EVALUATION OF MICROSATELLITE MARKERS FOR PATERNITY TESTING IN CATTLE

PREETHY. M. S.

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

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur

2004

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

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DECLARATION

I hereby declare that this thesis entitled "EVALUATION OF MICROSATELLITE MARKERS FOR PATERNITY TESTING IN CATTLE" 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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PREETHY M.S.

CERTIFICATE

Certified that the thesis entitled **"EVALUATION OF MICROSATELLITE MARKERS FOR PATERNITY TESTING IN CATTLE"** is a record of research work done independently by Dr. Preethy M.S., 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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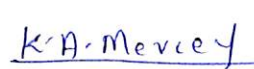
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Introduction

1. INTRODUCTION

Improvement of livestock for various traits of economic importance to mankind is dependent upon selection for future breeding stock with performance better than population average. The data for selection of breeding stock are taken from the records of their pedigree, sibs and progenies. In progeny testing programme of cattle, the bulls are evaluated, ranked and selected based on the records of their daughters. The value of such information depends on whether the parentage records are accurate or not. If there are errors in records, such ranking would be misleading, resulting in genetic gain lesser than expected.

With the adoption of artificial insemination for cows, there is always chance of inseminating a cow with semen from two different bulls in consecutive heats. When estrus cycle is shorter than 15 days or resulting calving is earlier than predicted dates, it may not be possible to decide the actual sire of the newborn calf on the basis of records. Exchange of semen samples and misidentification of semen used are probable stages where errors crop up. Wrong entry of dam number of newly born calf may also lead to disputed parentage, particularly in farms where day old weaning is practised. Hence, it is very necessary to establish the parentage of individual animals to verify their pedigree.

The genetic material in each individual is contributed equally from both parents. This is the basis for parentage studies. For decades, blood typing has proved to be the efficient tool for verifying parentage. Blood grouping is based on detection of antigens of red blood cells with the help of antibodies. There are about 11 blood group systems in cattle. The low level of polymorphism and finite number limit the accuracy of blood typing for parentage testing.

Biochemical polymorphism is another tool for parentage identification. Polymorphic forms of a particular protein are controlled by multiple alleles.

Polymorphism of serum proteins like transferrin, albumin, serum amylase, alkaline phosphatase and esterase revealed through electrophoresis can be used in parentage verification in domestic animals. But the low concentration of serum proteins, their relative instability, poor resolution techniques, age and disease dependent variations in protein expression pose problems of misinterpretation.

Recent developments in DNA technologies have overcome the technical limitations in serological methods and protein polymorphisms. The bovine diploid genome map consists of approximately 6000 million base pairs of DNA, distributed over 29 pairs of acrocentric autosomes and a pair of sex chromosome. The average bovine chromosome is approximately 100 million basepairs long containing 2000 to 5000 genes, which consist of coding sequences known as exons, interrupted by intervening sequences referred to as introns. Only about five percent of the genome actually code for proteins, rRNA and tRNA. The remaining 95% of the DNA consist of various noncoding sequences. The non-coding regions of DNA are often repetitive and are generally very polymorphic. DNA based technologies have made it possible to uncover a large number of polymorphisms at the DNA level and to use them as molecular markers. These markers have several advantages over the conventional blood typing and serological methods. DNA can be extracted from putrified blood samples, dead tissues and semen straws stored for a long time. Moreover, DNA based systems are not affected by age of animals. Different types of DNA polymorphisms have been characterized viz, RFLPs, RAPDs and VNTRs.

RFLPs are nucleotide changes in the genome, resulting in creation or abolition of a restriction endonuclease recognition site generating different electrophoretic patterns. These markers were initially used for typing DNA polymorphisms. It is expensive to develop probes and detection involves laborious techniques. RFLPs usually have a limited number of alleles with low heterozygosity and polymorphic information content. Relatively large amounts of DNA are required for detecting RFLPs and several days to weeks are

necessary to detect allelic patterns following hybridization with radiolabelled probes. Hence, RFLPs are less suitable for routine parentage testing.

RAPDs involve random amplification of DNA segments by PCR using short oligonucleotide pairs of length 8-10 bp. These markers are able to detect DNA polymorphism without any prior sequence information of genome DNA. RAPDs are dominant markers and heterozygotes are typically scored as homozygotes, which decreases their information content. Moreover, even under carefully controlled conditions, there can be ambiguity in the scoring of bands separated on a gel. Nonparental bands frequently found in the offspring also limit the choice of the technique for routine typing.

VNTRs are sequences that occur repeatedly throughout the genome and are highly polymorphic due to variation in the number of repeats at a particular locus in different individuals. Polymorphic information content is generally higher for VNTRs. VNTR loci are divided into two classes: minisatellite and microsatellites. Minisatellites consist of 15-60 bp repeat units, clustered in the telomeric regions. PCR analysis of length variation at minisatellite loci is possible, but alleles at highly polymorphic loci are too large (20-25 Kb) to amplify efficiently. Therefore detection is expensive and relatively laborious relying on southern blotting.

Microsatellites overcome the difficulties encountered in typing the markers mentioned above. Microsatellites consist of tandem repeats of very short nucleotide motifs from one to six base pairs long and are widespread in eukaryotic genomes. Microsatellite sequences can be amplified *in vitro* by PCR using primers flanking the chosen repeat sequence and specific for a locus. The alleles can be differentiated by fractionating the amplified DNA product on a high-resolution gel. Even though their functional significance is not yet clearly known, they are considered to be hotspots for recombination and may have a role in packaging and condensing DNA in eukaryotes. They are proved to be efficient

markers for the mapping of economic trait loci, for parentage determination, gene mapping and medical genetics.

In Kerala, more than 75 per cent of the cattle are crossbreds. No significant research work has been done till date to characterize microsatellite polymorphism in this population. The present study was undertaken with the objectives to determine the polymorphicity of three microsatellite markers viz. DRB3, ETH131 and FSHβ in these cattle and to evaluate the efficacy of these markers for paternity testing. The results of this study will pave way to select a panel of microsatellite markers and apply them for routine parentage testing in cattle population of Kerala.

Review of Literature

2. REVIEW OF LITERATURE

Breeding programmes have been of considerable importance to improve productivity in animal industry. In dairy cattle, as a result of the rapid development of artificial insemination, there is always a high risk of uncertain pedigree. The practice of mating cows to different bulls during the same or consecutive heat periods, exchange and misidentification of semen samples, poor record keeping and practice of natural service in a herd with several bulls lead to unknown pedigree. The risk of such errors makes it necessary to establish the parentage of individual animals. Blood group studies, protein polymorphisms and at present DNA based technologies have been recommended for parentage verification in cattle.

2.1 BLOOD GROUP STUDIES

Landsteiner's discovery of the ABO blood group in 1900 established the basis for modern day parentage studies. The discovery of MN system in 1927, Rh-Hr systems in 1939 and Mendelian inheritance of the blood groups systems resulted in their application as first generation tests in paternity testing (Stormont, 1958).

Hall (1959) reported 11 blood group systems in cattle, which could be detected either by haemolysis or haemagglutination. He suggested that errors in statements of pedigree could be exposed by examination of blood samples from animals concerned.

Spooner (1967) reported that the presence of more than 70 recognized blood groups in cattle could be used as valuable tools for checking the parentage of bulls for use in artificial insemination.

Stormont (1967) detailed the importance of cattle blood typing in solving problems of questionable parentage. He pointed out that blood typing by itself

could not be used to prove parentage, even though it could weed out those animals that were not the true parents.

Silver (1989) reviewed that the human leukocyte antigen discovered during the late 1960s when combined with the multiple blood group systems provided a practical means for excluding 95 per cent of alleged paternity cases.

Wenk *et al.* (1992) defined Probability of Exclusion (PE) as the probability that tests for alleles of one genetic system will exclude a falsely accused parent. They calculated the probability of exclusion using different blood group systems and red cell markers. The presence of rare alleles for the red cell markers was reported to have caused misinterpretation.

2.2 PROTEIN POLYMORPHISMS

Similar to antigenic variations among red cells, variations among serum proteins like plasma amylase and transferrins detected by electrophoretic techniques were valuable in parentage control (Spooner, 1967). About 40 percentage of cases of incorrect parentage was reported to have been detected by transferrins alone.

Stormont (1967) reported a net improvement of five per cent efficiency when blood typing tests were supplemented with transferrin tests for resolving problems of questionable parentage.

Wenk *et al.* (1992) reviewed that while electrophoretic separation of protein variants for parentage analysis had several advantages like small sample volume requirements, possible simultaneous separation of alleles at different loci, simultaneous testing of many individuals etc., several factors like relative instability of some enzymatic proteins, poor resolution of the techniques, age and disease dependent variations in protein expression and presence of silent alleles and variants posed problems of misinterpretation.

2.3 DNA BASED TECHNOLOGIES

DNA based technologies for paternity tests were developed in the early 1980s. These include the Restriction Fragment Length Polymorphism (RFLP) analysis or multiple-locus probes and the PCR-based technologies involving the Random Amplified Polymorphic DNA (RAPD), Variable Number of Tandem Repeats (VNTRs) or minisatellites, Short Tandem Repeats (STRs) or microsatellites, and of late the Single Nucleotide Polymorphisms (SNPs).

2.3.1 Restriction Fragment Length Polymorphisms (RFLPs)

The RFLPs, inherited as codominant Mendelian traits being plentiful in the genome, became candidates for parentage tests as early as 1983. Soller and Beckmann (1983) reviewed the applications of RFLPs as genetic markers for parentage identification. They opined that exclusion of parentage by any particular individual required a set of 20 polymorphic loci, while identifying the true parent in a group of potential parents, required twice that number.

The methods used to demonstrate and distinguish RFLPs is extremely time consuming and in some phases are labour intensive. The endonuclease producing the greatest degree of polymorphism may also produce fragments of similar lengths, which can make distinction and interpretation difficult (Weber and May, 1989).

Allen *et al.* (1990) reported the use of single locus and multi-locus RFLP mapping in paternity testing. They concluded that the large number of polymorphic restriction fragments detected with a multilocus probe and the Mendelian inheritance of these fragments in large pedigrees made RFLP mapping a powerful method for establishing paternity.

2.3.2 Random Amplified Polymorphic DNA (RAPDs)

Random Amplified Polymorphic DNA markers are based on amplification of DNA through the PCR technique using random sequence primers (Williams *et al.,* 1990).

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

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

Chung *et al.* (1995) in their study on Holstein dairy cattle using 12 RAPD primers on three sire families established that the amplified products in offspring were always present in one or both parents.

Anilkumar (2003) observed non-parental bands in nine out of the 12 RAPD primers used and concluded that the presence of non parental bands in the offspring of the parent limit the use of RAPD-PCR technique for parentage verification.

2.3.3 Variable Number Tandem Repeats (VNTRs)/Minisatellites

VNTRs or Minisatellites are tandem repetitive regions dispersed throughout the genome and are highly polymorphic due to allelic variation in repeat copy number (Jeffreys *et al.*, 1985a).

Jeffreys *et al.* (1985b) reported that multilocus variability in restriction fragment length polymorphisms produced DNA fingerprints or minisatellite patterns, which were completely individual specific and could be applied directly to problems of human identification, including parenthood testing.

Kashi *et al.* (1990b) illustrated that DNA fingerprints with minisatellites could be a highly effective means of parentage identification in domestic animals especially in dairy cattle progeny testing programmes.

Pena and Chakraborty (1994) reviewed that multilocus fingerprints, because of their great informativeness, somatic stability and Mendelian inheritance were the most powerful tools for paternity testing.

2.3.4 Microsatellites

Microsatellites are short (1-6 bp), simple tandemly repeated nucleotide sequences present as multiple copies in the genome (Miesfeld *et al.,* 1981; Litt and Luty, 1989). They are also referred to as short tandem repeats or simple repeats.

The hypervariability of the simple sequence stretches are due to slippage events occurring during DNA replication. These are flanked by unique DNA sequences enabling their amplification using PCR (Tautz, 1989).

The dinucleotide repeats may be located within the protein coding regions, within introns or between genes and are inherited in normal Mendelian fashion (Weber and May, 1989).

According to Troyer *et al.* (1990) microsatellites were extremely repetitive short sequences with each repeat two or three bases long located at every 10,000 bases of DNA sequences and that they could be used to assess the degree of inbreeding in populations, to identify animals heterozygous for simple recessive traits and in gene mapping research.

Fries *et al.* (1990) performed a preliminary database search to identify bovine sequences containing $(CA)_n$, $(AC)_n$, $(GT)_n$ or $(TG)_n$ blocks with $n \geq 6$ and concluded that dinucleotide blocks may be an abundant source of DNA polymorphism in cattle.

Weber (1990) examined over 100 human (dC-dA)$_n$, (dG-dT)$_n$ sequences and divided the sequences into three categories: perfect repeat sequences without interruptions in the runs of CA or GT dinucleotides (64 per cent of total), imperfect repeat sequences with one or more interruptions in the run of repeats (25 per cent), and compound repeat sequences with adjacent tandem simple repeats of a different sequence (11 per cent).

Stallings *et al.* (1991) determined the distribution of GT repetitive sequences in the human genome by analyzing over 3700 cosmid clones containing human DNA and found that on average, a GT repetitive sequence occurred every 30kb in DNA from euchromatinic regions and were significantly underrepresented in centric heterochromatin.

