GENETIC DIVERGENCE IN RABBITS USED FOR BREEDING IN KERALA

NISHA VALSAN

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

2007

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

DECLARATION

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

Mannuthy
26.06.07

Nisha
NISHA VALSAN

CERTIFICATE

Certified that this thesis, entitled **"GENETIC DIVERGENCE IN RABBITS USED FOR BREEDING IN KERALA"** is a record of research work done independently by **Dr. Nisha Valsan**, under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Mannuthy
26.06.07

Dr. K. A. Bindu
(Chairperson, Advisory Committee)
Assistant Professor (Sr. Scale)
Department of Animal Breeding and Genetics,
College of Veterinary and Animal Sciences,
Mannuthy

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Dr. Nisha Valsan**, a candidate for the degree of Master of Veterinary Science in Animal Breeding and Genetics, agree that the thesis entitled **"Genetic divergence in rabbits used for breeding in Kerala"** may be submitted by Dr. Nisha Valsan, in partial fulfilment of the requirement for the degree.

Dr. K.A.Bindu
(Chairperson, Advisory Committee)
Assistant Professor (Sr. Scale)
Department of Animal Breeding and Genetics
College of Veterinary and Animal Sciences
Mannuthy.

Dr. K.V.Raghunandanan,
Director
Centre for Advanced Studies in
Animal Genetics and Breeding
College of Veterinary and Animal Sciences
Mannuthy.
(Member)

Dr.T .V.Aravindakshan,
Assistant Professor (Sr. Scale)
Department of Animal Breeding
and Genetics,
College of Veterinary and Animal
Sciences
Mannuthy.
(Member)

Dr. Sisilamma George,
Associate Professor and Head
Department of Veterinary Biochemistry,
College of Veterinary and Animal
Sciences,
Mannuthy.
(Member)

26/09/07
External Examiner

ACKNOWLEDGEMENT

With immense pleasure I depict my sincere and heartfelt gratitude to the Chairperson of the Advisory committee, ***Dr. K. A. Bindu,*** *for her creative suggestions, sustained encouragements, meticulous guidance and timely help in each step of my research programme.*

I am indebted to ***Dr. K. V. Raghunandanan,*** *Director, Centre for Advanced Studies in Animal Genetics and Breeding for the inspiration and unreserved regard rendered to me throughout the study.*

I am grateful to ***Dr. T. V. Aravindakshan,*** *Assistant Professor, Department of Animal Breeding and Genetics, for his expert advice, valuable guidance and timely help in laboratory techniques.*

I humbly place on record my respect and gratitude to ***Dr. Sisilamma George,*** *Associate Professor and Head, Department of Veterinary Biochemistry, for her helpful suggestions, critical evaluation and cordial help as a member of the Advisory committee.*

I owe my sincere gratitude to ***Dr. K. C. Raghavan.*** *Associate Professor, Department of Animal Breeding and Genetics for his suggestions and inspiring professional guidance.*

With exquisite pleasure and gratitude, I acknowledge ***Dr. A. P. Usha,*** *Associate Professor, Department of Animal Breeding and Genetics for her affectionate encouragement and cordial help.*

I am extremely thankful to ***Dr. Stephen Mathew,*** *Associate Professor, Department of Animal Breeding and Genetics for his immense generosity and help.*

I thank ***Dr. A. K. Surridge,*** *School of Biological Sciences, University of East Anglia, Norwich, UK, for providing me valuable suggestions through e-mails.*

The co-operation and help extended by the staff members of the Department of Animal Breeding and Genetics are thankfully acknowledged.

I thank the Dean-in-charge, ***Dr. E. Nanu,*** *for providing necessary facilities in carrying out this research work.*

I am in short of words to express my deep sense of gratitude to my colleagues and best friends, ***Dr. Jimcy Joseph, Dr. Reshmi R Chandran and Dr. Rajeev.M,*** *for their tireless help, unconditional support and constant encouragement.*

My sincere thanks to ***Mr. Binoy A.M*** *and* ***Dr. Biju S,*** *Senior Research Fellows, Department of Animal Breeding and Genetics, for the help rendered by them during my research work.*

The care and concern shown by my senior colleagues ***Dr. Seena T. X*** *and* ***Dr. Ariprasath*** *are gratefully acknowledged. A special bouquet of thanks to my*

loving and cheerful juniors ***Dr. Rojan, Dr. Abraham*** *and* ***Dr. Bipin*** *for their company and help.*

With great fondness, I acknowledge the sincere helping hand extended to me by my friends ***Dr. Chitra R Nair, Dr. Pramod S, Dr. Ranjini A. R, Dr. Safna Issac, Dr. Priya A.R*** *and all others who have directly or indirectly helped me in completing this research programme.*

I cannot confine my feelings towards my husband ***Mr. Paul P.R*** *to a mere gratitude. Without his prayers, constant encouragement, understanding and support, I would not have been able to complete this study successfully.*

No phrase or words can express my deep sense of gratitude to my loving ***daddy, mummy, Niji*** *and* ***in-laws*** *for their love, affection and moral support.*

Above all, I bow my head before ***God Almighty,*** *for the blessings showered on me..... for helping me to reach the shore safely.....*

Nisha Valsan

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

1. INTRODUCTION

In recent years, there has been a rise in global awareness on the virtues of rabbit meat, especially in developing countries, depicting it as an alternative means of alleviating world food shortages.

India, which faces enormous shortages in meat supply, has the greatest chance to tap the potentials of rabbit production. Due to the fact that India doesn't have a rabbit breed of its own, the Central Sheep and Wool Research Institute (CSWRI) of the ICAR imported rabbits for meat from the United Kingdom in 1978 and for wool in 1979 from the erstwhile USSR to study their adaptability and performance in different agroclimatic conditions of India.

Of the imported meat-type rabbit breeds in Kerala, Newzealand White, Soviet Chinchilla and Grey Giant are the most common. Although some decline in fertility has been observed, these exotic breeds have performed relatively well under our diversified environmental conditions, under proper feeding, housing, management and health measures.

The population of valuable pure bred rabbits in the State is facing a rapid decline due to the adoption of indiscriminate crossbreeding programme. To prevent this genetic erosion, a possible way is to characterize rabbit breeds precisely with a view to formulate a proper breeding programme. It is in this context that the differences among the breeds and the genetic distances existing between them need be investigated.

Though there is no dearth of reports on the productive and reproductive performance of various breeds of rabbits, the molecular characterization works thereon are scarce. The genetic characterization of a species in turn allows the assessment of its genetic variability, which is an important element in determining breeding strategies and genetic conservation programmes.

Some of the recent developments in molecular biology help to identify and utilize the genetic variability for genetic improvement of different species. Molecular techniques allow the detection of variability or polymorphism of specific regions of DNA, among individuals in a population and also between populations. Among the various molecular markers identified, the microsatellite markers have found widespread applications in the analysis of population structure and genetic diversity among populations.

Microsatellites are short, simple, tandemly repeated nucleotide sequences abundantly present in the genome. Polymerase Chain Reaction (PCR) based microsatellite markers have been very useful because of their abundance and ubiquitous distribution in the genome, specificity of primers, high degree of polymorphisms that yield several alleles and complemented by easy detection and automation of the technique.

In order to set up an appropriate breeding programme for rabbits in the state with a view to evolve a suitable meat breed, genetic characterization of rabbit breeds should precede. The present study was undertaken with the following objectives:

1. Characterization of different breeds of rabbits using microsatellite markers,

2. Estimation of allele and genotype frequencies at the polymorphic loci and

3. Estimation of the genetic relationship among different breeds of rabbits.

Review of Literature

2. REVIEW OF LITERATURE

2.1 GENETIC DIVERSITY ANALYSIS

Genetic diversity is the 'raw material' that enables species to adjust to a changing world. The level of similarity (homogeneity) or difference (heterogeneity) in the genetic makeup (genome) of populations of the same species indicates the extent to which genetic material can be exchanged between populations, keeping a species-specific gene pool intact.

Understanding the mechanisms by which genes are exchanged within a species can provide insight into the role of diverse populations in maintaining a species' genetic diversity, or leading to the isolation and creation of distinctive new genomes and, potentially, species. Studies on genetic diversity within a species also has the potential to reveal that a single species, perhaps classified as such for morphological reasons, may in fact be two or more species.

Notter (1999) stressed the importance of maintenance of genetic variation within livestock species. He supported the global programmes to determine genetic distances among livestock breeds and encouraged public co-operation in maintenance of animal genetic diversity.

A genetic diversity measure to permit ranking of breeds and thereby providing useful information concerning the relative contribution of 18 local cattle breeds from Spain, Portugal and France to genetic diversity was defined by Canon *et al.* (2001)

2.2 MOLECULAR MARKERS

Recent developments in molecular biology have opened up new vistas in identifying and using genomic variation for genetic improvement of livestock.

The molecular techniques detect variation or polymorphisms among individuals in specific regions of DNA. Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Amplification Fragment Length Polymorphism (AFLP), Single Nucleotide Polymorphism (SNP), Minisatellites, Microsatellites *etc* are some of the DNA genetic markers (Montaldo and Meza-Herrera, 1998).

Studies based on genetic markers suggest that cattle breeds are significantly differentiated at the genetic level. Therefore genetic markers could provide a potentially powerful way of identifying the breed to which an individual animal belongs, when pedigree information is missing (Blott *et al.*, 1999).

Molecular markers are highly polymorphic, ubiquitously distributed throughout the genome, follow Mendelian inheritance and are unaffected by environmental factors (Arora *et al.*, 2003)

Hai-Guo *et al.* (2005) suggested that the discriminating power of blood group typing and biochemical polymorphisms is less than that of DNA markers.

