest genetic distance indicating the dissimilarity between Highrange dwarf and Kasargode group.

21. A dendrogram of genetic relationship between 52 animals of the four genetic groups was prepared. Mixing of animals irrespective of genetic groups was observed.

22. The four genetic groups of dwarf cattle in Kerala are having a common gene pool.

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*Originals not seen

Annexures

ANNEXURE – 1

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

1. EDTA (0.5 *M*, pH 8.0)

Dissolved 18.61 g of EDTA (disodium, dihydrate) in 80 ml of distilled water. Adjusted pH to 6.0 with NaOH and made the volume to 100 ml. The solution was filtered, autoclaved and stored at room temperature.

2. RBC Lysis Buffer

Ammonium chloride	150 m*M*	8.0234 g
Potassium chloride	10 m*M*	0.7455 g
EDTA	0.1 m*M*	0.0372 g

Added distilled water upto 1000 ml, stirred, filtered and autoclaved. Stored at 4°C.

3. Tris Buffered Saline (pH 7.4)

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

Dissolved in 900 ml distilled water and adjusted the pH to 7.4. Made up the volume to 1000 ml, filtered, autoclaved and stored at room temperature.

4. Saline EDTA buffer (pH 8.0) (SE buffer)

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

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

5. Sodium Dodecyl Sulphate (SDS) 20%

SDS 20 g

Added water to made upto 100 ml, stirred on a magnetic stirrer, filtered and stored at room temperature.

6. Phenol (saturated, pH 7.8)

Crystalline phenol was melted at 68°C in a water bath. Added hydroxyquinolone to a final concentration of 0.1%. To the molten phenol, added equal volume of 0.5 *M* Tris hydrochloride (pH 8.0). Stirred the mixture for 30 minutes on a magnetic stirrer. Transferred the content into a separating funnel and when the two phases have separated, collected the lower phenolic phase. Added an equal volume of 0.1 *M* Tris hydrochloride (pH 8.0) to the phenol. Stirred the mixture on a magnetic stirrer for 30 minutes. Collected the phenolic phase as above.

Repeated the extraction with 0.1 *M* Tris hydrochloride (pH 8.0), until the pH of the phenolic phase is more than 7.8. Finally 0.1 volume of 0.001 *M* Tris hydrochloride (pH 8.0) was added and stored in a dark bottle at 4°C.

7. Sodium chloride (5 *M*)

For 100 ml, dissolved 29.22 g of sodium chloride in 80 ml of distilled water, made up the volume to 100 ml, filtered and stored at room temperature.

8. Sodium acetate (3 M, pH 5.5)

For 100 ml, dissolved 40.824 g of sodium acetate in 70 ml of distilled water. Adjusted the pH to 5.5 with glacial acetic acid. Made up the volume to 100 ml, autoclaved and stored at 4°C.

9. Phenol: Chloroform: isoamyl alcohol mixture

Phenol: chloroform isoamyl alcohol mixture was prepared by mixing 25 parts phenol; 24 parts chloroform and 1 part isoamylalcohol.

10. Chloroform: isoamyl alcohol mixture

The mixture was prepared by mixing 24 parts chloroform and 1 part isoamyl alcohol.

11. Tris-EDTA (TE) buffer (pH 8.0)

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

Dissolved in 900 ml distilled water and adjusted the pH to 8.0. Made up the volume to 1000 ml, filtered, autoclaved in batches of 100 ml and stored at 4°C.

12. TAE buffer (50 x)

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

Autoclaved and stored at room temperature

13. Ethidium bromide (10 mg/ml)

Dissolved 100 mg of ethidium bromide in 10 ml distilled water and store in a dark bottle at 4°C.