According to Moore *et al.* (1992) DNA microsatellites are important markers for gene maps in many species. The ease of isolation and characterization of these sequences coupled with the high levels of polymorphism and the method of analysis make them attractive gene markers.

Steffen *et al.* (1993) screened a partial plasmid library with bovine genomic inserts of about 500 base pairs with a (dC-dA)$_n$, (dG-dT)$_n$ oligonucleotide probe for the repeated nucleotide motif (CA)$_n$. Eleven positive clones were discovered and were subsequently isolated and sequenced.

The degree of polymorphism and heterozygosity detected by microsatellites is much greater than that of protein markers (Arranz *et al.*,1996).

Hirano *et al.* (1996) isolated 42 highly polymorphic microsatellite (GT/CA)$_n$ markers from Japanese black cattle Wagyu. Fortyone of the markers were mapped to bovine autosomes through linkage analyses and the remaining marker was found to show X-linked inheritance.

Ma *et al.* (1996) isolated and characterized 45 $(CA)_n$ polymorphic microsatellites from the bovine genome out of which 40 markers were found to exhibit autosomal Mendelian inheritance.

Heterologous microsatellite markers i.e., markers derived from one species and tested in another species can reveal syntenic relationship among different species that have been conserved during evolution (Arora *et al.,* 2003).

2.4 APPLICATION OF MICROSATELLITES

Microsatellites had been widely used to estimate the genetic distance between different populations of cattle (MacHugh *et al.*, 1994; Arranz *et al.*, 1996; Moazami-Goudarzi *et al.*, 1997; MacHugh *et al.*, 1998; Burriel *et al.*, 1999), buffaloes (Barker *et al.*, 1997; Arora *et al.,* 2003), goats (Yang *et al.,* 1999), pigs (Kaul *et al.*, 2001; Li *et al.*, 2004), dogs (Irion *et al.*, 2003) and horses (Tozaki *et al.*, 2003).

The usefulness of microsatellites as markers to establish the genetic structure of different breeds of cattle (MacHugh *et al.,* 1998), sheep (Sodhi *et al.*, 2003), and horse (Bjornstad and Roed, 2001) has been reported.

Smith and Simpson (1986) reviewed the potential application of QTL map in marker-assisted selection. Several microsatellite markers had been found to be associated with QTL affecting important economic traits in cattle (Ron *et al.,* 1994; Arranz *et al.*, 1998; Wiener *et al.*, 2000), and sheep (Chu *et al.*, 2003). These QTL maps could be used for improving the production performance by marker-assisted selection (Kashi *et al.*, 1990a).

Microsatellites had been proved to be efficient markers for mapping of several disease genes in man (Stallings, 1994) and animals (Holmes, 1994; Geldermann *et al.*, 2003).

Polymorphic microsatellites had been used as tools for construction of genetic linkage maps in cattle (Georges *et al.*, 1990; Barendse *et al.*, 1994; Bishop *et al.*, 1994).

Microsatellite markers being highly polymorphic are widely used as efficient tools for paternity testing in several species. They obviate most of the difficulties associated with all other types of markers (Glowatzki-Mullis *et al.*, 1995; Usha *et al.*, 1995; Baron *et al.*, 2002; De Nise *et al.*, 2004).

The above works show that microsatellite markers are powerful tools for genetic studies like genetic distance between breeds, mapping of quantitative trait loci and disease genes, parentage testing and marker assisted selection.

2.5 GENOMIC DNA ISOLATION

The first and foremost requirement to carryout microsatellite analysis is the isolation of genomic DNA.

2.5.1 Blood

The standard protocol involving Proteinase-K digestion and phenol: chloroform extraction followed by ethanol precipitation is an efficient method for isolation of genomic DNA from whole blood (Andersson *et al.*, 1986; Oliver *et al.*, 1989; Sambrook *et al.*, 1989; Trommelen *et al.*, 1993; Arora *et al.*, 2003; Chenyambuga *et al.*, 2004).

2.5.2 Semen

Genomic DNA can be isolated from spermatozoa of fresh semen by mercaptoethanol-Proteinase-K treatment followed by phenol:chloroform extraction method (Andersson *et al.*, 1986; Trommelen *et al.*, 1993).

Frozen semen can also yield good quality DNA by phenol:chloroform extraction (Lien *et al.*, 1990).

Aravindakshan *et al.* (1998) developed a simple and efficient modified phenol:chloroform method for extraction of genomic DNA from fresh bovine semen for genetic analysis.

2.5.3 Yield and purity of DNA

Andersson *et al.* (1986) using the phenol:chloroform method obtained a yield of 150-200 μg and 100-200 μg of DNA per sample from blood and semen, respectively.

Lien *et al.* (1990) obtained an yield of 300-500 μg of DNA from 2.5 x 10^8 sperm cells using phenol:chloroform method with the same quality as DNA prepared from blood.

Apparao *et al.* (1994) isolated DNA from venous blood with an yield of 250 to 300 μg per 15 ml using the phenol:chloroform method.

Senthil *et al.* (1996) obtained an average yield of 615.55 ± 0.72 μg of DNA by high salt method and 444.58 ± 21.54 μg by phenol:chloroform method from 15 ml of blood.

Chitra (2002) and Suprabha (2003) using a modified phenol:chloroform method obtained yields of 231.097 ± 11.65 μg and 110.1 ± 9 μg from 5 ml of venous blood, respectively.

2.6 MICROSATELLITE ANALYSIS

2.6.1 DRB3

The gene coding for the bovine major histocompatibility complex class II antigen, DRB3 was found to contain a microsatellite within second intron (Muggli-Cockett and Stone, 1988).

The DRB3 microsatellite is composed of three repeat motifs, a stretch of atleast 10 uninterrupted $(TG)_n$ dinucleotides, a long but interrupted stretch of $(GA)_n$ dinucleotides and a few $(CAGA)_n$ tetranucleotides (Ellegren *et al.*, 1993).

Gwakisa *et al.* (1994) through their studies on linkage between DRBP1 and CYP21 microsatellites in MHC of cattle demonstrated the extensive polymorphism in these two microsatellites and suggested that they could be applied for breed comparison and paternity testing.

Usha *et al.* (1995) observed 19 alleles across 14 breeds of cattle for DRB3 loci and used this marker for parentage verification in cattle.

Haeringen *et al.* (1999) reported the presence of 25 DRB3 alleles with sizes ranging from 143 to 215 bp.

2.6.2 ETH131

The microsatellite ETH131 with a $(CA)_{23}$ dinucleotide repeat was mapped to syntenic group U4 by PCR analysis of somatic cell hybrid panel and assigned to chromosome 21 (Steffen *et al.*, 1993).

Ron *et al.* (1994) reported significant association of D21S4 locus with QTL affecting milk and protein production in commercial dairy cattle population and recommended its use in marker-assisted selection.

Glowatzki-Mullis *et al.* (1995) obtained 21 alleles for ETH131, most of them not well separated and differed by less than the repeat unit of 2 bp. They analysed the sequence of some alleles of the locus, which revealed that the allelic variation was based on a variable number of CG and CA dinucleotides.

Usha *et al.* (1995) detected 24 alleles with sizes ranging from 137-171 bp for ETH131 locus in 14 breeds of cattle.

Arranz *et al.* (1996) in a study to compare protein markers and microsatellites in differentiation of cattle populations obtained 13-20 alleles at ETH131 locus.

2.6.3 FSHβ

The β subunit of bovine Follicle Stimulating Hormone locus (FSH β) was reported to contain a $(AT)_{20}$ stretch of repeats in intron β (Kemp and Teale, 1991). Six alleles were distinguished in a group of 9 N'Dama cattle.

Moore *et al.* (1992) reported 11 alleles at the FSH β microsatellite locus.

Ron *et al.* (1994) in their study to find association of microsatellite markers with QTL affecting milk production traits in seven Israeli- Holstein grand sire families, obtained eight alleles at the FSHβ locus.

Usha *et al.* (1995) obtained 19 alleles at the locus across 14 breeds of European cattle.

2.6.4 Incorporation of radioactivity

Visualisation of PCR products by autoradiography can be done by incorporating radioactivity in the PCR reaction. Basically this is achieved by two means.

2.6.4.1 *Direct incorporation*

Radioactivity can be directly incorporated into the PCR reaction mixture in the form of α ^{35}S-dATP, α ^{32}P-dCTP or α ^{32}P-dATP.

Litt and Luty (1989) incorporated 3.5 μCi of α ^{32}P-dCTP in the PCR reaction mix for amplification of $(TG)_n$ microsatellites in the human cardiac actin gene. The aliquots of amplified samples were run in alternate lanes of DNA sequencing gels along with end-labeled Sau 3A fragments of pBR 322 sequence ladders as size standards and the products were visualized by autoradiography.

Weber and May (1989) amplified (dC-dA)$_n$. (dG-dT)$_n$ repeat blocks of DNA by incorporating 1-2 µCi of α ^{32}P-dATP at 800 Ci/mmol or α ^{35}S-dATP at 500 Ci/mmol in the standard PCR reaction mix and electrophoresed the products in standard denaturing polyacrylamide DNA sequencing gels with dideoxy sequencing ladders produced by M13 as gel standards.

Fries *et al.* (1990) amplified a microsatellite locus from steroid 21 hydroxylase gene by incorporating 1.5 µCi of α ^{32}P- dCTP (3000Ci/mmol) into the PCR reaction mix. The amplified DNA fragments were resolved on a 6 per cent polyacrylamide sequencing gel and found that CA strands in the autoradiographs were more intense due to labeling with α ^{32}P-dCTP.

Kaukinen and Varvio (1993) incorporated 1 µCi of α ^{32}P-dCTP (3000 Ci/mmol) into 20 µl PCR reaction mixture for amplification. The products were run on 6 per cent polyacrylamide sequencing gels and relative sizes of different alleles were determined by running *Msp*I digested pBR322 sequencing ladder in the same gel along with amplified products.

Vankan *et al.* (1994) detected autoradiographic products by direct incorporation of α ^{32}P-dCTP (374 Bq/ml) into the PCR reaction mix followed by electrophoresis of the products in 6 per cent polyacrylamide gels along side standard size markers.

Usha *et al.* (1995) carried out PCR amplification of five microsatellites by direct incorporation of α ^{35}S-dATP in the PCR reaction mixture, electrophoresed the products in 6 per cent denaturing polyacrylamide gels and visualized the alleles by autoradiography. The apparent sizes of alleles were assigned by comparison with a sequencing ladder from M13.

Arranz *et al.* (1996) amplified microsatellites from genomic DNA using α ^{35}S-dATP. PCR products were electrophoresed on standard polyacrylamide

sequencing gels and genetic variants were visualized by autoradiography. The samples of M13 mp18 were used as a size standard.

Ma *et al.* (1996) directly incorporated α ^{32}P-dCTP into PCR reaction mix. The amplified products were electrophoresed in 7 per cent denaturing polyacrylamide gels and visualized by autoradiography.

Barker *et al.* (1997) incorporated α ^{32}P-dCTP during the PCR cycles and electrophoresed the amplified samples in sequencing gels. The products were detected by autoradiography of dried gels. Allele sizes were determined by comparison with pUC 19 sequencing ladders.

Moazami-Goudarzi *et al.* (1997) carried out PCR analysis of microsatellites by two methods using direct incorporation of α ^{32}P-dATP and fluorescently labelled PCR primers. In the former technique, the amplified products were electrophoresed in 6 per cent polyacrylamide denaturing sequencing gel and visualized by autoradiography while in the latter, size characterization of PCR products were carried out using an automated DNA fragment analyzer.

2.6.4.2 *End-labelling of Primers*

Alternatively one of the PCR primers can be end-labelled with γ ^{32}P-ATP using Polynucleotide Kinase (PNK).

Tautz (1989) end-labelled the PCR primers at the 5' end using γ ^{32}P-ATP and Polynucleotide Kinase for amplifying simple sequence loci within Notch gene of Drosophila. The amplified products were resolved in a 6 per cent denaturing polyacrylamide gel and visualized by autoradiography.

Ellegren *et al.* (1993) amplified DRB3 microsatellite locus by end-labelling one of the primers for PCR with γ ^{32}P-ATP (1 μCi for 10 p*M* primer) using T4 PNK. The amplified products were loaded on 6 per cent denaturing

polyacrylamide gels along side a sequencing ladder for determination of size differences between alleles.

Hughes (1993) described a method of typing microsatellite polymorphisms involving amplification by PCR. One primer of each pair was end-labelled with 10μCi of γ ^{32}P-ATP using T4 Polynucleotide Kinase (PNK), the end-labelling reaction was carried out at 37°C for 30 minutes. The alleles were separated by denaturing gel electrophoresis and detected by autoradiography.

Marklund *et al.* (1994) end-labelled the forward primers of nine equine microsatellite loci at the 5' end with γ ^{32}P-ATP and used those primers for subsequent amplification. The products were separated in sequencing gels and alleles detected by autoradiography.

Binns *et al.* (1995) kinase-labelled one of each primer pair with γ ^{32} P-ATP for amplifying six equine microsatellite loci. The products were resolved on 5 per cent polyacrylamide DNA sequencing gels using a M13 sequence ladder as size markers.

2.6.5 Polymerase Chain Reaction

Mullis *et al.* (1986) described the term polymerse chain reaction (PCR) as the reciprocal interaction of two oligonucleotides and the DNA polymerase extension products whose synthesis they prime, when they are hybridized to different strands of a DNA template in a relative orientation such that their extension products overlap. The method consisted of repetitive cycles of denaturation, hybridization and polymerase extension.