2.3 MICROSATELLITE MARKERS

Litt and Luty (1989) found that $(TG)_n$ repeats found abundantly in the human genome are polymorphic and since they are present in regions where minisatellites are rare, these microsatellite loci are very useful for linkage studies in humans.

Microsatellites are basically variable non-coding regions of nuclear DNA, consisting of short tandemly repeated sequences of typically 1-6 bp in length. Given their multi-allelic nature and therefore high Polymorphic Information

Content (PIC), microsatellite DNA profiling has become standard methodology for genetic analysis of natural population (Tautz, 1989).

Weber and May (1989) found out that the interspersed DNA elements of the form $(dC\text{-}dA)_n$. $(dG\text{-}dT)_n$ were polymorphic in length and they demonstrated the variations in lengths by amplifying the DNA within and immediately flanking the repeat blocks using PCR.

Ciampolini *et al.* (1995) detected 17 new alleles of larger sizes in eight of the microsatellites used in Italian cattle breeds compared with previous information collected. Specific breed alleles with high frequencies, particularly in Piemontese breed were also obtained.

A method for the estimation of relative mutation rates at microsatellite loci, grouped by the repeat motif, using generalized stepwise mutation model was put forward by Chakraborty *et al.* (1997). They suggested that the dinucleotides have mutation rates 1.5-2 times higher than the tetranucleotides.

Blott *et al.* (1999) recommended that the most powerful microsatellite markers for breed discrimination are those with high average heterozygosities and with greater mean numbers of observed alleles.

Of the 20 microsatellite markers used by Dorji *et al.* (2003) for the genetic diversity study of indigenous cattle population in Bhutan, 17 loci deviated from Hardy-Weinberg equilibrium. They suggested that the reason for this disequilibrium is population subdivision following sampling from a range of distinct location within the same broad geographic area.

Hai-Guo *et al.* (2005) used ten microsatellites to assess the genetic diversity of five native cattle breeds of China and found that the variability of

microsatellite DNA loci is much larger than that of traditional genetic markers based on unique sequence DNA mutation.

2.3.1. Applications of Microsatellite Markers

The evolutionary relationships and molecular biogeography of 20 different cattle population were determined by surveying microsatellite loci by MacHugh *et al.* (1997). These markers proved to be very useful for the investigation of gene flow and admixture in African population.

Surridge *et al.* (1999) used polymorphic microsatellite loci to study the genetic structure of a population of European wild rabbits and found that the formation of stable breeding groups with low levels of gene flow between them influenced the genetic structure of population.

Microsatellite markers have potential use in genetic characterization of animals. Using the allele frequency data obtained from microsatellite analysis, Chenyambuga *et al.* (2004) classified the goat population of Sub-Saharan Africa into distinct genetic groups or breeds.

Cho and Cho (2004) investigated the validity of 16 microsatellite markers for routine Korean Native Horse parentage testing and polymorphisms in the population of Korean Native Horse. DNA based methods offer several potential advantages compared with conventional parentage testing system because of their accuracy and specificity. They could give basic information for developing accurate parentage verification and individual identification system in Korean Native Horse.

The genetic relationship of 10 Chinese indigenous Pig breeds was evaluated by Li *et al.* (2004) using microsatellite markers.

Wiener *et al.* (2004) described and defined breeds in terms of their cohesiveness based on the evaluation using microsatellite markers on samples chosen at random from the United Kingdom breeding pool of eight cattle breeds.

Chantry-Darmon *et al.* (2005) constructed an integrated cytogenetic and genetic map based on microsatellites homogenously anchored to the rabbit genome. The study revealed that rabbit genome manifested a higher proportion of $(TC)_n$ repeats and a nonhomogenous distribution of $(TG)_n$ and $(TC)_n$ repeats compared to those in human. They reported a reservoir of 305 new rabbit microsatellites.

In Bhutan, the formulation of conservation strategies and programmes for the indigenous cattle population was based on the analysis of microsatellite markers among them (Dorji *et al.*, 2003).

2.3.2. Conservation of Microsatellite Across Species

Surridge *et al.* (1997) reported that six polymorphic microsatellite markers developed for European wild rabbit (*Oryctolagus cuniculus*) amplified with 20 other species of lagomorphs. The amplification indicated that the flanking regions of the microsatellite loci have been strongly conserved over the last 50 million years.

Andersson *et al.* (1999) used five microsatellite primer pairs, developed for the European wild rabbit (*Oryctolagus cuniculus*) to amplify microsatellites in two species of hare in Sweden and could detect a significant level of differentiation between the two species. They found that the conservation of microsatellite loci across taxa usually enables PCR amplification of the microsatellites in closely related species with the same primers.

Flagstad *et al.* (1999) utilized heterologous primers for the Bovidae family to assess the effect of habitat fragmentation of hartebeest (*Alcelaphus buselaphus swaynei*) using microsatellite analysis. They used 15 ovine and 28 bovine primer sets, as there were no reports in the literature on microsatellite primer sets designed for Alcelaphine species. 26 of the 43 microsatellite markers tested amplified successfully and about 75 % of the amplifiable markers were polymorphic.

Pang *et al.* (1999) tested 48 primer pairs for chicken (*Gallus gallus*) microsatellite loci in PCR amplification of Japanese Quail (*Coturnix japonica*) genomic DNA. Amplification products were obtained from 28 primer pairs (58.3%) after optimizing the PCR conditions.

Many of the bovine microsatellite markers had been adapted for analysis in sheep and goat. The genetic relationships in goat and buffalo breeds were widely estimated using bovine microsatellite markers (Saitbekova *et al.*, 1999; Arora *et al.*, 2003; Chenyambuga *et al.*, 2004; Martinez *et al.*, 2004; Tantia *et al.*, 2004; Sukla *et al.*, 2006 and Triwitayakorn *et al.*, 2006).

2.3.3. Microsatellites and Genetic Divergence

Ciampolini *et al.* (1995) investigated the genetic variability within and between cattle breeds using 17 polymorphic microsatellites. They observed 181 alleles and reported that the average number of alleles per microsatellite was 10.59.

The genetic relationship among eight Swiss goat breeds was established by estimating genetic distances from the analysis of 20 microsatellite markers by Saitbekova *et al.* (1999). Their findings were in perfect agreement with the history of Swiss goat breeding.

Microsatellite markers have found widespread application in the analysis of population structure and genetic diversity among various species like cattle, goat, buffalo, horse, pig, dog and goose. (Canon *et al.*, 2001; Maudet *et al.*, 2002; Arora *et al.*, 2003; Dorji *et al.*, 2003; Chenyambuga *et al.*, 2004; Cho and Cho, 2004; Li *et al.*, 2004; Martinez *et al.*, 2004; Cho, 2005; Arora and Bhatia, 2006; Cervini *et al.*, 2006; Sukla *et al.*, 2006 and Tu *et al.*, 2006).

2.3.4. Sol 03

The rabbit microsatellite marker, Sol 03, was first reported by Rico *et al.* (1994) by screening a size-selected library for the rabbit (*Oryctolagus cuniculus*) containing 200 to 600 bp inserts. The interrupted repeat was found to be $(TC)_{14}(T)_4(TC)_6$ and had a size-range of 237 to 252 bp. The marker proved to be polymorphic with five alleles with a heterozygosity and PIC of 0.800 and 0.700, respectively.

Surridge *et al.* (1997) used this marker for the intergeneric amplification study of microsatellite loci in the Laporidae family and detected 12 alleles with a size range of 225 to 249 bp in *Oryctolagus cuniculus*.

This marker had been used by several workers for genetic variability studies in rabbit and had obtained varying number of alleles. (Surridge *et al.*, 1999a, Surridge *et al.*, 1999b and Burton *et al.*, 2002).

2.3.5 Sol 33

Surridge *et al.* (1997) reported this microsatellite marker from a size-selected rabbit genomic library. The repeat array was found to be $(TG)_3GG(TG)_{18}$ and on PCR amplification revealed 15 alleles of size range 189 to 219 bp.

This marker had been used by Surridge *et al.* (1999) in two different studies and obtained 16 and nine alleles with a heterozygosity of 0.581 and 0.605.

The polymorphism of Sol 33 was also reported by Burton *et al.* (2002).

2.3.6 Sol 44

Sol 44 was detected to be a polymorphic marker composed of 17 GT repeats in rabbit. On PCR amplification, this marker was found to be polymorphic with nine alleles with a size range of 178 to 208 bp (Surridge *et al.*, 1997). The polymorphism of Sol 44 locus was also reported by Surridge *et al.* (1999a and 1999b) with 15 and four alleles, respectively.

2.4 ISOLATION OF GENOMIC DNA

Any genetic study requires pure, high molecular weight DNA as the first step. Phenol-chloroform extraction technique is the most extensively used method for the isolation of genomic DNA.

Blin and Stafford (1976) introduced a new method for the isolation of high molecular weight DNA from eukaryotes. According to them, DNA could be prepared from a variety of tissues such as calf thymus or human placenta.

A simple salting out procedure for extracting DNA from human nucleated cells was put forward by Miller *et al.* (1988). They introduced a rapid, safe and inexpensive method involving salting out of the cellular proteins by dehydration and precipitation with a saturated sodium chloride solution.

Grimberg *et al.* (1989) described a simple and efficient non-organic procedure for isolation of genomic DNA from blood which eliminated the use of hazardous organic reagents.

Jackson *et al.* (1990) compared several DNA extraction techniques quantitatively and qualitatively using both fresh and paraffin wax embedded tissue and reported that incubation with proteinase K was the most efficient extraction procedure.