14. Gel loading buffer (6 X)

Bromphenol blue	0.25%	50 mg
Xylene cyanol	0.25%	50 mg
Sucrose	40%	8 g

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

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 Fine, Chemicals, Mumbai
Methanol	-	SRL Bombay
Potassium chloride	-	SRL Bombay
Sodium acetate	-	SRL Bombay
Sodium chloride	-	SRL Bombay
Sodium dodecyl sulphate	-	SRL Bombay
Tris base	-	SRL Bombay

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.
PCR buffer	-	Bangalore Genei Pvt. Ltd.

MOLECULAR GENETIC DIVERSITY IN DWARF CATTLE OF KERALA

SUPRABHA, P.

ABSTRACT OF A THESIS

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for the degree of

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ABSTRACT

This study was undertaken for finding the molecular genetic diversity among dwarf cattle groups of Kerala namely Vechur, Highrange dwarf, Vatakara and Kasargode using RAPD-PCR technique.

Genomic DNA was isolated from 101 animals belonging to the four cattle groups using phenol: chloroform method. The mean yields of DNA isolated from five ml of whole blood was 110.07 ± 9.4 µg and the average ratio between optical densities at 260 and 280 nm was 1.6. The PCR conditions were standardized with respect to annealing temperature (38°C), magnesium chloride concentration (1.5 m*M*) and the amount of *Taq* DNA polymerase (0.5 units/20 µl). RAPD-PCR analysis was performed in pooled DNA samples using 26 random oligonucleotides. Individual sample analysis was done by selecting ten primers. The degree of polymorphism obtained for the primers varied from 0 to 100 per cent in different populations. The polymorphism of primers ILO 876, ILO 1127, OPA 20, OPA 12, OPA 09 were higher for all groups tested. ILO 526, OPA 10, OPA 01 and OPA 18 gave moderate levels of polymorphism whereas the polymorphism given by OPA 06 was very low.

The primer ILO 1127 produced a distinct band which was present in half of Vechur animals and in one Kasargode cattle. ILO 526 gave a low degree of polymorphism in Kasargode cattle and moderate levels in other groups tested. A predominant population specific band of 2.5 kb was identified in Highrange dwarf cattle with primer OPA 06. When amplified with OPA 20, a band (OPA 20 c) was noticed in Vechur animals with a frequency 0.07 and it was absent in other animals. The primer OPA 18 produced two common bands and the primer OPA 06 produced eight common bands. OPA 014, OPA 09 g and OPA 12 g were the common bands present in all the animals tested when respective primers were used.

The band sharing values obtained were higher for Vechur and Vatakara animals (0.83 ± 0.00) and lower for Highrange dwarf and Kasargode animals (0.81 ± 0.00), when within group band sharing was considered. The between group band sharing value varied from 0.80 ± 0.00 (between Highrange dwarf and Kasargode) to 0.83 ± 0.00 (between Vechur and Vatakara). This indicates that Vechur and Vatakara populations are more similar than Highrange dwarf and Kasargode cattle. The least squares analysis of variance of band sharing value showed significant effects ($P<0.01$) of genetic group, gel and GC content of primer on band sharing. The percentage difference values calculated as a measure of genetic divergence between groups were not significantly different between Vechur and Vatakara and between Highrange dwarf and Vatakara. They differed significantly between comparisons of Vechur-Highrange dwarf, Vatakara-Kasargode, Vechur-Kasargode and Highrange dwarf-Kasargode combinations. The mean average percentage difference calculated as a measure of genetic dissimilarities between groups, ranged from 17.93 (between Vechur and Vatakara) to 20.92 (between Highrange dwarf and Kasargode). The genetic distance calculated was lowest between Vechur and Vatakara (0.006) and highest between Highrange dwarf and Kasargode (0.046). The dendrogram prepared showed mixing of animals of different genetic groups.

In the present study the molecular genetic diversity among four genetic groups of dwarf cattle in Kerala was estimated using the RAPD-PCR technique. It was observed that the Vechur and the Vatakara animals are genetically more similar than the Highrange dwarf and Kasargode animals.