Saiki *et al.* (1988) detailed 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 reaction mixture consists of 10X Amplification Buffer (500mM KCl, 100mM Tris HCl, 15mM MgCl$_2$, 0.1 per cent Gelatin), four dNTPs each at a concentration of 1.25mM, 100pM of each primer 1 and 2, Template DNA, 0.5µl of *Taq* DNA Polymerase (5U/µl) and water to a final concentration of 100µl (Sambrook *et al.,* 1989).

According to Appelhans (1991) the basic principle of a PCR cycle was (1) heat denaturation of the target DNA at 95°C (2) annealing of the primary molecules to appropriate DNA strands flanking the target DNA at 50°C and (3) extending the primers across the template by a thermostable DNA polymerase at 72°C. After 30-40 cycles, the target DNA was reported to have amplified 10^9 folds.

Erlich *et al.* (1991) detailed the various applications of PCR including introduction of new sequences in DNA via PCR primers, analysis of DNA protein interaction, physical and genetic mapping, identification of mutation, diagnosis of several diseases and in forensics.

Kemp and Teale (1991) amplified FSHβ microsatellite locus with a 20 µl reaction mix consisting of 200 ng template DNA, 38 µM each dNTPs, 2 mM MgCl$_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 20 µM each of the primers and 0.25U of *Taq* DNA polymerase. The temperature cycling was optimized as denaturing at 94°C, annealing at 55°C, extension at 72°C each for 60 seconds, followed by a final extension of 10 min at 72°C.

A 20µl volume reaction consisting of 25ng genomic DNA, 10 pM of each primer, 200 µM dNTPs, 1.5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris and 1.5 U *Ampli Taq* polymerse was carried out by Ellegren *et al.* (1993) for amplifying the DRB3 microsatellite locus. 30 cycles were performed each comprising 94°C for

60 seconds, 50°C for 30seconds and 72°C followed by a prolonged extension for five minutes.

Usha *et al.* (1995) carried out PCR in 20 µl volume containing 20 ng template DNA, 20 p*M* each oligonucleotide primers, 200 µ*M* each dNTPs, 10 m*M* Tris (pH 8.3), 10 m*M* KCl, 1.25 m*M* MgCl$_2$ and 0.5U of *Taq* DNA polymerase for amplification of DRB3, FSHβ and ETH131 microsatellites. The PCR protocol consisted of denaturation at 94°C for a minute followed by annealing and extension (5 min) for 30 cycles. An annealing temperature of 60°C was used for DRB3 and FSHβ while a temperature of 55°C was used for amplification of ETH131.

Haeringen *et al.* (1999) used 50 ng DNA, 200 µ*M* dNTPs, 2.5 m*M* MgCl$_2$, 83 m*M* KCl, 16.6 m*M* Tris HCl, 5 p*M* of the forward and reverse primer and 1U of *Amplitaq* DNA polymerase for PCR amplification of DRB3 locus. Twenty five cycles each consisting of 94°C for 50seconds, 50°C and 72°C each for 60seconds followed by a final extension of 72°C for five minutes was performed.

2.7 STATISTICAL ANALYSIS

2.7.1 Heterozygosity

Nei (1978) defined heterozygosity per locus as the mean population heterozygosity over all structural loci in the genome. The statistical formula for arriving at population heterozygosity at a locus was also given as $1 - \sum P_i^2$ where Pi was the frequency of the ith allele at a locus in a population.

Observed heterozygosity is the percentage of all individuals assayed that are heterozygous at a particular locus (O'Reilly, 1998).

2.7.2 Polymorphic Information Content

Botstein *et al.* (1980) described the term Polymorphic Information Content (PIC) as the probability that a given offspring of a parent carrying the rare allele at an index locus will allow deduction of the parental genotype at the marker loci.

Weber (1990) reported that microsatellite sequences with 12 or fewer repeats were found to be non polymorphic and informativeness of the markers increased as the average number of repeats increased especially in the range of 11-17 repeats.

2.7.3 Probability of Parentage Exclusion

The efficacy of markers of varying allele frequencies in paternity identification is evaluated by the Probability of Exclusion (PE) values.

Soller and Beckmann (1983) described the term Combined Probability of Exclusion (CPE) as the probability that a randomly chosen male of a known genotype will be excluded as the parent of a given offspring on the basis of genotype of both the offspring and its female parent at one or more polymorphic loci.

Hayasaka *et al.* (1986) calculated the probability of paternity exclusion in six troops of Rhesus and Japanese macaques housed in open enclosures and 68 wild troops of Japanese, crab eating and toque macaques using 33 genetic loci encoding blood proteins. They observed probability of paternity exclusion values of 0.2214, 0.4655 and 0.7382 respectively.

Chakraborty *et al.* (1988) detailed the use of polymorphic genetic markers in resolving genealogical relationship among individuals in a natural population. They reported that the average exclusion power depends upon the number of alleles as well as their frequencies.

Jamieson (1994) defined probability of parentage exclusion as the probability that a series of co-dominant alleles of known frequency should detect a falsely recorded father.

Marklund *et al.* (1994) evaluated eight microsatellite loci in four breeds of horses for parentage test and found that the combined probability of exclusion values varied between 0.96 and 0.91.

Vankan *et al.* (1994) determined the allelic frequencies and exclusion probabilities of 11 microsatellite loci in 10 different cattle breeds and found that the overall exclusion probability for parentage was above 99 per cent in all breeds except Poland Hereford (98.54 per cent)

Glowatzki-Mullis *et al.* (1995) obtained an average probability of exclusion of 0.999 with six microsatellite loci using two multiplex co-amplification PCR systems for parentage control in cattle. Thirty-five bovine parentage control cases not solvable by conventional typing were reported to be resolved using microsatellites.

Usha *et al.* (1995) developed a new method for calculating the efficiency of markers for paternity testing, termed as 'probability of random sire exclusion', which was defined as the proportion of sires in a population that could be excluded from paternity for a randomly selected dam-calf pair. They evaluated the use of five microsatellite markers for parentage verification in 14 breeds of cattle and reported that five markers gave on an average across breeds, a probability of 99.99 per cent of excluding an incorrect sire.

Bowling *et al.* (1997) reported that 15 loci of blood groups and protein polymorphisms and 11 loci of dinucleotide repeat microsatellites together gave 99.99 per cent theoretical effectiveness of determining incorrect parentage, validating DNA markers for horse pedigree verification.

Heyen *et al.* (1997) evaluated six fluorescent multiplexes containing primer pairs for the amplification of 22 microsatellites on 17 bovine autosomes developed for semi automated fluorescence genotyping for parentage testing. They reported that the 22 markers excluded at least 99.86 per cent of non-parents when genotypes were known for only one alleged parent and an offspring and at least 99.999 per cent if the genotype of a confirmed parent also was known.

Mommens *et al.* (1998) tested a set of 33 cattle microsatellite primer pairs with DNA of American bison and evaluated for usefulness in parentage testing and concluded that cattle microsatellites were useful in bisons for identification, parentage testing and phylogenic studies.

Sutton *et al.* (1998) determined band share estimates of unrelated, first degree and second degree relationships in dogs using *Hinf*1/33-6 DNA fingerprinting and microsatellite allele frequencies were effective in resolving parentage.

Koskinen and Bredbacka (1999) used 10 polymorphic microsatellite markers combined in three multiplex-PCR reactions for parentage analysis test for dogs and obtained exclusion probabilities ranging from 99.34 per cent to 99.93 per cent.

Luikart *et al.* (1999) developed two multiplex systems each containing 11 microsatellite loci for semi-automated parentage testing in goats. Probability of exclusion values of 0.994 to 0.999 were obtained for the first set of 11 loci and exclusion probabilities of 0.920 to 0.996 were obtained for the second set. These results were found similar to commercially available multiplexing systems for cattle.

Vankan and Faddy (1999) reported that in paternity testing in 39 multiple sire mating groups, a reliability of 99.99 per cent was achieved when the exclusion probability was 0.99 or greater and when the exclusion dropped below 0.9, the reliability was poor. This highlighted the need for DNA testing

laboratories to offer paternity tests with an exclusion power of at least 90 per cent.

Schnabel *et al.* (2000) evaluated 15 bovine microsatellites for use in parentage testing in 107 domestic cattle from five different breeds and reported that a core set of 12 loci provided an average exclusion probability of 0.9995 across all populations tested.

Janik *et al.* (2001) identified a total of 79 alleles at 11 microsatellite loci and calculated the probability of sire exclusion for the 11 loci to be 99.96 per cent.

Kakoi *et al.* (2001) developed a microsatellite DNA typing with 17 microsatellites for parentage verification of race horses and obtained exclusion probabilities over 0.9999 suggesting the ability of DNA typing for individual identification and parentage verification.

Baron *et al.* (2002) used six microsatellite markers in progeny tests to assess the paternity of 71 probable offspring of nine Gir dairy sires. The combined probability of exclusion for all the markers together was found to be much higher than exclusion based on single locus. A 30 per cent misidentification rate was obtained suggesting the need to conduct paternity evaluation in progeny testing programmes to assess accuracy of the genetic values of the sires.

Curi and Lopes (2003) evaluated nine microsatellites for paternity testing in families of Gir breed of bovine population. They found that the markers recommended for paternity test by ISAG in European cattle showed low probability of exclusion values in Gir cattle and those markers not commonly used in paternity testing showed high exclusion probabilities, suggesting the need for characterization of microsatellites for different populations within a breed for paternity testing.

De Nise *et al*. (2004) determined the power of exclusion for parentage verification and probability of match for identity in American Kennel club using 17 microsatellite markers.

Thus, the above-published reports strongly prove the effectiveness of microsatellites over the conventional approaches in paternity testing with high exclusion probabilities.

Materials and Methods

3. MATERIALS AND METHODS

3.1. SOURCE OF SAMPLES

DNA samples from crossbred cattle of Kerala constituted the materials for the study. Blood and semen were used as the source of DNA.

Initially for determining the polymorphisity of the markers, 100 genetically unrelated animals were sampled from University Livestock Farm, Mannuthy, Cattle Breeding Farm, Thumburmuzhi, Regional Agricultural Research Station, Pilicode and other different areas in the field.

Samples of known pedigree were collected from the University farms to test the inheritance of markers. Blood samples from dam-progeny pairs were collected immediately after calving to avoid misidentification.

Five milliliter of venous blood was collected into 15 ml sterile disposable polypropylene tubes containing Ethylene Diamine Tetra Acetic Acid (EDTA) as anticoagulant (1 mg/ml of blood).

Semen was used as source of DNA in case of bulls. Frozen semen of bulls included under the ICAR Progeny Testing Scheme and fresh semen of bulls of the Kerala Agricultural University Bull station, Mannuthy were used for DNA isolation.

Five frozen semen straws each containing 30 million sperms were used for extraction of DNA from every bull sampled. In case of fresh semen, about 1-2 ml containing approximately 300-500 million sperms was obtained on the day of ejaculation itself and transported to the laboratory in a chilled condition.

3.2. ISOLATION OF GENOMIC DNA

3.2.1. Extraction from Whole Blood

DNA was prepared from whole blood using the standard phenol chloroform method (Andersson *et al.,* 1986) with modifications. The procedure followed was as follows

1. Five ml of blood collected in a 15ml centrifuge tube was centrifuged at 4000 rpm for 10 min and the plasma was discarded leaving erythrocytes and leukocytes.

2. Two to three volume of ice-cold RBC lysis buffer (150 mM NH$_4$Cl, 10 mM KCl, 0.1 mM EDTA) was added, mixed well and kept in ice with occasional mixing for 10 minutes for complete lysis of RBCs.

3. The leukocytes were resuspended by centrifuging at 3500 rpm for 15 min. and the supernatant containing lysed RBCs was discarded.

4. Steps 2 and 3 were repeated till the cell pellet was clear without any unlysed erythrocytes.

5. The cell pellet was washed twice with 10 ml of Tris Buffered Saline (TBS-140mM NaCl, 0.5mM KCl, 0.25mM Tris) by vigorous vortexing followed by centrifugation at 3000 rpm for 10 minutes.

6. The washed white cell pellet was suspended completely by vortexing in 5 ml of saline EDTA buffer (SE- 75mM NaCl, 35mM EDTA). To this mixture 25 μl of Proteinase-K (20 mg/ml) and 0.25 ml of 20 per cent SDS were added, mixed well and incubated at 50°C for a minimum of three hours.

7. To the digested sample, 300μl of 5M NaCl was added and mixed by vortexing. An equal volume of phenol (pH 7.8) saturated with Tris-HCl, was added, mixed and centrifuged at 3500 rpm for 15 minutes.

8. The aqueous phase containing DNA was collected in fresh tubes, to which an equal volume of saturated phenol:chloroform:isoamylalcohol (25:24:1) was added. The contents were mixed thoroughly by inversion for 10 minutes and centrifuged at 3500 rpm for 15 minutes.

9. To the aqueous phase collected in fresh tubes, equal volume of chloroform:isoamylalcohol (24:1) was added, mixed and centrifuged at 3500 rpm for 15 minutes.

10. The supernatant was transferred to a sterile 50 ml beaker and $1/10^{th}$ volume of $3M$ Sodium acetate (pH 5.5) was added.

11. An equal volume of chilled isopropyl alcohol was added and the precipitated DNA was spooled out using a fresh micropipette tip, washed in 70 per cent ethanol and air-dried.