Surridge *et al.* (1999) performed DNA extraction using the standard Phenol-Chloroform method for the amplification of genomic DNA by PCR. The isolated DNA was then used for the genetic diversity studies using microsatellite markers among European wild rabbits in East Anglia.

2.5 POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) is a rapid technique for the enzymatic *in vitro* amplification of DNA. The technique is very sensitive and can tolerate small quantity of poor quality template DNA. (Jackson *et al.*, 1990)

Holland *et al.* (1991) described that the 5'-3' exonuclease activity of the thermostable enzyme *Thermus aquaticus* (Taq) DNA polymerase might be employed in a PCR product detection system to generate a specific detectable signal concomitantly with amplification.

A 'touch down' PCR programme which decreased the specificity of primers by lowering the annealing temperature 0.5°C for each cycle was used by Andersson *et al.* (1999) when the amplification proved unspecific at high annealing temperatures.

The PCR conditions should be optimized for each primer set before testing the microsatellite markers for polymorphisms using DNA samples (Korstanje *et al.*, 2001; Arora *et al.*, 2003 and Vijh *et al.*, 2004).

According to Burton *et al.* (2002), the amplified products could be visualized under ultraviolet light after electrophoresing on an ethidium bromide stained two percent agarose gel.

The PCR products could be resolved on polyacrylamide sequencing gels. (Andersson *et al.*, 1999 and Canon *et al.*, 2001). The separated PCR products could be visualized by silver staining (Bassam *et al.*, 1991; Canon *et al.*, 2001; Arora *et al.*, 2003 and Tu *et al.*, 2006), autoradiography or by means of an automated sequencer (Canon *et al.*, 2001; Maudet *et al.*, 2002 and Martinez *et al.*, 2004).

2.6 AUTORADIOGRAPHY

The PCR products can be visualized by incorporating radiolabelled nucleotide or by endlabelling one of the primers used.

Biggin *et al.* (1983) proposed that the use of deoxyadenosine 5′-[α- (^{35}S) thio] triphosphate as the label incorporated in dideoxynucleotide sequence reaction increases the sharpness of the bands on an autoradiograph and so increases the resolution achieved.

Hite *et al.* (1996) labeled the 5′- end of the forward primer of each primer pair with γ-^{33}P or γ-^{32}P using T_4 polynucleotide kinase to study the factors affecting the fidelity of DNA synthesis during PCR and they visualized the results by autoradiography.

Burton *et al.* (2002) visualized the PCR products by exposing the dried gels to autoradiographic film for 24-48 hours after endlabeling the forward primer using 9.25 kBq [γ^{32}P] ATP to study the genetic structure of cyclic snowshoe hares in Canada.

Sukla *et al.* (2006) detected various microsatellite bands for identification of different alleles present in six buffalo population by autoradiography after adding α-^{32}P dCTP to the PCR mix.

2.7 SEQUENCING

Maxam and Gilbert (1977) sequenced DNA by a chemical procedure that breaks the terminally labelled DNA molecule partially at each repetition of a base. They found that the technique permitted sequencing of at least 100 bases from the point of labelling.

Sanger *et al.* (1977) described a new method for determining nucleotides in DNA. This method made use of inhibitors that terminate the newly synthesized chains at specific residues. The inhibitory activity of 2'3'-dideoxythymidine triphosphate (ddTTP) on DNA polymerase 1 depended on its being incorporated into the growing oligonucleotide chain in the place of thymidylic acid (dT).

Flagstad *et al.* (1999) resolved the PCR products by electrophoresis on a six percent denaturing gel and determined the allele sequences by comparison with M13 mp 18 DNA sequencing ladder.

Sukla *et al.* (2006) sequenced M13 DNA by chain-termination method and the sequencing ladder (G, A, T and C) was run on the gel to size mark the PCR products.

2.8 STATISTICAL ANALYSIS

2.8.1. Heterozygosity

Nei (1978) evaluated the magnitudes of the systematic biases involved in sample heterozygosity and obtained the unbiased estimate of average heterozygosity for a single locus which may be written as,

$$H=2n\,(1-\Sigma x_i^2)\,/2n-1$$

where 'n' is the number of individuals and 'x_i' is the allele frequency.

In a sample of individuals, heterozygosity could be estimated as the binomial proportion of heterozygotes in the sample. (Ott, 1992).

Heterozygosity is given by,

$$H_e = 1-\sum_{i=1}^{k} P_i^2$$

where p_i is the frequency of i^{th} allele at a locus.

2.8.2. Polymorphic Information Content (PIC)

The polymorphism at a given locus influences the probability of detection of linkage of that locus to another. This could be found out by a measure called polymorphic information content (Botstein *et al.*, 1980). It is given by,

$$PIC = 1-\left[\sum_{i=1}^{k} P_i^2\right] - \sum_{i=1}^{k-1}\sum_{j=i+1}^{k} 2\,P_i^2P_j^2$$

where P_i and P_j stands for frequency of band i and band j respectively in one population and 'k' is the number of alleles from a certain locus.

2.8.3. Genetic Distance

Genetic distance calculated using allele frequency is the basis of genetic diversity research. It should be an index for group structure and breed diversity when breed conservation decisions are made.

The number of individuals to be used for estimating the genetic distance can be very small if the genetic distance is large and average heterozygosity of the two species compared is low. (Nei, 1978)

Takezaki and Nei (1996) studied the efficiencies of genetic distance measures in phylogenetic reconstruction by using computer simulation. They found that Nei's genetic distance (D_A) and Cavalli-Sforza and Edwards' chord distance (D_C) are the most efficient.

Andersson *et al.* (1999) used the obtained allelic variation from microsatellite markers to construct a genetic distance tree based on the amount of shared alleles between all pairs of individuals. This method offered sufficient differences to arrange all individuals in two groups.

Vijh *et al.* (2004) calculated several genetic distances based on the assumption of infinite allele model. The genetic distance between populations provided a relative estimate of the time that had elapsed since the breeds existed as one entity.

2.8.4. Dendrograms or Phylogenetic Trees

When a dendrogram for a group of species is constructed from genetic distance estimates, the reliability of the topology of dendrogram depends on the difference in genetic distance among different pairs of species. If these differences are small the genetic distance must be estimated accurately ie, a considerable concentration of individuals should be examined for each locus (Nei, 1978).

Vijh *et al.* (2004) prepared dendrogram using distance matrix and using unweighted pair group method with arithmetic mean and neighbour joining (NJ) algorithm. The results of dendrogram were similar to those obtained using Nei's genetic distance.

Materials and Methods

3. MATERIALS AND METHODS

A total of 75 genetically unrelated animals, belonging to three breeds, viz. Newzealand White, Soviet Chinchilla and Grey Giant formed the material for this study. The animals were sampled from the Rabbit farm attached to the Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

3.1 COLLECTION OF BLOOD SAMPLES

Blood samples (5 ml each) were collected from the ear vein aseptically using sterile disposable syringes and immediately transferred into sterile disposable centrifuge tubes containing Ethylene Diamine Tetraacetic Acid (EDTA-1 mg/ml of blood) as anticoagulant. The samples were transported on ice to the laboratory and stored at 4°C till processed.

3.2 ISOLATION OF DNA FROM WHOLE BLOOD

DNA was extracted from whole blood using the standard Phenol-Chloroform extraction procedure (Sambrook *et al.*, 1989) with modifications. The procedure was as follows;

1. To 5 ml of blood collected in a 15 ml centrifuge tube, two volumes of ice-cold RBC lysis buffer (150m*M* NH_4Cl, 10m*M* KCl, 0.1m*M* EDTA) was added, mixed well and kept on ice with occasional mixing for ten minutes for the complete lysis of erythrocytes.

2. The leukocytes were recovered by centrifuging at 3500 rpm for 15 minutes and the supernatent containing lysed erythrocytes was discarded.

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

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

5. The washed white cell pellet was resuspended completely by vortexing in 5 ml of saline EDTA buffer (SE buffer- 75 m*M* NaCl, 35 m*M* EDTA). To this mixture, 25 µl of proteinase-K (20 mg/ml) and 0.25 ml of 20 % SDS were added, mixed well and incubated at 50°C for a minimum of 3 hours.

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

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

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

9. The supernatent was transferred to a sterile 50 ml beaker and 1/10th volume of 3*M* sodium acetate (pH 5.5) was added and mixed.

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

11. Dried DNA was resuspended in 0.5 ml of Tris EDTA buffer (TE buffer- 10m*M* Tris, 0.1m*M* EDTA) and stored at -20°C.

3.3 PCR ANALYSIS

3.3.1 Template DNA

Working solutions of DNA samples were prepared from the DNA stock solution by diluting with sterile double distilled water to get a final concentration of 50 ng/µl. One microlitre of this working solution was used in every 10 µl PCR assay.

3.3.2 Selection of primers

A set of 12 microsatellite markers (INRA 005, INRA 063, TGLA 53, TGLA 63, ILSTS 030, HUJ 1177, BM 6121, EMX 1, LA 4, Sol 03, Sol 33 and Sol 44) was selected from available literature and the primers were custom synthesized. Three markers, viz. Sol 03, Sol 33 and Sol 44 which exhibited comparatively higher degree of polymorphism, among those typed, were chosen for the study. The sequences of the forward and reverse primers for each locus were as follows.

Locus	Primer sequence (5′ -3′)
Sol 03	F 5′- TACCGAGCACCAGATATTAGTTAC-3′
	R 5′- GTTGCCTGTGTTTTGGAGTTCTTA-3′

Sol 33	F	5'- GAAGGCTCTGAGATCTAGAT-3'
	R	5'- GGGCCAATAGGTACTGATCCATGT-3'
Sol 44	F	5'- GGCCCTAGTCTGACTCTGATTG-3'
	R	5'- GGTGGGGCGGCGGGTCTGAAAC-3'

The primers obtained in lyophilized form were reconstituted in sterile double distilled water to make a stock solution of 200 p*M*/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 stock.

3.3.3 Incorporation of Radioactivity: End-Labeling of Primers

For visualizing the PCR products by autoradiography, the forward primer of each marker was radio-labeled at the 5' end with γ^{32} P-ATP. The reaction was carried out with the DNA end-labeling kit (Bangalore Genei Pvt. Ltd).

The procedure for end-labeling was as follows:

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

10X Polynucleotide kinase (PNK) buffer	-	1 µl
Forward primer (200 p*M*/µl)	-	1 µl
T_4 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 30 minutes. Five picomoles of the diluted end-labeled primer (10p*M*/µl) was used for every 10 µl PCR assay.

3.3.4 PCR Conditions

The PCR conditions for each microsatellite loci were standardized separately. Each reaction was carried out in 10 µl volume. PCR reaction was set up with 1 µl of 10X PCR buffer (15 m*M* $MgCl_2$, 100 m*M* Tris-pH 8.3, 500m*M* KCl), 200µ*M* dNTP, 5p*M* of end-labeled forward primer, 5p*M* of reverse primer and 0.3 U of Taq DNA polymerase. The concentrations of $MgCl_2$ used were 1.5 m*M* for Sol 03 and 1.25m*M* for Sol 33 and Sol 44. The reaction mixture was mixed well and subjected to amplification in a thermal cycler (Techne Flexigene). The thermal cycling involved an initial denaturation of 94 °C for 3 minutes followed by 35 cycles each consisting of denaturation at 94 °C for one minute, annealing at 60.9 °C, 60.5 °C and 67.7 °C for one minute for Sol 03, Sol 33 and Sol 44, respectively and extension at 72 °C for one minute. This was followed by a final extension at 72 °C for five minutes. The samples were then cooled down to 4 °C and stored at -20 °C till further analysis.

3.4 SEQUENCING M13 BACTERIOPHAGE DNA

Determination of the exact size 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 (Amersham Biosciences Corporation, USA) according to the manufacture's instructions as follows.

1 Preparation of annealing mixture.

The composition of the mixture is as follows:

M13 phage DNA (0.2 µg/µl)	-	5µl
5 X Sequenase reaction buffer	-	2µl
Primer	-	1µl

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

2. To the four tubes labeled G, A, T and C, 2.5 µl of each dideoxy termination mixtures (ddGTP, ddATP, ddTTP and ddCTP) were added.

3. Dilution of labeling mix.

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

5X Labeling mix	-	0.5µl
Water	-	2.0µl

4. Dilution of enzyme.

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

Sequenase enzyme (13U/µl)	-	0.5µl
Sequenase dilution buffer	-	3.5µl

1. Labeling reaction.

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

Dithiothreitol (DTT 0.1*M*)	-	1µl
Diluted labeling mix	-	2µl
Diluted enzyme	-	2µl
α ^{35}S dATP (10µ Ci/µl)	-	2µl

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

2. To each termination tubes (G, A, T and C), 3.5µl of labeling reaction mixture was transferred, mixed well and incubated 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.

3.5 ELECTROPHORESIS

3.5.1 Checking of Amplification of Target DNA

The PCR products were checked in 1.5% agarose gels in 1X Tris Acetate EDTA (TAE) (0.04 m*M* Tris acetate and 0.001 m*M* EDTA) buffer in a horizontal submarine electrophoresis unit. Appropriately sized gel tray was cleaned and sealed. The tray was placed on a leveled surface and comb was kept in proper position in the tray. 1.5 % agarose in TAE buffer was heated in a microwave oven until it was a clear solution, cooled to 60°C and 0.5µg/ml of Ethidium bromide was added and mixed well. The mixture was cooled to 60°C and carefully poured into the gel tray avoiding air bubbles. After polymerization, the comb and sealing were removed gently and the tray was immersed in the buffer tank (Amersham Pharmacia Biotech, USA) containing 1X TAE buffer. One microlitre of the PCR product was mixed with equal volume of 6X gel loading buffer (Bromophenol blue 0.25%, Xylene cyanol 0.25%, Sucrose 40%) and loaded into the well carefully. *HaeIII* digested pBR322 was used as the DNA size marker. 1.5 µl of the marker was mixed with equal volume of gel loading buffer and was loaded into one of the wells.

Electrophoresis was carried out at 2V/cm until the Bromophenol blue dye migrated more than two-third of the length of gel. The gel was visualized under a UV transilluminator (Hoefer, USA) and checked amplification of target DNA. Those samples with amplified PCR products were subjected to polyacrylamide gel electrophoresis (PAGE).

3.5.2 Denaturing Polyacrylamide Gel Electrophoresis

The radioactively labeled PCR products were subjected to electrophoresis on six percent denaturing polyacrylamide gels for better resolution. Denaturing PAGE was performed on the Vertical Sequencer (Consort, Belgium) as described by Biggin *et al.* (1983). The gels were set between two glass plates (41 x 33 cm) separated by 0.35 mm thick spacers.

3.5.2.1 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 chloroform and acetone. 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% acrylamide, 6*M* urea, 0.5X TBE) and 125 µl each of 10% 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) was inserted on top with the toothed surface facing upwards. The gel was allowed to set for half an hour before electrophoresis. The tapes and clamps were removed and assembled in the sequencer. The upper and lower electrode tanks were filled with 1X Tris Borate EDTA (pH 8.3) buffer (TBE 0.045 *M* Tris borate, 0.001 *M* EDTA) to the required level. The comb was removed, wells cleaned

with buffer solution and comb was then reinserted in opposite direction with the toothed surface now facing downwards to form sample-loading wells.

3.5.2.2 Loading of Samples

The PCR products were mixed with 4 µl formamide loading buffer (0.02% Xylene Cyanol, 0.02% Bromophenol blue, 10 m*M* EDTA and 98% deionised formamide), denatured at 94°C for five minutes and cooled immediately on ice. About 4 µl each of this mixture was loaded into each well with care to avoid mixing up of the samples from adjacent wells. The prepared M13 DNA sequencing ladder, which was also denatured at 94°C for five minutes, was loaded simultaneously in the middle or side wells.

3.5.2.3 Electrophoresis

The gels were electrophoresed at 30W for three hours. 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 approximately equivalent to a 100 base fragment.

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 cling film and dried in a gel drier at 80°C for one and a half hours.

3.7 AUTORADIOGRAPHY

The cling film was removed after drying and the gel was set for autoradiography with X-ray film (Kodak, 35.6 x 43.2cm) 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 the 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, one percent 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 genotypes of the animals were determined for each microsatellite loci by comparing the sizes of alleles 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 at each locus was determined by direct counting.

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).

Heterozygosity is given by;

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

where p_i is the frequency of i^{th} 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. The PIC values of the markers were calculated as;

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

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

3.11 GENETIC DISTANCE

Genetic distance measures provide the best description of genetic differentiation among breeds or genetic groups. The standard genetic distance between the four populations studied were computed using Nei's method as given by Nei (1978). The distance measures were derived using the POPGENE version 1.31 program (Yeh *et al.*, 1999).

Nei's standard genetic distance is given by;

$$Ds = -\ln [J_{XY} / \sqrt{J_X J_Y}]$$

where J_X, J_Y and J_{XY} are averages of Σx_i^2 , Σy_i^2 and $\Sigma x_i y_i$ over r loci and x_i and y_i are the sample allele frequencies of the i^{th} allele in populations X and Y, respectively.

3.12 DENDROGRAM

A dendrogram representing the relationship between the animals belonging to the three rabbit populations was constructed using the POPGENE version 1.31 program (Yeh *et al.*, 1999). The UPGMA method was used for plotting the dendrogram.

Results

4. RESULTS

4.1 ISOLATION OF GENOMIC DNA

A total of 75 DNA samples were isolated from whole blood collected from three breeds of rabbit, viz. Newzealand White, Soviet Chinchilla and Grey Giant belonging to the rabbit farm attached to the Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

4.2 PCR ANALYSIS

Microsatellite markers, selected from among published literatures, were custom synthesized and used for PCR amplification of the samples. Out of the 12 microsatellite markers tested (INRA 005, INRA 063, TGLA 53, TGLA 63, ILSTS 030, HUJ 1177, BM 6121, EMX 1, LA 4, Sol 03, Sol 33 and Sol 44), six markers (INRA 063, TGLA 53, TGLA 63, ILSTS 030 and BM 6121) failed to amplify. Among the markers which had successful amplification, three (INRA 005, HUJ 1177 and EMX 1) were found to be monomorphic. Three markers (Sol 03, Sol 33 and Sol 44) which showed polymorphism were used for further analysis.

4.2.1 Optimization of PCR

PCR reactions, temperature and time of the cycles were optimized separately for each primer pair for efficient amplification and accurate typing of the products, the details of which are presented in Tables 4.1, 4.2 and 4.3.

The PCR products were checked for amplification by electrophoresis on 1.5 % agarose gels using *Hae III* digested pBR 322 as the size standard. Amplified products of the expected size range were observed for all loci.

4.3 NUMBER, SIZE AND FREQUENCY OF ALLELES

The amplified products were fractionated by denaturing polyacrylamide gel electrophoresis. The pattern of bands at each locus was visualized by autoradiography. Direct counting was employed for detecting the number of alleles at each locus. The size of alleles were approximated by comparing with the sequence of M13 phage DNA run along with the samples. The different alleles and their frequencies for the three markers studied are presented in Tables 4.4 to 4.6. The different genotypes and their frequencies are presented in Tables 4.7 to 4.9. The number of alleles, size range, heterozygosity and PIC values for the different loci tested are summarized in Tables 4.10 to 4.12. The autoradiograph of the three polymorphic loci and the monomorphic markers are depicted in Figures 1 to 6.