12. Dried DNA was resuspended in 0.5 ml of Tris EDTA buffer (TE-10mM Tris, 0.1mM EDTA) and stored at $-20°C$.

3.2.2. Extraction from Semen

1. Fresh semen containing around 300-500 million sperms (1-2 ml) was used for isolation of DNA. The sample was taken in a centrifuge tube and was washed twice with 10 ml of Phosphate Buffered Saline (PBS- 138mM NaCl, 4mM NaH$_2$PO$_4$, and 6mM Na$_2$HPO$_4$) by centrifugation at 3500 rpm for 10 minutes. In case of frozen semen, five straws were thawed in water and the semen collected in centrifuge tubes was washed with PBS.

2. The semen pellet was resuspended in 2 ml of PBS by vortexing.

3. 6ml of warm sperm lysis buffer (100mM Tris, 500mM NaCl, 10mM EDTA, 1 per cent SDS, 2 per cent Mercaptoethanol) was added, mixed well and incubated at 50°C in a water bath for 30 minutes with occasional mixing.

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

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

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

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

8. To the aqueous phase collected, equal volume of chloroform: isoamylalcohol (24:1) was added, mixed well for 10 minutes and then centrifuged at 3500 rpm for 15 minutes.

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

10. Equal volume of chilled isopropylalcohol was added and the precipitated DNA was spooled using a fresh micropipette tip.

11. The spooled out DNA was immediately washed with 70 per cent ethanol and was air-dried. The air dried DNA was resuspended in 500µl TE buffer and stored at −20°C.

3.2.3. Determination of Yield and Purity of DNA

20μl of the DNA stock solution was diluted to 2 ml with sterile distilled water giving a dilution of 100 times. Optical Densities (OD) were measured at 260 nm and 280 nm wavelengths using a 2-ml cuvette in a UV spectrophotometer (Genway, UK). Sterile distilled water was used as blank. Yield and purity of DNA samples were estimated as follows.

3.2.3.1. *Yield*

An OD of 1 at 260 nm wavelengths corresponds to approximately 50μg/ml of double stranded DNA. Concentration of the DNA stock solution was calculated as follows.

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

The total yield of DNA obtained from 5ml blood was calculated by multiplying the concentration and the volume of DNA stock solution.

3.2.3.2. *Purity*

Purity of DNA sample was assessed by estimating the ratio between the readings at 260 and 280nm wavelengths**.** The quality of DNA samples was first assessed electrophoretically on 0.7 per cent agarose gel in 1X TAE buffer in horizontal submarine gel electrophoresis unit.

3.3. PCR ANALYSIS

3.3.1. Template DNA

Working solution of DNA was prepared from the DNA stock solution by diluting with sterile triple distilled water to get a final concentration of 50 ng/μl. One microliter of this working solution was used in every 10 μl reaction.

3.3.2. Selection of Primers

A set of microsatellite markers was selected from various available literatures and the primers were custom synthesized. These markers were typed for their polymorphicity. Three markers viz. DRB3, ETH131 and FSHβ, which exhibited high degree of polymorphism, were chosen for the study. The sequences of the forward and reverse primers for each locus are as follows.

Locus		Primer sequence (5'-3')
DRB3	F	GAGAGTTTCACTGTCGAG
	R	CCAGAGTGAGTGAAGTATCTC
ETH131	F	GTGGACTATAGACCATAAGGTC
	R	GCTGTGATGGTCTACGAATGA
FSH β	F	TGGGATATAGACTTAGTGGC
	R	CAGTTTCTAAGGCTACATGGT

The primers obtained in lyophilized form were reconstituted in sterile triple distilled water to make a stock solution of 200pM/μl concentration. The solutions were incubated at room temperature for one hour and then stored at -20°C. Working solutions of the primers were prepared by dilution from the stocks.

3.3.3. Incorporation of Radioactivity: End-labelling of Primers

For visualizing the PCR products by autoradiography, one of the primers was radio-labelled. The forward primer for each marker was radio-labelled at the

5' end with γ ^{32}P-ATP. The reaction was carried out with the DNA End-labelling Kit 1 (Genei).

The procedure for end-labelling was as follows.

The following components were added to a 0.2ml-microcentrifuge tube in the order.

10 X Polynucleotide Kinase (PNK) buffer	-	1 μl
Forward primer (200 pM/μl)	-	1 μl
T4 Polynucleotide kinase (5U/μl)	-	0.5 μl
γ ^{32}P-ATP (10mCi/ml)	-	1 μl
Nuclease free water	-	6.5 μl

The mixture was incubated at 37°C for half an hour. The final volume was made upto 40 μl. One microliter of this end-labelled primer was used for every 10 μl PCR reaction.

3.3.4. PCR Conditions

The PCR conditions for each microsatellite loci were standardized separately. Each reaction was carried out in a 10 μl volume. PCR reaction was set up with 1μl of 10X PCR buffer (100mM Tris-pH 8.3, 500mM KCl) , 200μM dNTP, 1μl of end-labelled and diluted forward primer, 5pM of reverse primer and 0.3U of *Taq* DNA polymerase. Concentration of MgCl$_2$ used varied with primers (1.25mM each for DRB3 and FSHβ, and 1.5mM for ETH 131). The reaction mixture was mixed well and subjected to amplification in a thermal cycler (Techne Flexigene). The thermal cycling involved 35 cycles each consisting of denaturation at 94°C for one minute, annealing at 60°C for DRB3 and FSHβ and 55°C for ETH131 for one minute and extension at 72°C for one

minute. This was followed by a final extension for five minutes at 72°C. The samples were then cooled down to 4°C and stored at −20°C till further analysis.

3.4. ELECTROPHORESIS

3.4.1. Agarose Gel Electrophoresis

The PCR products were checked in 1 per cent agarose gels in 1X Tris Acetate EDTA (TAE) buffer in a horizontal submarine electrophoresis unit. Appropriate sized gel tray was cleaned and sealed. The tray was placed on a leveled surface and comb kept in proper position in the tray. One percent Agarose in TAE buffer (0.04mM Tris acetate, 0.001mM EDTA) was heated in a microwave oven until it was a clear solution, cooled to 60°C and 0.5µl /ml of Ethidium bromide added and mixed well. The mixture was cooled to hand bearable temperature and carefully poured into the gel tray avoiding air bubbles. After solidification, the comb and sealing was removed gently and the tray was immersed in the buffer tank (Amersham Pharmacia Biotech, USA) containing 1X TAE buffer. 1µl of the PCR product was mixed with 1/6th volume of 6X gel loading buffer (Bromophenol Blue 0.25 per cent, Xylene cyanol 0.25 per cent, Sucrose 40 per cent) was loaded into the wells carefully. *Hae* III digested pBR322 DNA was mixed with gel loading buffer and loaded into one of the wells as marker.

Electrophoresis was carried out at 2V/cm until the Bromophenol blue dye migrated more than 2/3rd of length of the gel. The gel was visualized under UV transilluminator and products detected. Those samples with amplified PCR products were subjected to Polyacrylamide gel electrophoresis.

3.4.2. Denaturing Polyacrylamide Gel Electrophoresis

The radioactively labelled PCR products were fractionated using 6 per cent denaturing polyacrylamide gels for better resolution. Denaturing Polyacrylamide Gel Electrophoresis (PAGE) was performed on the Vertical

Sequencer (Consort) as described by Biggin *et al.* (1983). The gels were set between two glass plates (41x33cm, 39x33cm) separated by 0.35 mm thick spacers.

3.4.2.1. Casting the gel

The glass plates were cleaned thoroughly with soap solution and dried. Traces of grease and oil were removed by repeated wiping with alcohol. One of the plates was gently coated with Dimethyl dichlorosilane solution (BDH) to prevent the gel from adhering to both the plates. The plates were assembled with 0.35 mm thick spacers in between and the sides and bottom sealed with sealing tape.

The gel was prepared by mixing 60 ml of 0.5X TBE gel mix (6 per cent Acrylamide, $6M$ urea, 0.5X TBE) and 125 µl each of 10 per cent Ammonium persulphate solution (APS) and N,N,N',N', Tetra Methyl Ethylene Diamine (TEMED) in a beaker. The mixture was poured between the glass plates avoiding air bubbles. The plates were clamped and the comb (Shark toothed comb) inserted on top with the toothed surface facing upwards. The gel was allowed to set for an hour before electrophoresis. The tapes were removed, plates cleaned and assembled in the sequencer. The upper and lower electrode tanks were filled with 1X Tris Borate EDTA (pH 8.3) buffer (TBE- $0.045M$ Tris-borate, $0.001M$ EDTA) to the required level. The comb was removed, wells cleaned with buffer solution and comb was then reinserted in opposite direction to form sample-loading wells.

3.4.2.2. Loading of samples

The PCR products were mixed with 3.5 µl formamide loading buffer (0.02 per cent Xylene Cyanol, 0.02 per cent Bromophenol Blue, 10m*M*M EDTA, 98 per cent deionised formamide) denatured at 95°C for 5 min and cooled immediately on ice. About 4 µl each of this mixture was loaded into each well with great care to avoid mixing up of the samples from adjacent wells.

Sequenced products of M13 DNA were loaded simultaneously in the middle or side wells.

3.4.2.3. Electrophoresis

The gels were electrophoresed at 40W for three hours maintaining a temperature of around 50°C. The bromophenol dye in the loading buffer acted as indicator of the mobility of DNA fragments and had a mobility equivalent to a 25 base fragment and the Xylene cyanol dye had a mobility equivalent to an approximately 100 base fragment.

3.5. SEQUENCING M13 BACTERIOPHAGE DNA

Determination of apparent sizes of alleles necessitated comparison with a sequencing ladder from M13. Single stranded M13 phage DNA was sequenced using the DNA Sequencing Kit Version 2.0 (M/s Amersham Biosciences Corporation, USA). Manufacturers' instructions were followed.

1. Preparation of annealing mixture

   The composition of the mixture was as follows.

M13 phage DNA (0.2 µg/µl)	-	5 µl
5X Sequenase reaction buffer	-	2 µl
Forward primer (0.5p*M*/µl)	-	1 µl

   The volume was made up to 10 µl with distilled water. The mixture was centrifuged, incubated at 65°C for two minutes and then slowly cooled to room temperature over 15-30 min and chilled on ice.

2. Four tubes labelled G, A, T and C were filled with 2.5 µl of each dideoxy termination mixtures (ddGTP, ddATP, ddTTP and ddCTP).

3. Dilution of labelling mix

The labelling mix provided in the kit was diluted five fold as follows:

5X Labelling mix   -   0.5 µl

Water   -   2.0 µl

4. Dilution of enzyme

The enzyme (Sequenase version 2.0) was diluted eight fold with Sequenase enzyme dilution buffer as follows.

Sequenase Enzyme (13 U/µl) -   0.5 µl

Sequenase dilution buffer -   3.5 µl

5.   Labelling reaction

To the ice cold annealed DNA mixture, the following components were added.

Dithiothreitol (DTT 0.1 *M*) -   1 µl

Diluted labeling mix   -   2 µl

Diluted enzyme   -   2 µl

α ^{35}S dATP (10 µCi/ml)   -   2 µl

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

6.   3.5 µl of labelling reaction mixture was transferred to each termination tubes (G, A, T, C), mixed well and incubation continued at 37°C for five minutes.

7.   The reaction was stopped by addition of 4µl of stop solution provided in the sequencing kit and stored at -20°C.

3.6. DRYING OF GELS

After electrophoresis the glass plates were removed from the sequencer, and carefully separated. The gel adhering to one of the plates was transferred to a filter paper. The position of the first well was marked by cutting out a small portion of the corresponding corner. The gel was covered with a Klin film and dried in a gel drier at 80°C.

3.7. AUTORADIOGRAPHY

The Klin wrap was removed after drying and the gel was set for autoradiography with X-ray film (Kodak, 35.6 x 43.2 cm) in a cassette (Kiran Hypercassette) fitted with an intensifying screen. The X-ray film was developed after 24 to 48 hours depending on the intensity of radioactive signal.

3.8. DEVELOPMENT OF X-RAY FILM

The X-ray film was developed in the dark room. Developing was done by transferring the film serially into 1X developer solution (Kodak) for three to five minutes, 1 per cent acetic acid for a minute followed by washing in distilled water and finally into fixer solution (Kodak) for six to ten minutes.   The developed film was washed thoroughly in running water and dried.

3.9. MICROSATELLITE TYPING

The number of alleles for each marker was counted and their apparent sizes were determined by comparing with M13 sequencing ladder. The G, A, T and C sequences were read from the bottom to the top in order. The allele sizes were assigned corresponding to the G, A, T, C bands. The frequency of each allele was worked out.

3.10. STATISTICAL ANALYSIS

3.10.1. Heterozygosity (He)

Heterozygosity is a measure of usefulness of the marker. Heterozygosity was calculated by the method of Ott (1992).

$$He = 1 - \sum_{i=1}^{k} p_i^2$$

where p_i is the frequency of ith allele at a locus. Markers with higher heterozygosity values are more useful.

3.10.2. Polymorphic Information Content (PIC)

The polymorphic information content expresses informativeness or usefulness of a marker for linkage studies. PIC values of the markers were calculated as

$$PIC = 1 - \left[\sum_{i=1}^{k} p_i^2 \right] - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2 p_i^2 p_j^2$$

Where p_i and p_j are the frequencies of ith and j^{th} alleles respectively. (Botstein *et al.* 1980.)

3.10.3. Probability of Parentage Exclusion

Probability of exclusion of an incorrect sire is the probability that a series of co-dominant alleles of known frequency should detect a falsely recorded sire. PE for each marker was calculated using the method of Jamieson, (1994).

$$PE = \sum_{i} p_i (1-p_i)^2 - \sum_{i>j} (p_i p_j)^2 \{ 4 - 3 (p_i + p_j) \}$$

3.10.4. Combined Probability of Exclusion (CPE)

Combined probability of exclusion is the cumulative probability of exclusion using two or more markers. CPE was calculated using the method of s Wenk *et al.* (1992).

$$CPE = 1- (1-PE_1) (1-PE_2) (1-PE_3)$$

Where PE is the probability of exclusion of each independent locus.

Results

4. RESULTS

4.1 ISOLATION OF GENOMIC DNA

DNA was isolated from whole blood, fresh and frozen semen samples using phenol: chloroform extraction procedure.

4.1.1 Yield and Quality of DNA

The mean yield of DNA obtained from blood, fresh and frozen semen samples are presented in Table 4.1. The Optical Density ratios at 260/280 nm scale ranged from 1.64 to 1.81 for blood samples and that from semen samples ranged from 1.42 to 1.73 for fresh semen and 1.54 to 1.76 for frozen semen, respectively. On agarose gel electrophoresis, the DNA samples were found to be of high molecular weight and appeared as single band without sheared fragments.

4.2 PCR ANALYSIS

A set of microsatellite primers were selected, the primers were custom synthesized and used for PCR amplification of DNA samples. Based on the polymorphisity and ease of typing, three markers viz., DRB3, ETH131 and FSHβ were chosen for the study. These markers were typed on 100 DNA samples collected randomly from University Livestock Farms. PCR conditions were optimized separately for each of the primers. The reaction components, temperature and time optimized for each pair of primers are presented in Table 4.2.

4.3 NUMBER, SIZE AND FREQUENCIES OF ALLELES

The PCR products were first checked by agarose gel electrophoresis (as shown in plate 1) and then fractionated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography as band patterns. The number of alleles was counted manually and their apparent sizes were assigned by

comparison with the sequence of M13 DNA run alongside the samples in the gel. The banding patterns differed for the three markers typed, as indicated in Plates 2 to 4. Each allele of DRB3 and ETH131 appeared as double bands, one darker and another lighter band below. Each allele of FSHβ was represented by more than two bands not clearly distinguishable and hence appeared as a broad and thick band.

The frequencies of the alleles for each locus were worked out separately. The number, size ranges and frequencies of alleles for each locus are as follows.

4.3.1 DRB3

The DRB3 locus was highly polymorphic with 17 alleles ranging in size from 138-192 bp. The allele sizes and frequencies obtained are presented in Table 4.3.

4.3.2 ETH131

Eleven alleles with sizes ranging from 134-168 bp were detected for ETH131. The allele sizes and frequencies are given in Table 4.4.

4.3.3 FSHβ

FSHβ had nine alleles with size range of 184-214 bp. The corresponding allele frequencies are given in Table 4.5.

4.4 HETEROZYGOSITY

The heterozygosity values (He), which indicate the usefulness of the markers, were calculated by the method of Ott (1992). Heterozygosity values obtained were 0.8938 for DRB3, 0.8385 for ETH131 and 0.8519 for FSHβ, respectively.

4.5 POLYMORPHIC INFORMATION CONTENT

The level of informativeness of the markers as measured by polymorphic information content was calculated using the method of Botstein *et al.* (1980). Highest PIC value was obtained for DRB3 (0.8864) followed by FSHβ (0.8392) and ETH131 (0.8157).

4.6 PROBABILITY OF PARENTAGE EXCLUSION

The probability of exclusion of an incorrect sire, given by PE was worked out for each locus separately using the method of Jamieson (1994). The exclusion probabilities obtained were 0.7913 for DRB3, 0.6788 for ETH131 and 0.7035 for FSHβ, respectively.

The heterozygosity, PIC and PE values for the three markers are given in Table 4.6.

4.7 COMBINED PROBABILITY OF EXCLUSION

The combined probability of exclusion using the three markers was arrived using the method of Wenk *et al.* (1992). DRB3 together with ETH131 yielded a combined exclusion probability of 0.9329, and with FSHβ gave an exclusion probability of 0.9381. The combined exclusion probability with ETH131 and FSHβ was 0.9047. The three markers together yielded a cumulative exclusion probability of 0.9801.

4.8 VERIFICATION OF PARENTAGE

The inheritance of markers used in the study was tested on known sire families. In case of correct sire, in all the three markers, the allele sizes of the progeny matched with that of the sire and dam. In case of incorrect sire, the allele sizes of the progeny were found to match with the dam, but not with the sire. Thus incorrect sire was clearly identified. The inheritances of the markers are shown in Plates 5 to 7.

Table 4.1 Yield of DNA extracted from different sources

Whole blood	Frozen semen	Fresh semen
388.2 ± 14.3 μg/5 ml	116.95 ± 25.2 μg/ 150 million sperms	182.15 ± 6.2 μg/ 400 million sperms

Table 4.2. PCR parameters

	Parameters	Primers		
		DRB3	ETH131	FSHβ
REACTION MIX		Quantity		
1	Template DNA	50ng	50ng	50ng
2	MgCl$_2$	1.25mM	1.5mM	1.25mM
3	10X Reaction Buffer	1μl	1μl	1μl
4	dNTPs	200μM	200μM	200μM
5	Forward Primer	5pM	5pM	5pM
6	Reverse Primer	5pM	5pM	5pM
7	*Taq* DNA Polymerase	0.3U	0.3U	0.3U
8	Reaction Volume	10μl	10μl	10μl
CYCLE PARAMETERS		Temperature/time		
9	Denaturation	94ºC/1min	94ºC/1min	94ºC/1min
10	Annealing	60ºC/1min	55ºC/1min	60ºC/1min
11	Extension	72ºC/1min	72ºC/1min	72ºC/1min
12	Number of cycles	35	35	35
13	Final Extension	72ºC/5min	72ºC/5min	72ºC/5min

Table 4.3 Allele sizes and frequencies at DRB3 locus

Sl. No.	Size in base pairs	Allele frequencies
1.	138	0.015
2.	148	0.040
3.	160	0.080
4.	162	0.015
5.	164	0.025
6.	166	0.020
7.	168	0.025
8.	170	0.075
9.	172	0.115
10.	174	0.015
11.	176	0.065
12.	178	0.075
13.	180	0.230
14.	182	0.025
15.	184	0.025
16.	186	0.100
17.	192	0.055

Table 4.4 Allele sizes and frequencies at ETH131 locus

Sl. No.	Size in base pairs	Allele frequencies
1.	134	0.005
2.	138	0.005
3.	142	0.035
4.	144	0.060
5.	148	0.215
6.	150	0.220
7.	152	0.170
8.	156	0.165
9.	158	0.060
10.	162	0.040
11	168	0.025

Table 4.5 Allele sizes and frequencies at FSHβ locus

Sl. No.	Size in base pairs	Allele frequencies
1.	184	0.045
2.	188	0.040
3.	190	0.050
4.	192	0.145
5.	198	0.185
6.	200	0.225
7.	204	0.125
8.	210	0.140
9.	214	0.045

Table 4.6 Number of alleles, Heterozygosity, PIC and PE

Observations	DRB3	ETH131	FSHβ
No. of alleles	17	11	9
Size range of alleles	138-192	134-168	184-214
Heterozygosity	0.8938	0.8385	0.8519
PIC	0.8864	0.8157	0.8392
PE	0.7913	0.6788	0.7035

A
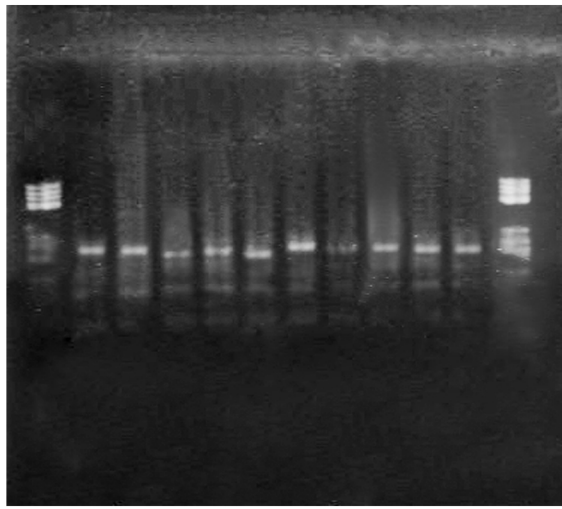
B
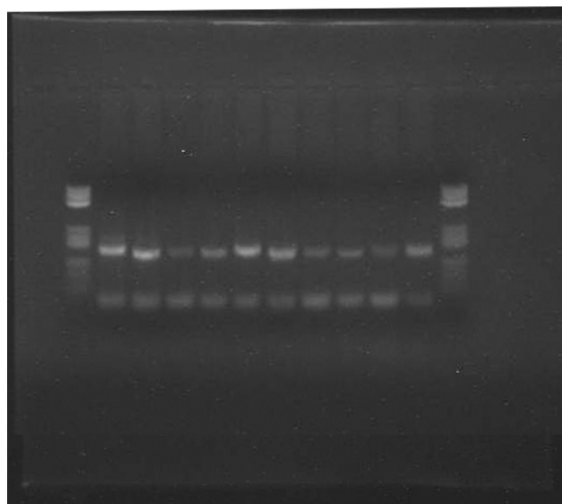
C
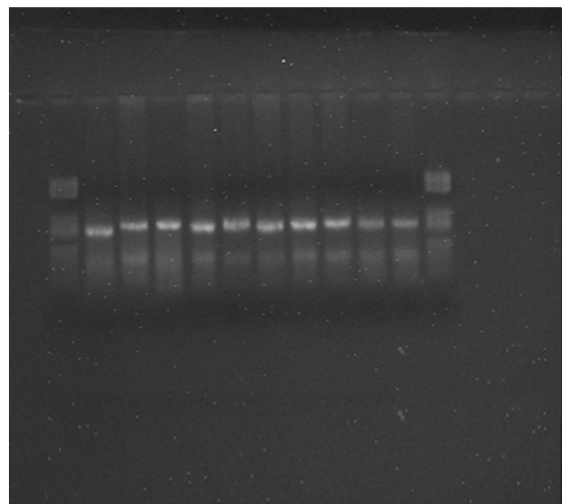
Plate.1. Agarose gel electrophoresis

A- DRB3 locus

B- ETH131 locus

C- FSHβ locus

First and Last lane- pBR322 *Hae*lll digest as marker
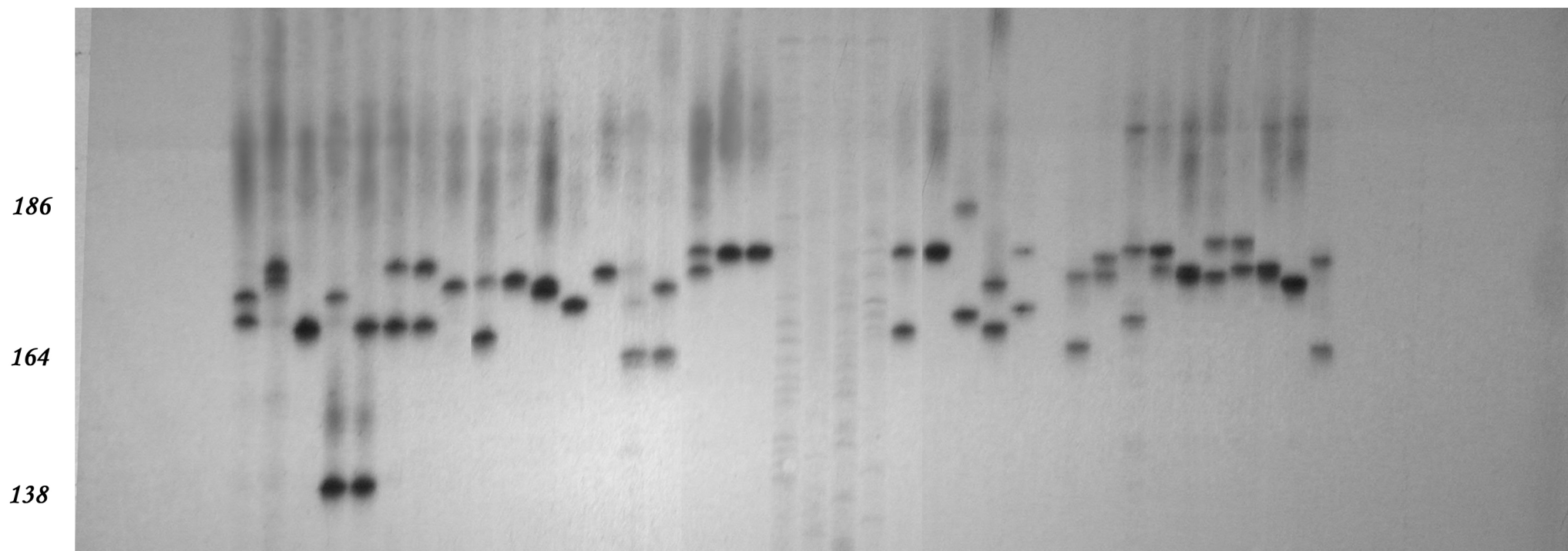
Plate.2 . Autoradiograph of gel showing polymorphism at DRB3 locus