4.3.1 Sol 03

A total number of eight, eleven and seven alleles were observed in Newzealand White, Soviet Chinchilla and Grey Giant for Sol 03 locus, respectively. Out of the three polymorphic markers, maximum polymorphism was observed at this locus (Fig.1). The allele size ranged from 221 to 235 bp in Newzealand White, 205 to 247 bp in Soviet Chinchilla and 209 to 247 bp in Grey Giant. Three alleles 205, 219 and 237 bp were specific to Soviet Chinchilla while the allele 217 bp was found exclusively in Grey Giant. The allele size of 235 bp was found in the highest frequency (0.250) in Newzealand White while the allele 223 bp topped in Soviet Chinchilla with a frequency of 0.438. In Grey Giant, the allele 229 bp was found to be the most frequent (0.350). The allele size and frequencies are summarized in Table 4.4.

A total of thirteen, eleven and eight genotypes were recorded in Newzealand White, Soviet Chinchilla and Grey Giant, respectively. The genotype 223/209 occurred in both Soviet Chinchilla and Grey Giant.

Newzealand White and Soviet Chinchilla revealed the presence of three common genotypes (231/223, 233/223 and 235/223). The genotype 235/231 was found with maximum frequency (0.250) in Newzealand White. In Soviet Chinchilla, both genotypes 223/223 and 235/223 were found to be more frequent (0.167). 229/229 genotype was observed in the highest frequency of 0.250 in Grey Giant. The genotypes and their frequencies are presented in Table 4.7.

4.3.2 Sol 33

A total of eight alleles could be observed for Sol 33 locus in Grey Giant while four alleles each were detected in Newzealand White and Soviet Chinchilla. The allele size and frequencies of Sol 33 locus are presented in Table 4.5. The allele size ranged from 206 to 216 bp in Newzealand White, 208 to 216 bp in Soviet Chinchilla and 206 to 224 bp in Grey Giant. The maximum number of alleles (eight) was observed in Grey Giant. Three alleles found exclusively in Grey Giant were 218 bp, 222 and 224 bp. The alleles 208, 212 and 216 bp were common to all the breeds. The allele size of 216 bp occurred in highest frequency both in Newzealand White (0.480) and Soviet Chinchilla (0.409). Two alleles 214 bp and 222 bp topped with a frequency of 0.190 in Grey Giant. The polymorphism at Sol 33 locus is depicted in Fig.2.

A total of 12 genotypes were observed for Sol 33 locus in Grey Giant while six each in Newzealand White and Soviet Chinchilla contributing to 16 different genotypes in the pooled population. The genotypes and their frequencies are presented in Table 4.8. The genotype 216/206 was found exclusively in Newzealand White. Eight genotypes (214/208, 218/214, 218/216, 222/218, 222/222, 224/216, 224/218 and 224/222) were specific to Grey Giant. The genotype 216/208 was present in all the populations. Both in Newzealand White and Soviet Chinchilla, 216/208 genotype was observed with maximum frequency of 0.280 and 0.318, respectively while in Grey Giant, the genotypes 208/206 and 214/212 recorded the highest frequency (0.143).

4.3.3 Sol 44

A maximum number of four alleles at this locus was observed in Grey Giant while three each were recorded in Newzealand White and Soviet Chinchilla. The allele size ranged from 205 to 211 bp in Newzealand White and Grey Giant while the size was from 205 to 209 bp in Soviet Chinchilla. The alleles 205, 207 and 209 bp were common to all the three breeds. The allele 209 bp topped in Newzealand White, Soviet Chinchilla and Grey Giant with frequencies of 0.480, 0.500 and 0.375, respectively. The allele size and frequencies are summarized in Table 4.6. The polymorphism of Sol 44 locus is depicted in Fig.3.

Three different genotypes were recorded for Sol 44 locus in Newzealand White, two in Soviet Chinchilla while Grey Giant recorded four. The genotype 207/205 was specific to Newzealand White while 211/205 was found in Grey Giant only. All the three populations had 209/207 genotype in common and was observed in maximum frequency of 0.800, 0.840 and 0.350 in Newzealand white, Soviet Chinchilla and Grey Giant, respectively. The genotype and their frequencies are summarized in Table 4.9.

4.4 HETEROZYGOSITY AND POLYMORPHIC INFORMATION CONTENT

The values for heterozygosity and PIC in Newzealand White at the Sol 03 locus were recorded as 0.840 and 0.836, in Soviet Chinchilla as 0.766 and 0.764, while in Grey Giant, the heterozygosity and PIC values stood at 0.775 and 0.765, respectively. The heterozygosity and PIC values at Sol 03 locus are presented in Table 4.10. The maximum values for heterozygosity were obtained in Newzealand White while Soviet Chinchilla recorded the minimum.

At the Sol 33 locus, the maximum values for heterozygosity (0.858) and PIC (0.854) were observed in Grey Giant while Newzealand White recorded the

lowest (0.672 and 0.667, respectively). In Soviet Chinchilla, values for heterozygosity and PIC were 0.691 and 0.680 respectively. The details of heterozygosity and PIC are summarized in Table 4.11.

The highest values for heterozygosity (0.728) and PIC (0.702) at the Sol 44 locus were recorded in Grey Giant, while the lowest (0.567 and 0.477) in Soviet Chinchilla. The heterozygosity and PIC values were 0.586 and 0.502, respectively in Newzealand White. The heterozygosity and PIC values of Sol 44 locus are presented in Table 4.12.

The mean values for the heterozygosity and PIC obtained were 0.794 and 0.788 at Sol 03 locus, 0.740 and 0.734 at Sol 33 locus and 0.627 and 0.560 at Sol 44 locus. The values are summarized in Table 4.13.

4.5 GENETIC DISTANCE

The genetic distance between Newzealand White and Soviet Chinchilla was found to be 0.2022, between Soviet Chinchilla and Grey Giant was 0.6942 and between Newzealand White and Grey Giant recorded as 0.6130. The maximum genetic distance was recorded between Soviet Chinchilla and Grey Giant, while the minimum between Newzealand White and Soviet Chinchilla. Genetic distance matrix is presented in Table 4.14.

4.6 DENDROGRAM

The dendrogram of relationship between the three rabbit breeds based on POPGENE program (Nei, 1978) showed one cluster, grouping Newzealand White and Soviet Chinchilla. The dendrogram is presented in Fig.7.

Table 4.1 Standardised conditions for the PCR assay

Sl. No.	Parameter	Value
1.	Template DNA (ng)	50
2.	$MgCl_2$ (m*M*)	Sol 03 – 1.5
		Sol 33 – 1.25
		Sol 44 – 1.25
3.	10X Reaction Buffer (µl)	1
4.	dNTPs (µ*M*)	200
5.	Forward Primer (p*M*)	5
6.	Reverse Primer (p*M*)	5
7.	Taq DNA Polymerase (U)	0.3
8.	Reaction Volume (µl)	10

Table 4.2 Standardised temperature and cycling conditions for the PCR at different polymorphic microsatellite loci analysed

Sl. No.	Parameter	Temperature/Time		
		Sol 03	Sol 33	Sol 44
1.	Initial denaturation (°C/3min)	94	94	94
2.	Denaturation (°C/min)	94	94	94
3.	Annealing (°C/min)	60.9	60.5	67.7
4.	Extension (°C/min)	72	72	72
5.	No. of Cycles	35	35	35
6.	Final extension (°C/5 min)	72	72	72

Table 4.3 Standardised conditions for the PCR at different monomorphic microsatellite loci analysed

Marker	$MgCl_2$ (m*M*)	Annealing temperature (°C/minute)
INRA 005	1.25	58.6
HUJ 1177	1.25	55
EMX 1	1.1	62

Table 4.4 Alleles, their size and frequency at the Sol 03 locus in the rabbit populations investigated

Sl. No.	Allele (bp)	Frequency		
		Newzealand White	Soviet Chinchilla	Grey Giant
1	205	0.000	0.042	0.000
2	209	0.000	0.083	0.025
3	217	0.000	0.000	0.125
4	219	0.000	0.042	0.000
5	221	0.042	0.042	0.000
6	223	0.104	0.438	0.025
7	225	0.042	0.000	0.075
8	227	0.146	0.042	0.000
9	229	0.104	0.000	0.350
10	231	0.188	0.021	0.200
11	233	0.125	0.021	0.000
12	235	0.250	0.125	0.000
13	237	0.000	0.104	0.000
14	247	0.000	0.042	0.200

Table 4.5 Alleles, their size and frequency at the Sol 33 locus in the rabbit populations investigated

Sl.No.	Allele (bp)	Frequency		
		Newzealand White	Soviet Chinchilla	Grey Giant
1	206	0.120	0.000	0.071
2	208	0.240	0.273	0.143
3	212	0.160	0.250	0.071
4	214	0.000	0.068	0.190
5	216	0.480	0.409	0.143
6	218	0.000	0.000	0.095
7	222	0.000	0.000	0.190
8	224	0.000	0.000	0.095

Table 4.6 Alleles, their size and frequency at the Sol 44 locus in the rabbit populations investigated

Sl.No.	Allele (bp)	Frequency		
		Newzealand White	Soviet Chinchilla	Grey Giant
1	205	0.020	0.080	0.225
2	207	0.420	0.420	0.175
3	209	0.480	0.500	0.375
4	211	0.080	0.000	0.225

Table 4.7 Genotypes and frequencies at the Sol 03 locus in the different rabbit populations.