Lanes 1-18, 23-38; PCR products

19-22; G,A,T,C sequences of M13 DNA
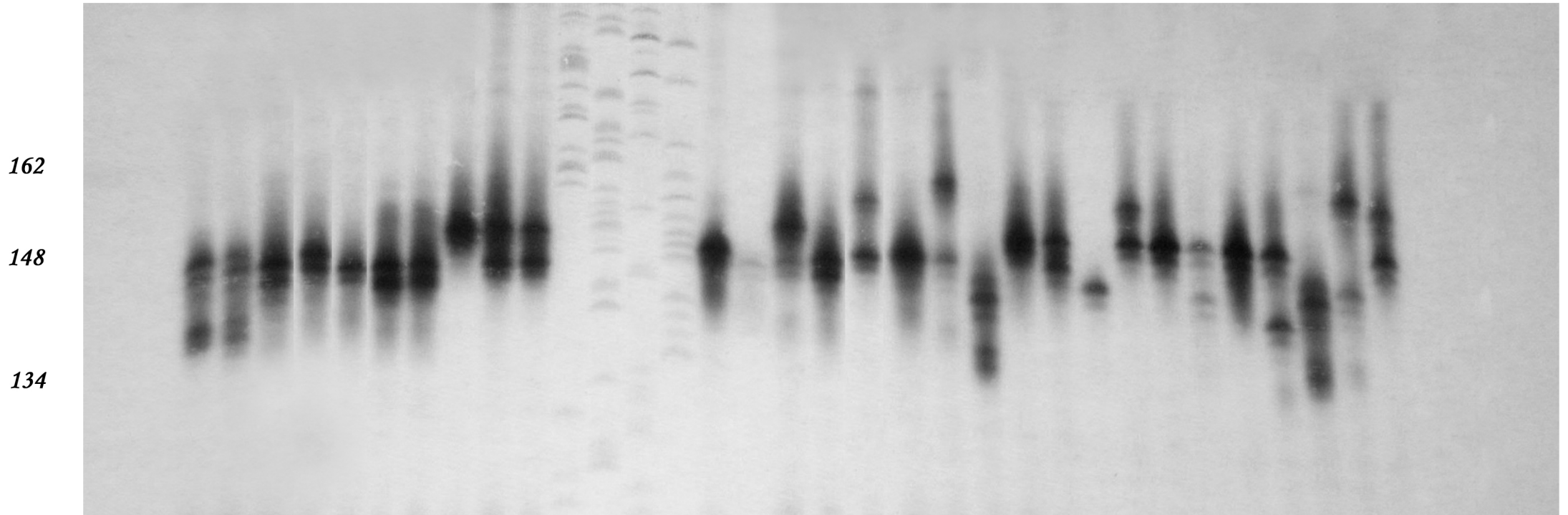
Plate.3. **Autoradiograph of gel showing polymorphism at ETH131 locus**

Lanes 1-10,15-33; PCR products

11-14; G,A,T,C sequences of M13 DNA.
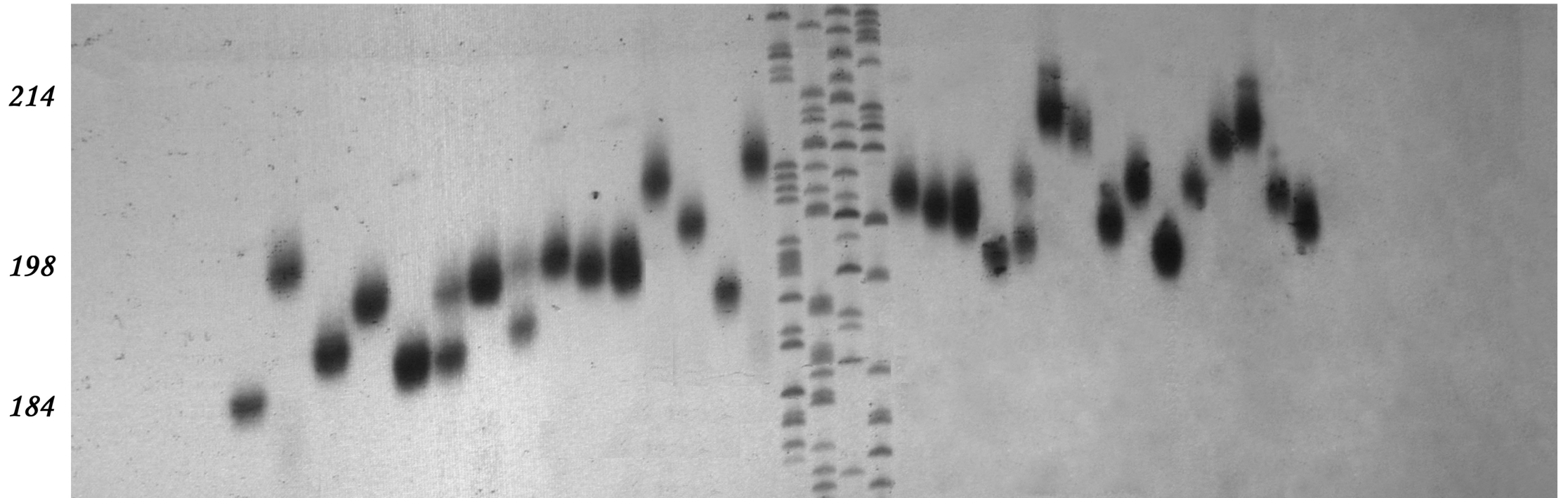
Plate .4. Autoradiograph of gel showing polymorphism at FSHβ locus

Lanes 1-15,20-34; PCR products

16-19; G,A,T,C sequences of M13 DNA
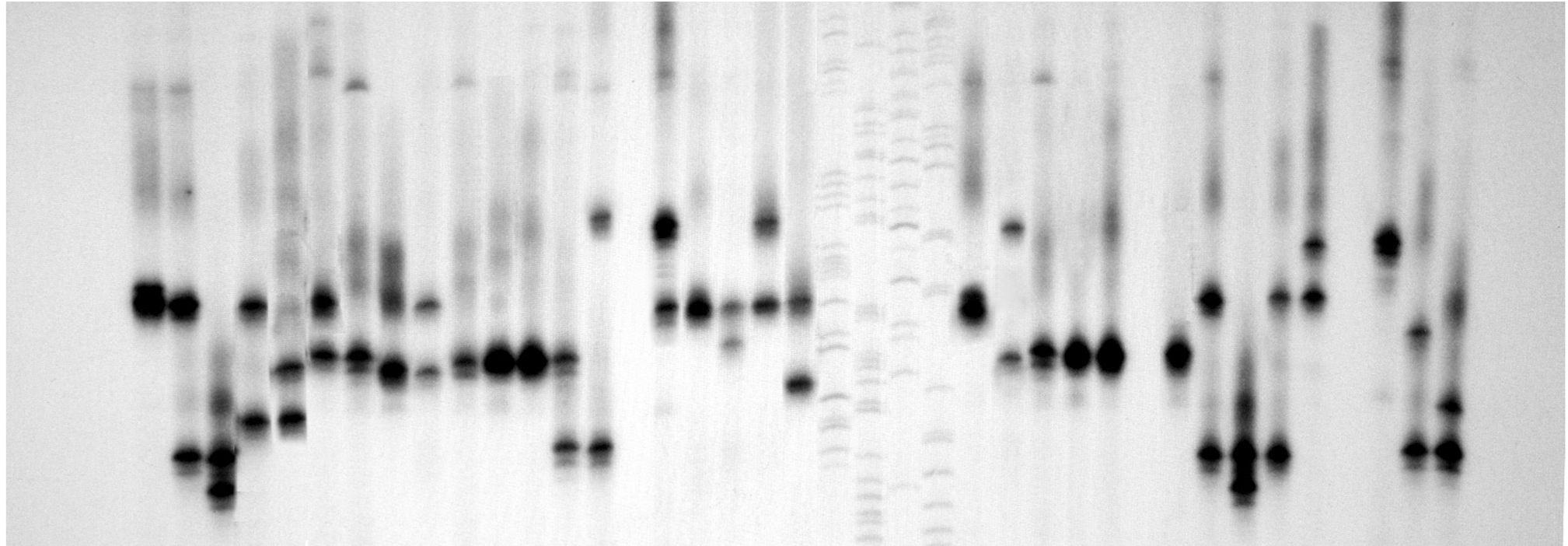
Plate.5. **Autoradiograph of gel verifying parentage using DRB3 locus**

Lanes 1-20; correct parentage

1,8,16,-sires; 2,4,6,9,11,13,17,19-progeny; 3,5,7,10,12,14,18,20-dams.

21-24; G,A,T,C sequences of M13 DNA

25-39; wrong parentage

25,31,37-sires; 26,28,32,34,38-progeny; 27,29,33,35,39-dams.
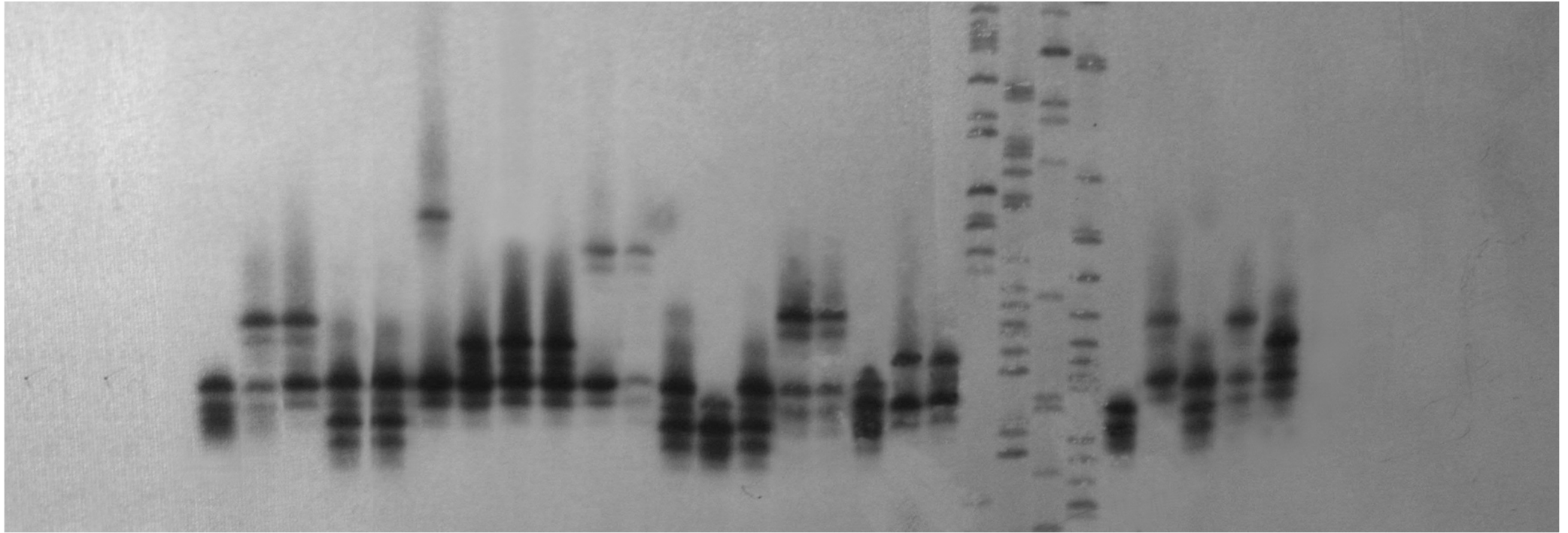
Plate.6. **Autoradiograph of gel verifying parentage using ETH131 locus**

Lanes 1-19; correct parentage

1,8,12,17-sires; 2,4,6,9,11,13,15,18-progeny; 3,5,7,10,14,16,19-dams.

20-23; G,A,T,C sequences of M13 DNA

24-28; wrong parentage

24-sire; 25,27-progeny; 26,28-dams.
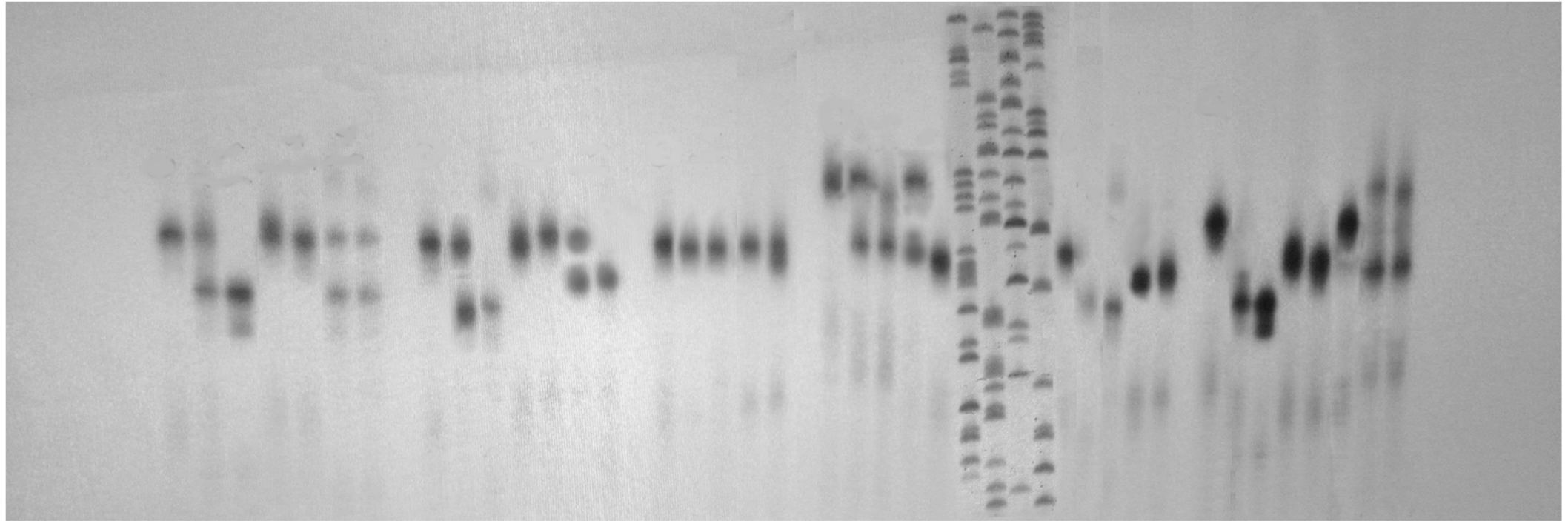
Plate. 7. Autoradiograph of gel verifying parentage using FSHβ locus

Lanes 1-27; correct parentage

1,9,17,23-sires; 2,4,6,10,12,14,18,20,24,26-progeny;

3,5,7,11,13,15,19,21,25,27-dams.