Sl. No.	Genotype	Frequency		
		Newzealand White	Soviet Chinchilla	Grey Giant
1	219/205	0.000	0.083	0.000
2	221/209	0.000	0.083	0.000
3	223/209	0.000	0.083	0.050
4	223/223	0.000	0.167	0.000
5	227/223	0.000	0.083	0.000
6	229/217	0.000	0.000	0.050
7	229/221	0.083	0.000	0.000
8	229/223	0.083	0.000	0.000
9	229/229	0.000	0.000	0.250
10	229/247	0.000	0.000	0.150
11	231/217	0.000	0.000	0.200
12	231/223	0.042	0.042	0.000
13	231/225	0.042	0.000	0.000
14	231/227	0.042	0.000	0.000
15	231/231	0.000	0.000	0.050
16	233/223	0.042	0.042	0.000
17	233/227	0.125	0.000	0.000
18	233/229	0.042	0.000	0.000
19	235/223	0.042	0.167	0.000
20	235/225	0.042	0.000	0.000
21	235/227	0.125	0.000	0.000
22	235/229	0.042	0.000	0.000
23	235/231	0.250	0.000	0.000
24	235/235	0.000	0.042	0.000
25	237/223	0.000	0.125	0.000
26	247/225	0.000	0.000	0.150
27	247/231	0.000	0.000	0.100
28	247/237	0.000	0.083	0.000

Table 4.8 Genotypes and frequencies at the Sol 33 locus in the different rabbit populations

Sl. No.	Genotype	Frequency		
		Newzealand White	Soviet Chinchilla	Grey Giant
1	208/206	0.120	0.000	0.143
2	212/208	0.080	0.227	0.000
3	214/208	0.000	0.000	0.095
4	214/212	0.000	0.045	0.143
5	216/206	0.120	0.000	0.000
6	216/208	0.280	0.318	0.048
7	216/212	0.240	0.227	0.000
8	216/214	0.000	0.091	0.095
9	216/216	0.160	0.099	0.000
10	218/214	0.000	0.000	0.048
11	218/216	0.000	0.000	0.095
12	222/218	0.000	0.000	0.048
13	222/222	0.000	0.000	0.095
14	224/216	0.000	0.000	0.048
15	224/218	0.000	0.000	0.048
16	224/222	0.000	0.000	0.096

Table 4.9 Genotypes and frequencies at the Sol 44 locus in the different rabbit populations

Sl. No.	Genotype	Frequency		
		Newzealand White	Soviet Chinchilla	Grey Giant
1	207/205	0.040	0.000	0.000
2	209/205	0.000	0.160	0.200
3	209/207	0.800	0.840	0.350
4	211/205	0.000	0.000	0.250
5	211/209	0.160	0.000	0.200

Table 4.10 Allele size range, Number of alleles, Heterozygosity and Polymorphic Information Content (PIC) at the Sol 03 locus in the rabbit populations investigated.

Observation	**Newzealand White (n=25)**	**Soviet Chinchilla (n=25)**	**Grey Giant (n=25)**
Allele size range (bp)	221-235	205-247	209-247
Number of alleles	8	11	7
Heterozygosity	0.840	0.766	0.775
PIC	0.836	0.764	0.765

Table 4.11 Allele size range, Number of alleles, Heterozygosity and Polymorphic Information Content (PIC) at the Sol 33 locus in the rabbit populations investigated.

Observation	**Newzealand White (n=25)**	**Soviet Chinchilla (n=25)**	**Grey Giant (n=25)**
Allele size range (bp)	206-216	208-216	206-224
Number of alleles	4	4	8
Heterozygosity	0.672	0.691	0.858
PIC	0.667	0.680	0.854

Table 4.12 Allele size range, Number of alleles, Heterozygosity and Polymorphic Information Content (PIC) at the Sol 44 locus in the rabbit populations investigated

Observation	Newzealand White (n=25)	Soviet Chinchilla (n=25)	Grey Giant (n=25)
Allele size range (bp)	205-211	205-209	205-211
Number of alleles	3	3	4
Heterozygosity	0.586	0.567	0.728
PIC	0.502	0.477	0.702

Table 4.13 Mean Heterozygosity and PIC of the three microsatellite markers tested in different rabbit populations

Parameter	Microsatellite loci		
	Sol03	Sol33	Sol44
Mean Heterozygosity	0.794	0.740	0.627
Mean PIC	0.788	0.734	0.560

Table 4.14 Nei's genetic distance matrix for the three rabbit breeds analysed

Breeds	Newzealand White	Soviet Chinchilla	Grey Giant
Newzealand White	******		
Soviet Chinchilla	0.0222	******	
Grey Giant	0.6130	0.6942	******

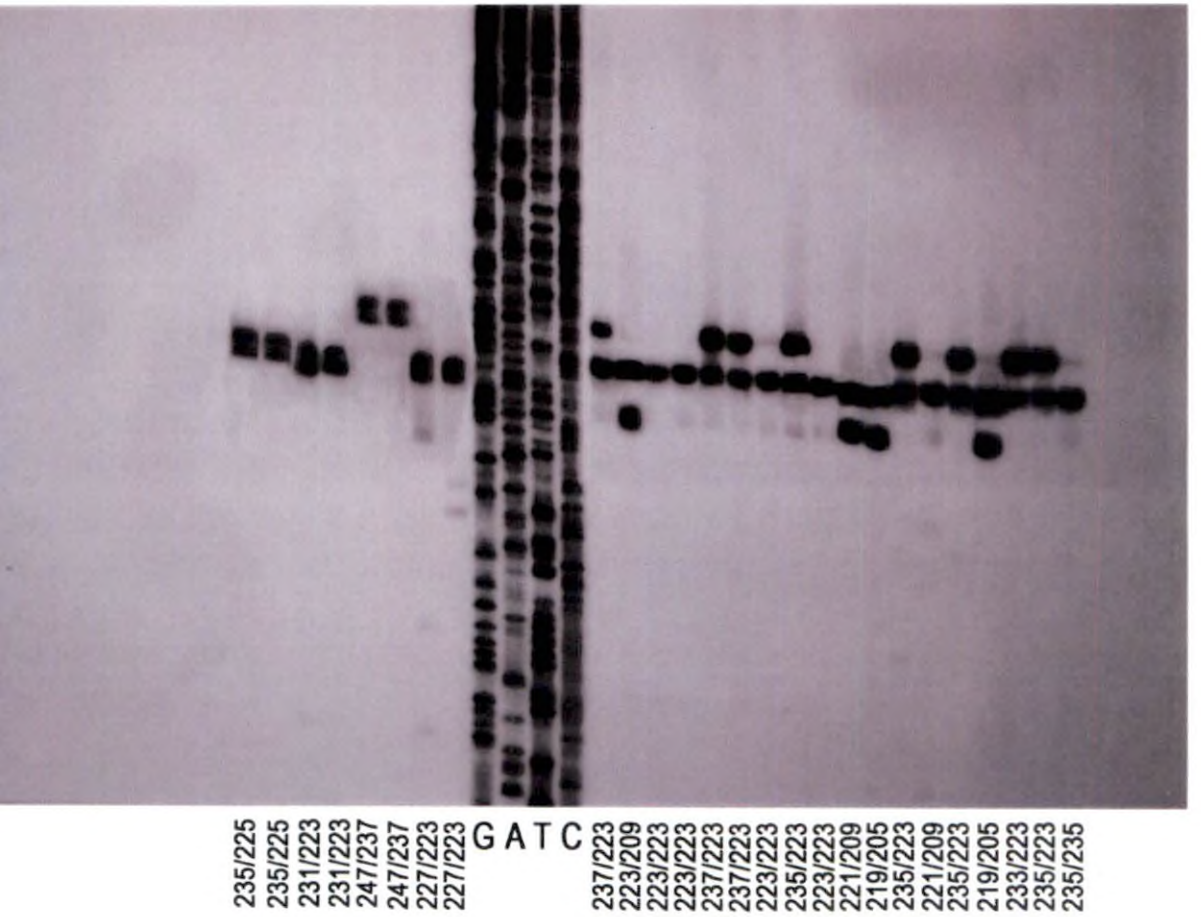

Fig.1. Autoradiograph showing polymorphism at Sol 03 locus GATC represents M13 sequence used as marker.