28-31; G,A,T,C sequences of M13 DNA

32-45; wrong parentage

32,38,43-sire; 33,35,39,41,44-progeny; 34,36,40,42,45-dams.

Discussion

5. DISCUSSION

Microsatellites represent one of the most abundant families of interspersed repetitive DNA in eukaryotic genome (Miesfeld *et al.*, 1981). The existence of short tandem repeats such as d(A)n, d(T)n, (dT-dG)n, (dA-dC)n have been described by several investigators (Litt and Luty, 1989; Tautz, 1989; Fries *et al.,* 1990; Stallings *et al.*, 1991). The ease in isolation and characterization of these sequences coupled with high levels of polymorphism and the method of analysis make them attractive genetic markers (Weber, 1990; Kemp and Teale, 1991).

Microsatellite markers had been proved to be an alternative to blood typing for parentage determination in cattle (Glowatzki-Mullis *et al.*, 1995; Usha *et al.*, 1995; Curi and Lopes, 2003).

5.1 YIELD AND QUALITY OF DNA

The mean yield of DNA obtained from 5 ml of whole blood was 388.2 ± 14.3 µg. This yield was comparable to that obtained by several researchers. Andersson *et al.* (1986), Apparao *et al.* (1994) and Senthil *et al.* (1996) reported yields of 150-200 µg/10 ml, 250-300 µg/15 ml and 444.58 ± 21.5 µg/15 ml of whole blood, respectively, using phenol: chloroform extraction method.

The mean yield of DNA obtained from fresh semen was 182.15 ± 6.2 µg/ 400 million sperms and that from frozen semen was 116.95 ± 25.2 µg/ 150 million sperms. Similar yields were reported by Lien *et al.* (1990) (300-500 µg/2.5 x 10^8 sperm cells) and Aravindakshan *et al.* (1998) (441.85 µg/3x10^8 sperm cells).

5.2 PCR ANALYSIS

The PCR parameters varied for the three primer sets. The concentration of different components and the time temperature parameters for PCR, which were best suited to obtain reproducible band patterns were identified after conducting a series of trials.

In polymerase chain reactions, the most variable factor was found to be of Mg^{2+} concentration, which had significant effects on polymerase activity and annealing efficiency. Excess concentrations produced nonspecific products, which made genotyping difficult, while decreased concentrations reduced the number of products. In the present study, 1.25 mM $MgCl_2$ was found optimal for both DRB3 and FSHβ while a higher concentration of 1.5 mM was found suitable for ETH131. Ellegren *et al.* (1993) recommended 1.5 mM $MgCl_2$ in a 20 µl reaction volume for DRB3. Kemp and Teale (1991) used 2 mM $MgCl_2$/20 µl reaction volume for FSHβ. Usha *et al.* (1995) found concentration of 1.25 mM $MgCl_2$ optimal for DRB3, FSHβ and ETH131.

Another important variable was the annealing temperature. Hybridization of primers to the specific sites requires specific annealing temperatures, which varies with the length and nucleotide composition of the primers. Specificity of primer annealing increases with increasing temperature. With too low annealing temperatures, one or both primers will anneal to sequences other than the true target as single base mismatches or partial annealing may be tolerated. This leads to non-specific amplification and consequent reduction in yield of desired product. Annealing temperatures ranging from 55-65°C were tried for each primer and a temperature of 60°C was found optimal for DRB3 and FSHβ while a slightly lower temperature of 55°C for ETH131. This was in accordance with the recommendation by Usha *et al.* (1995).

The other components of the PCR like concentrations of template DNA, dNTPs, primers and *Taq* DNA polymerase were constant for all the markers.

Template DNA was used at a concentration of 50 ng and forward and reverse primers at concentration of 5 pM per 10 µl reaction. Higher primer to template ratio resulted in non-specific amplification and primer dimer formation. dNTPS at 200µM concentration and 0.3 U of *Taq* DNA polymerase were found optimum.

5.3 NUMBER, SIZE AND FREQUENCIES OF ALLELES

Three markers used in the study were found to be polymorphic and informative. DRB3 was found highly polymorphic followed by ETH131 and FSHβ. In the study of Ellegren *et al.* (1993) involving three breeds of cattle, 14 alleles with sizes range of 159-219 bp were described for DRB3. Usha *et al.* (1995) reported 23 alleles ranging in size from 144-220 bp across 15 breeds of cattle and 25 alleles with size range of 143-215 bp were detected by Haeringen *et al.* (1999) for DRB3. In the present study, 17 alleles are obtained for DRB3 with size range of 138-192 bp.

The number of alleles obtained at ETH131 locus was lesser than those reported by various investigators. Eleven alleles with size ranging from 134-168 bp were obtained for ETH131. This was less than the 21 alleles obtained by Glowatzki-Mullis *et al.* (1995); 24 alleles detected by Usha *et al.* (1995) and 13-20 alleles obtained by Arranz *et al.* (1996). The number of alleles observed for FSHβ also showed similar variations. Nine alleles with sizes ranging from 184-214 bp were detected for FSHβ. This was comparable to the 11 alleles reported by Moore *et al.* (1992) and eight alleles reported by Steffen *et al.* (1993) and Ron *et al.* (1994). Usha *et al.* (1995) reported 19 alleles at the locus across all 17 breeds of cattle which was much higher than that observed in the present study.

One of the possible reasons for the lesser number of alleles recognized in this study is due to the smaller sample size. Another possibility is that the samples for the study were confined to a limited area thus giving a narrow genetic base. Reports of higher number of alleles obtained were from a larger number of breeds (Usha *et al.*, 1995).

Each allele of dinucleotide repeat consists of more than one fragment. These shadow bands obscure the position of allele fragments, which makes genotyping difficult while typing individuals heterozygous for alleles differing in length by only two nucleotides (Murray *et al.*, 1993).

In the sequence of FSHβ there is a stretch of $(T)_{13}$ adjacent to the dinucleotide repeats which can be responsible for single basepair differences in size (Fries *et al.,* 1990). The alleles varying in a single repeat often becomes indistinguishable through autoradiography. This could be a possible reason for the decreased number of alleles observed at the locus.

A heterozygote with two alleles separated in length by only two bases produces a characteristic triplet pattern in which the middle band is slightly stronger than the top band and these two bands represent the alleles (Hughes, 1993). Too long time of exposure or high volumes of loaded product results in a pattern of smears in autoradiography rather than distinct bands. Hence distinguishing homozygote from a heterozygote often becomes difficult.

During gel electrophoresis, gel distortion happens because of variation in mobility across the gel due to temperature gradients caused by leakage of buffer or uneven current or gel irregularities arising from preparation or handling. With this, the allele identification and sizing becomes problematic because of lane-to-lane variation of bands (Maryland *et al.*, 1992).

All these limitations could be overcome by using automatic fluorescent DNA typing methods (Ziegle *et al.*, 1992). With co-amplification and co-electrophoresis, the number of loci analysed on a gel can be increased. Amplification of different loci can be done with different markers and the products mixed together at a comparable concentration and loaded on a single lane for analysis. But the use of this approach is limited, as products cannot be distinguished if the loci overlap in size. This could be overcome by labelling the primers with different fluorescent dyes having different emission wavelengths

and the simultaneous analysis of microsatellite loci, which overlapped in size. This is more suitable for routine typing. This can also overcome the hazards of handling dangerous radioactive materials.

5.4 HETEROZYGOSITY

Heterozygosity gives a measure of usefulness of the marker. Higher the heterozygosity values, higher are the probabilities of paternity exclusion (Luikart *et al.*, 1999). In the present study, heterozygosity values of 0.8938, 0.8385 and 0.8519 were obtained for DRB3, ETH131 and FSHβ, respectively. These fairly high values indicate the suitability of the markers for parentage studies.

5.5 POLYMORPHIC INFORMATION CONTENT

Polymorphic information content (PIC) is the probability that a given offspring of a parent carrying the rare allele at an index locus will allow deduction of the parental genotype at the marker loci (Botstein *et al.*, 1980). Informativeness of the marker increases as the averge number of repeats increases (Weber, 1990). Microsatellites display a high degree of polymorphism with a mean PIC value of 0.6 (Vaiman *et al.,* 1994). In the current study, DRB3 locus was highly informative with PIC value of 0.8864 followed by FSHβ with 0.8392 and ETH131 with 0.8157. Steffen *et al* (1993) reported a PIC value of 0.80 for ETH 131 in four breeds of cattle while Glowatzki-Mullis *et al.* (1995) obtained PIC values ranging from 0.61 to 0.78 for ETH131 in 238 animals typed. Usha *et al.* (1995) reported an average PIC value of 0.93 for DRB3 and 0.91 for FSHβ in 15 breeds of cattle.

5.6 PROBABILITY OF PARENTAGE EXCLUSION

Probability of Parentage Exclusion indicates the usefulness of a particular marker in parentage testing. The probability of paternity exclusion is the probability that a series of co-dominant alleles of known frequency should detect a falsely recorded father (Jamieson, 1994). The average exclusion power

depends upon the number of alleles as well as their frequencies (Chakraborty *et al.*, 1988). In a study to evaluate microsatellites for paternity testing, Usha *et al.* (1995) reported exclusion probabilities of 0.72 for DRB3, 0.66 for ETH131 and 0.65 for FSHβ. In the present study, similar exclusion probabilities were obtained for the three markers 0.79 for DRB3, 0.68 for ETH131 and 0.70 for FSHβ, respectively.

Exclusion of paternity based on one marker may sometimes lead to misidentification because a mutation may generate a new allele in the offspring, wrongly suggesting exclusion of paternity (Halos *et al.*, 1999). In case of exclusion based on only one microsatellite polymorphism, special attention must be given to whether the offspring and sire in question are homozygous for their allele. In these cases non-paternity could be incorrectly diagnosed due to allele nonamplification (Glowatzki-Mullis *et al.*, 1995). Hence, paternity exclusion should never be based on one locus.

5.7 COMBINED PROBABILITY OF EXCLUSION

Combined Probability of Exclusion (CPE) is the cumulative probability of exclusion using two or more markers (Wenk *et al.*, 1992). It is the probability that a randomly chosen male of a known genotype will be excluded as the parent of a given offspring on the basis of genotype of both the offspring and its female parent at one or more polymorphic loci. In the present study two markers together yielded higher combined exclusion probabilities than individual marker. DRB3 together with ETH131 yielded CPE of 0.9329, while along with FSHβ gave a CPE of 0.9381. CPE obtained with ETH 131 and FSHβ was 0.9047. The three markers together yielded a CPE of 0.9801, which was more than CPE using any two markers.

The inheritance of markers used in the study was tested on known sire families. In case of correct sire, in all the three markers, the allele sizes of the progeny matched with that of the sire and dam. In case of incorrect sire, the allele sizes of the progeny were found to match with the dam, but not with the sire. Thus incorrect sire was clearly identified.

Polymorphic nature of the markers is inversely proportional to the number of markers to be used i.e., the effectiveness do not depend upon the number of microsatellites used, but on the level of informativeness that these markers provide (Curi and Lopes, 2003). Eventhough the markers included in this study were polymorphic, they were still not sufficient to obtain the desired maximum exclusion probability of 0.999. Inclusion of one or two similar polymorphic markers in this panel will yield the maximum probability of exclusion.

Although the efficiency of the microsatellite loci in this study was found high, they may still not represent the best combination of markers for parentage testing. On the contrary, the results presented in this study may stimulate the investigations of other microsatellite loci for their applicability to parentage analysis. The samples used in the study represent a general pool of crossbred animals. In addition to these crossbreds there are several native breeds of cattle in Kerala. The markers to be used for this purpose should be tested in these animals also, and a panel of markers, which gives the highest probability of exclusion, suitable for all genetic groups, has to be set to enable routine typing.

In conclusion, microsatellite loci with their high informativeness and ease in typing and standardization appear to be the simplest way to clarify family relationships and to provide genetic evidence of parentage.

Summary

6. SUMMARY

Parentage verification is important in the selection of future breeding stock. Microsatellite markers with their high degree of polymorphism and ease of typing have overcome the technical limitations in using conventional blood typing, protein polymorphisms and other molecular markers. In the present study, three microsatellite markers were evaluated for their efficiency in paternity testing.

Genomic DNA was isolated from whole blood, fresh and frozen semen samples using phenol: chloroform extraction procedures. DNA samples from 100 genetically unrelated animals were used for determining the polymorphisity of the markers. Samples of known pedigree collected from the University Livestock Farms were used to test the inheritance of the markers.

The mean yield of DNA obtained from 5 ml of whole blood was 388.2 ± 14.3 µg, from fresh semen was 182.15 ± 6.2 µg/400 million sperms and from frozen semen was 116.95 ± 25.2 µg/150 million sperms. The optical density ratio (260/280) ranged from 1.64 to 1.81, 1.42 to 1.73 and 1.54 to 1.76 for DNA obtained from blood, fresh and frozen semen, respectively.

A set of microsatellite markers from the bovine genome map were selected, the primers were custom synthesized and used for PCR amplification of DNA samples. Based on their polymorphisity and ease of typing, three markers viz., DRB3, ETH131 and FSHβ were chosen for the study.   PCR conditions were optimized separately for each of the primers. The forward primer of each primer pair was end-labelled with γ ^{32}P-ATP. 50 ng DNA, 1 µl of 10X reaction buffer, 200 µM dNTP, 5 pM each of forward (end-labelled) and reverse primer and 0.3 U of *Taq* DNA polymerase were used for PCR in 10µl volume. Concentration of MgCl$_2$ was standardized at 1.25 mM for DRB3 and FSHβ and 1.5 mM for ETH131. Thermal cycling was carried out at 94°C for one minute for

denaturation, 60°C for one minute for annealing (DRB3 and FSHβ), 55°C for ETH131and 72°C for one minute for extension for 35 cycles.

M13 phage DNA was sequenced by the dideoxy chain termination method using Sequenase Version 2.0 Sequencing Kit following the manufacturer's instructions. The G, A, T and C sequences were used as markers for allele sizing.

The amplified products checked by agarose gel electrophoresis were fractionated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. The number of alleles were counted and their sizes assigned by comparison with sequences of M13 DNA run along with the PCR products. The frequencies of alleles for each locus were worked out.

DRB3 locus was highly polymorphic with 17 alleles with sizes ranging from 138-192 bp. A heterozygosity value of 0.8938 and PIC value of 0.8864 was obtained.

Eleven alleles were identified at the ETH131 locus. The allele sizes ranged from 134 to 168 bp. The heterozygosity and PIC values obtained for the locus were 0.8385 and 0.8157 respectively.

Number of alleles identified at FSHβ locus was nine with allele sizes ranging from 184-214 bp. The heterozygosity and PIC values obtained for the locus were 0.8579 and 0.8392 respectively.

The probability of exclusion for each marker independently and the combined probability of exclusion using the three markers were calculated. The exclusion probabilities obtained with three markers independently were 0.7913, 0.6787 and 0.7035 for DRB3, ETH131 and FSHβ respectively. The combined probability of exclusion was worked out as 0.9329 with DRB3 and ETH131, 0.9381 with DRB3 and FSHβ, and 0.9047 with FSHβ and ETH131. The three markers together yielded a cumulative exclusion probability of 0.9801.

These markers were tested on known sire families to follow their inheritance. In known pedigree the offsprings clearly showed allele sizes matching the sire and dam. While in case of an incorrect sire, in all the three markers, the allele sizes of the progeny clearly matched with the dam but not with the sire, thus excluding the incorrect sire.

Microsatellite markers can be used efficiently for paternity testing. Few more polymorphic markers have to be used along with the markers studied to obtain a maximum probability of exclusion of 0.99.

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Annexures

ANNEXURE - 1
COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

40% Acrylamide

Acrylamide	380g
N_1N – Methylene bisacrylamide	20 g
Water to	1000 ml

1.5% Agarose

0.9 g of agarose powder weighed and mixed with 60 ml of 1 X TAE buffer in a conical flask. Solution heated in a microwave oven until boiling and cooled slowly.

10% Ammonium persulphate

APS	100 mg
Water to	1 ml

6% Denaturing Acrylamide Gel

0.5 X TBE Gelmix	-	60 ml
TEMED	-	0.125 ml
10% APS	-	0.125 ml

Mixed well without air bubbles

EDTA (0.5 *M*, pH 8.3)

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

Ethidium Bromide (10 mg/ml)

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

Formamide dye/Stop buffer

Deionised formamide	-	98%
Xylene cyanol	-	0.025%
Bromophenol blue	-	0.025%
0.5 M EDTA	-	10mM

Gel loading buffer

Bromophenol Blue	0.25%	50 mg
Xylene cyanol	0.25%	50 mg
Sucrose	40%	8 g

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

Phenol (Saturated, pH 7.8)

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

Phosphate Buffered Saline (PBS, pH 7.4)

Sodium chloride (NaCl)	138 mM	8.0647 g
Sodium Dihydrogen Phosphate (NaH$_2$PO$_4$2H$_2$O)	4 mM	0.6240 g
Disodium hydrogen phosphate (Na$_2$HPO$_4$12H$_2$O)	6 mM	2.1488 g

Contents weighed and mixed with 800 ml distilled water on a magnetic stirrer and pH adjusted to 7.4 using dilute HCl. Final volume made upto 1000 ml.

RBC lysis buffer

Ammonium chloride	150 mM	8.0235 g
Potassium chloride	10 mM	0.7455 g
EDTA	0.1 mM	0.0372 g

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

Sodium acetate (3 M, pH 5.5)

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

Sodium chloride (5 M)

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

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

Sodium chloride	75 mM	4.383 g
EDTA	35 mM	9.306 g

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

Sodium dodecyl sulphate (SDS) 20%

SDS   20 g

Distilled water to make up to 100 ml.

Stirred, filtered and stored at room temperature.

Sperm lysis buffer

Contents	Stock solution	For making 100 ml
0.5% SDS	10%	5 ml
10 m M Tris (pH 8.0)	1 M	1 ml
2% Mercaptoethanol	100%	2 ml
10 m M EDTA (pH 8.0)	0.5 M	2 ml
100 m M Nacl	5 M	2 ml

All reagents except mercaptoethanol added and volume made upto 98 ml. Heated to 65°C just before use and added mercaptoethanol.

Tris Acetate EDTA (TAE) buffer (50X)

Tris base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA (pH 8.0)	20 ml

Distilled water up to 1000 ml

Autoclaved and stored at room temperature

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

Tris base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g

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

0.5X TBE Gel mix

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

Mixed well in 700 ml distilled water

Volume made up to 1000 ml and stored at 4°C.

Tris Buffered Saline (TBS) pH 7.4

Sodium chloride	140 mM	8.18 g
Potassium chloride	0.5 mM	0.0373 g
Tris base	0.25 mM	0.0303 g

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

Tris EDTA (TE) buffer (pH 8.0)

Tris base	10 mM	1.2114 g
EDTA	0.1 mM	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 and stored at 4°C.

Tris 1M (pH 8.0)

Tris base	121.14 g

Distilled water up to 1 litre. pH adjusted to 8.0, filtered and stored at room temperature.

ANNEXURE – II

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

A. CHEMICALS

Acrylamide (Molecular Biology grade)	-	SRL, Bombay
Agarose (Low EED)	-	Bangalore Genei Pvt. Ltd.
Ammonium chloride	-	SRL, Bombay
Ammonium per sulphate	-	SRL, Bombay
N-N-Methylene Bis acrylamide	-	SRL, Bombay
Boric acid	-	SRL, Bombay
Chloroform	-	Merck
Crystalline phenol	-	Merck
Di-sodium hydrogen orthophosphate	-	SRL, Bombay
dNTPs	-	Finn Enzymes
EDTA	-	SRL, Bombay
Ethanol	-	Merk
Ethidum bromide	-	BDH lab, England
6 X gel loading buffer	-	Bangalore Genei Pvt. Ltd.
Glacial acetic acid	-	BDH-E, Merck (India) Ltd.
Hydroxy quinolone	-	Qualigens Chemicals Mumbai
Isoamyl alcohol	-	Merck
Methanol	-	SRL, Bombay
Potassium chloride	-	SRL, Bombay
Sodium acetate	-	SRL, Bombay
Sodium chloride	-	SRL, Bombay
Sodium dodecyl sulphate (SDS)	-	SRL, Bombay
TEMED	-	SRL, Bombay
Tris base	-	SRL, Bombay
Urea	-	SRL, Bombay

B. PRIMERS

   InVitrogen (India) Pvt. Ltd.

C. MOLECULAR MARKERS

pBR322 DNA/*Hae* III digest	-	Bangalore Genei Pvt. Ltd.
M13 sequencing ladder	-	Amersham Pharmacia Biotech, USA

D. ENZYMES

Taq DNA polymerase	-	Bangalore Genei Pvt. Ltd.
Proteinase-K	-	Bangalore Genei Pvt. Ltd.
PNK	-	Bangalore Genei Pvt. Ltd.

E. KITS

DNA-End-labelling kit	-	Bangalore Genei Pvt. Ltd.
Sequenase version 2.0 DNA sequencing kit	-	Amersham Pharmacia Biotech, USA

F. ISOTOPES

γ ^{32}P-ATP	-	BRIT, Bombay
α ^{35}S-dATP	-	BRIT (Jonaki), Hyderabad

ANNEXURE – III

ABBREVIATIONS

RFLP	Restriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
VNTR	Variable Number of Tandem Repeat
RAPD	Random Amplified Polymorphic DNA
DNA	Deoxy Nucleic Acid
PAGE	Polyacrylamide Gel Electrophoresis
STR	Short Tandem Repeat
PIC	Polymorphic Information Content
QTL	Quantitative Trait Loci
EDTA	Ethylene Diamine Teraacetic Acid
DTT	Dithiothretiol
TEMED	N,N,N,N Tetramethylethylenediamine
APS	Ammonium Persulphate
PNK	Polynucleotide Kinase
PE	Probability of Exclusion
CPE	Combined Probability of Exclusion
He	Heterozygosity
cM	Centimorgan
μl	microlitres
μg	microgram
mg	milligram
mM	millimolar
cm	centimetre
nm	nanometre
mCi	millicurie
Kb	kilo basepair
Rpm	revolutions per minute
SDS	Sodium Dodecyl Sulphate

dNTP	deoxy Nucleotide Triphosphate
ddATP	dideoxy Adenosine Triphosphate
ddCTP	dideoxy CytosineTriphosphate
ddGTP	dideoxy Guanosine Triphosphate
ddTTP	dideoxy Thymidine Triphosphate

ABSTRACT

A study was undertaken to evaluate the efficiency of microsatellite markers for paternity testing in cattle of Kerala. Genomic DNA was isolated from whole blood, fresh and frozen semen samples using phenol: chloroform method. DNA samples from 100 genetically unrelated animals were used to determine the polymorphisity of the markers and samples of known pedigree was used to test the inheritance of markers.

The mean yield of DNA obtained from 5 ml of whole blood was 388.2 ± 14.3 µg, from fresh semen was 181.15 ± 6.2 µg/400 million sperms and from frozen semen was 116.95 ± 25.2 µg/150 million sperm cells. The optical density ratios (260/280) ranged from 1.64 to 1.81, 1.42 to 1.73, 1.54 to 1.76 and for DNA obtained from blood, fresh and frozen semen respectively.

Three microsatellite markers viz., DRB3, ETH131 and FSHβ out of a panel of tested markers were chosen for the study based on their polymorphicity and ease of typing. The forward primer of each primer pair was end-labelled with γ ^{32}P-ATP. PCR parameters varied between the primers with respect to annealing temperature (60°C for DRB3 and FSHβ; 55°C for ETH131) and $MgCl_2$ concentration (1.25 mM for DRB3 and FSHβ; 1.5 mM for ETH131). The amplified products fractionated by denaturing polyacrylamide gel electropheresis were visualized by autoradiography. The number of alleles was counted and allele sizes assigned by comparison with sequences of M13 DNA run along with PCR products. The frequency of each allele was worked out.

Seventeen alleles with sizes ranging from 138-192 bp were identified for DRB3, 11 alleles of size ranges 134-168 bp for ETH131 and nine alleles of size ranges of 184-214 bp were observed for FSHβ. The heterozygosity values obtained for each locus were 0.8938, 0.8385 and 0.8519 for DRB3, ETH131 and

FSHβ respectively. DRB3 was highly informative with PIC value of 0.8864 followed by FSHβ (0.8392) and ETH131 (0.8151).

The probability of exclusion of incorrect sire was calculated independently for the three markers and the values were 0.7913, 0.6787 and 0.7035 for DRB3, ETH131 and FSHβ respectively. The combined probability of exclusion obtained with DRB3 and ETH131 was 0.9329 and DRB3 and FSHβ was 0.9381 and that with ETH131 and FSHβ was 0.9047. The three markers together yielded a cumulative exclusion probability of 0.9801. Thus the exclusion probability was found to increase with the number of markers.

The inheritance pattern of these markers was tested on known sire families. All the three markers agreed with each other in identifying the correct sire and excluding the incorrect one. Though the efficacy of the three markers for paternity testing was found satisfactory, it was concluded that one or two similarly polymorphic markers have to be used along with the markers studied to obtain maximum probability of exclusion of 0.99.