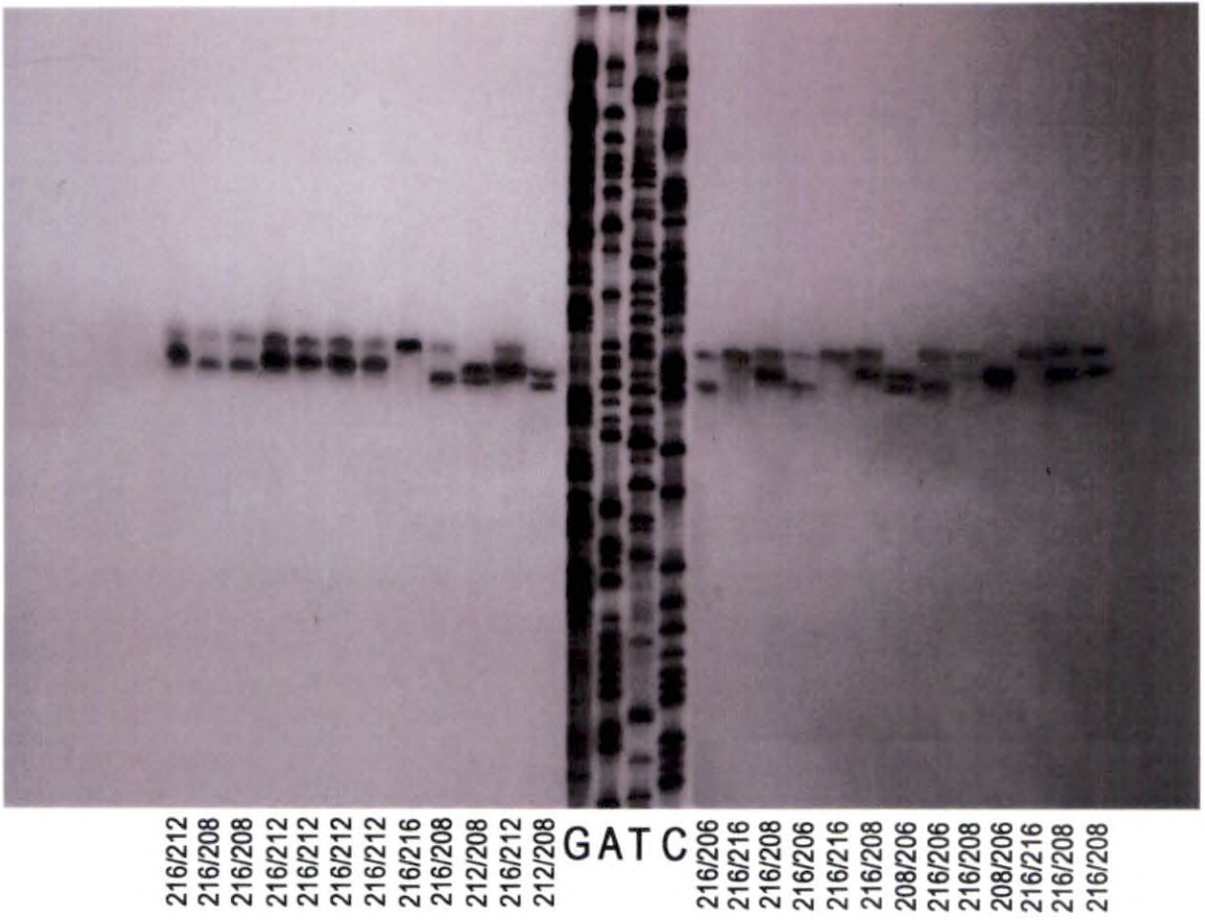

Fig. 2. Autoradiograph showing polymorphism at Sol 33 locus
GATC represents M13 sequence used as marker

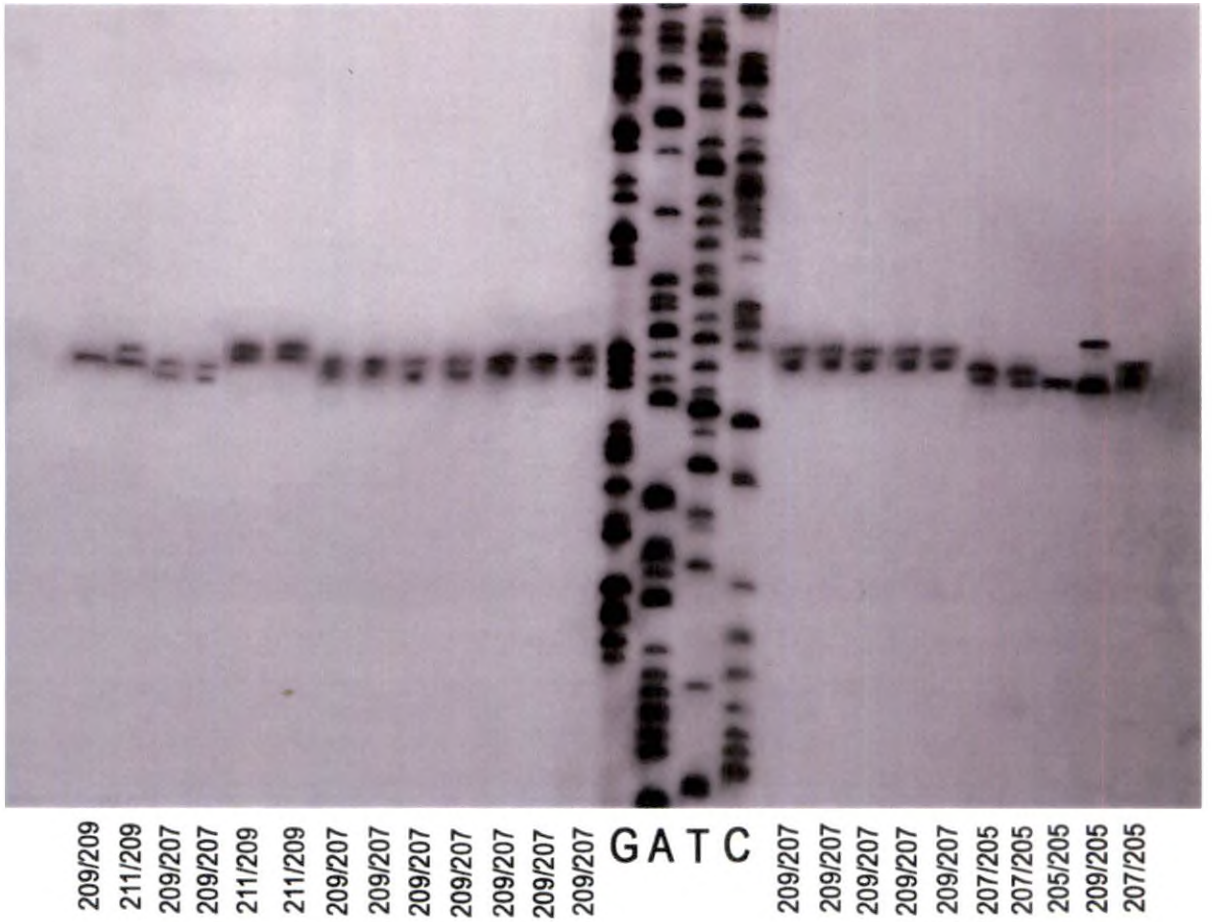

Fig. 3. Autoradiograph showing polymorphism at Sol 44 locus
GATC represents M13 sequence used as marker

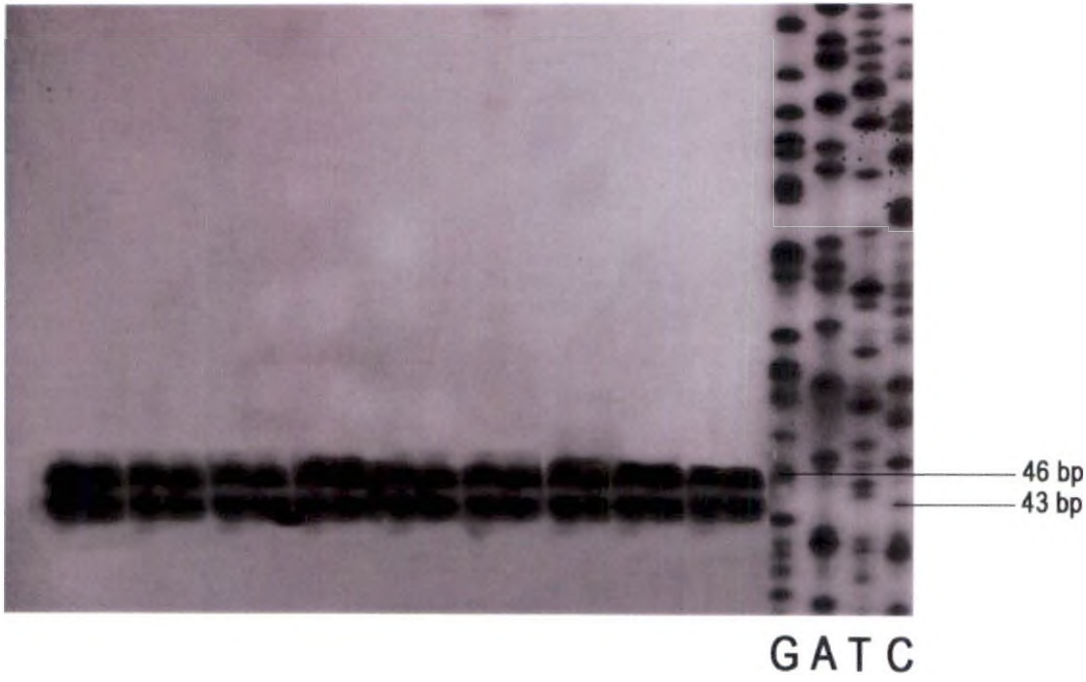

Fig.4. Autoradiograph showing monomorphic HUJ 1177 locus
GATC represents M13 sequence used as marker

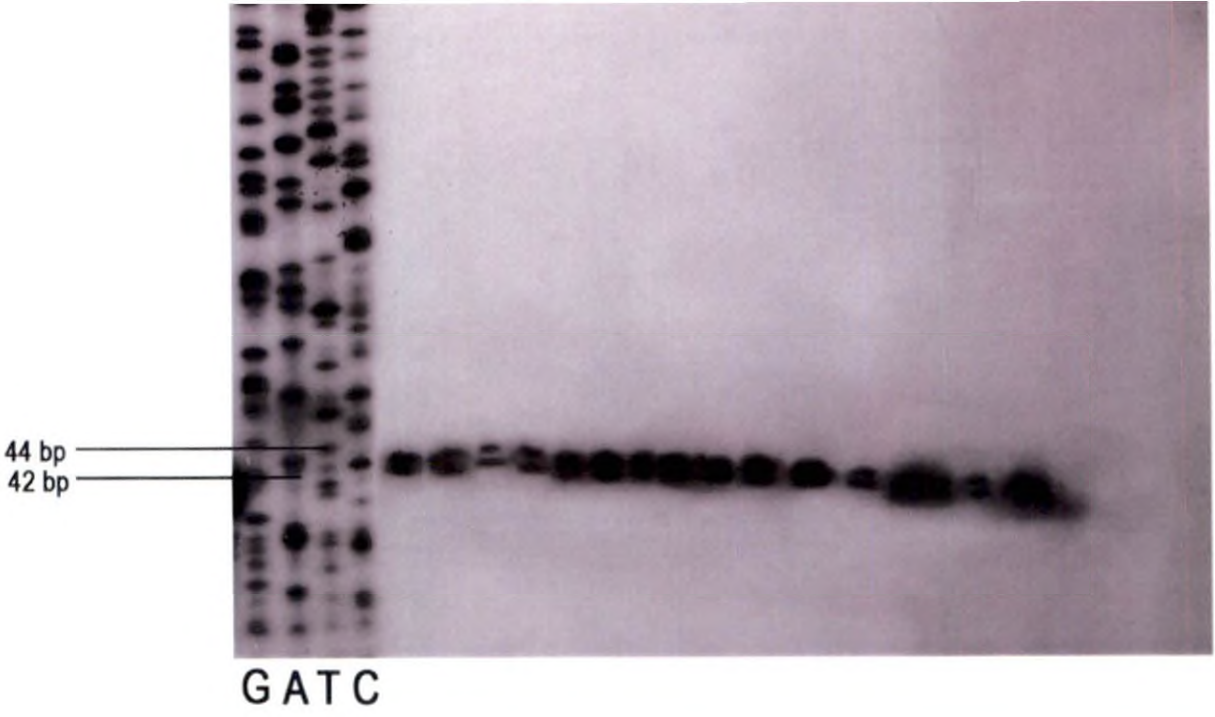

Fig.5 . Autoradiograph showing monomorphic EMX 1 locus
GATC represents M13 sequence used as marker

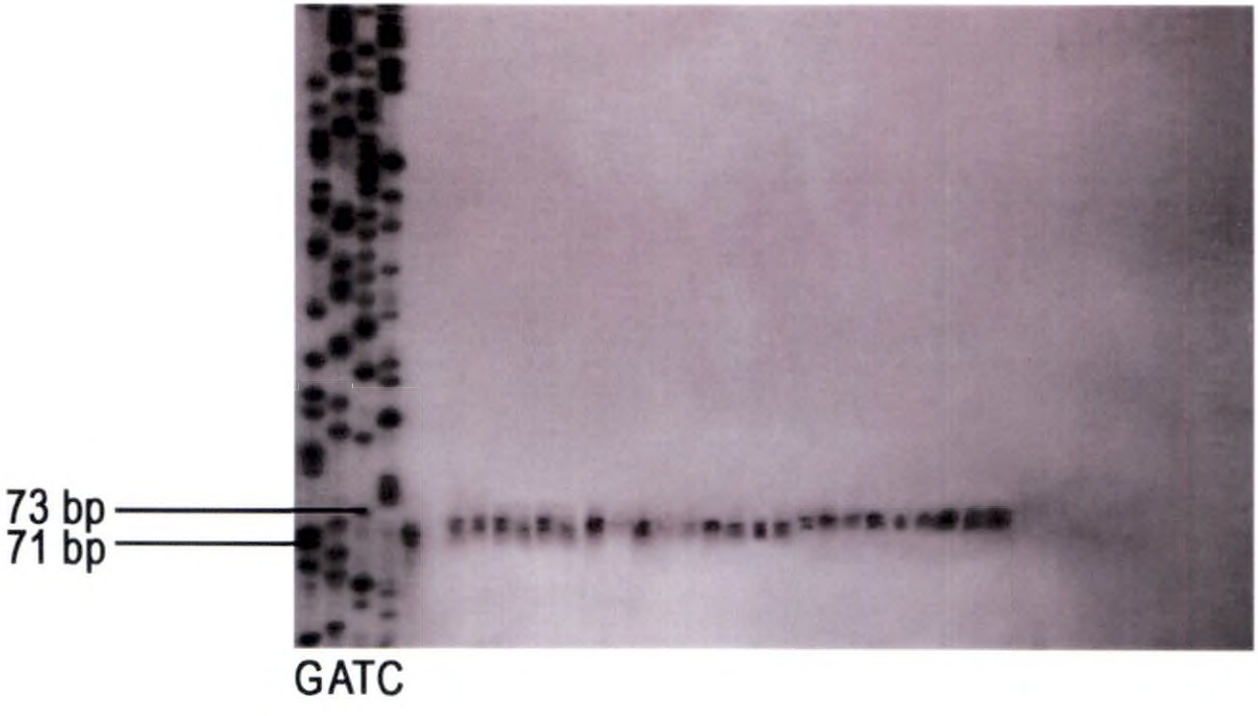

Fig.6. Autoradiograph showing monomorphic INRA 005 locus
GATC represents M13 sequence used as marker

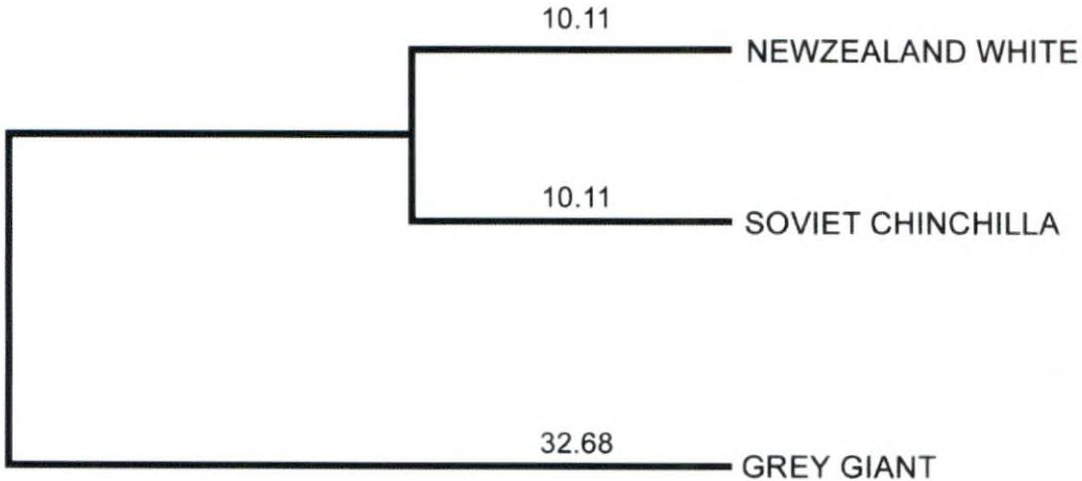

Fig.7 Dendrogram representing relationship between the three rabbit breeds studied constructed using the UPGMA method. Numbers indicate branch lengths

Discussion

Rabbit Breeds

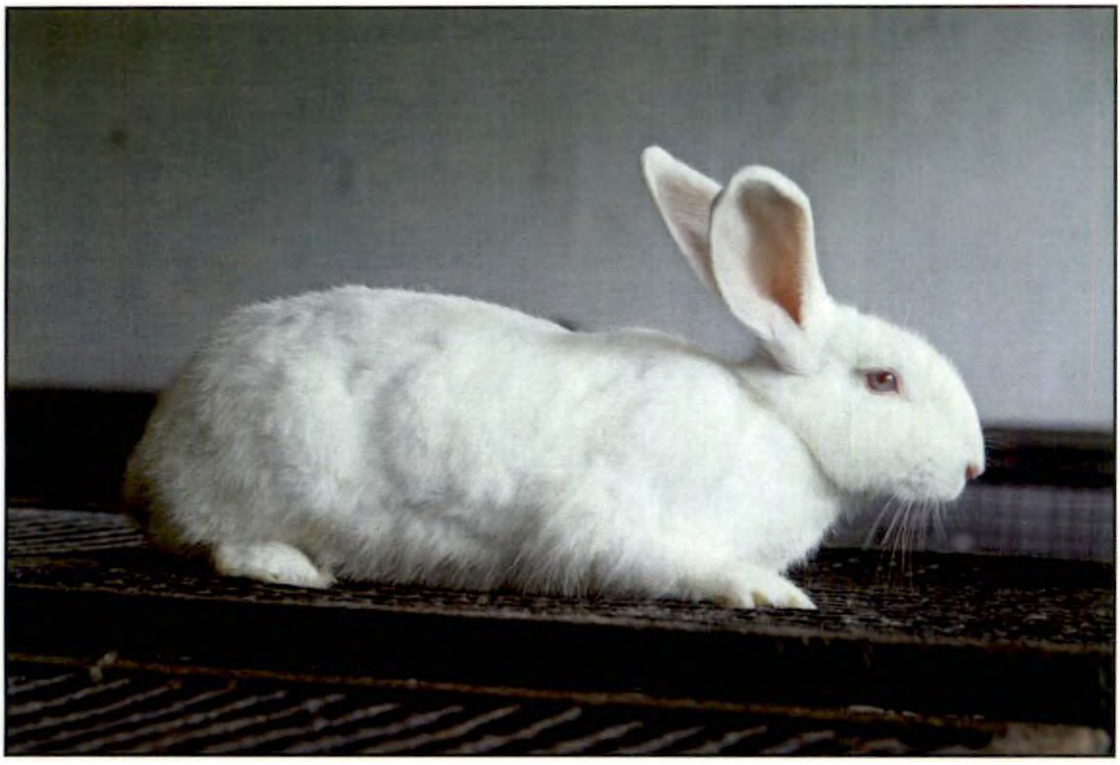

Fig: 8 Newzealand White

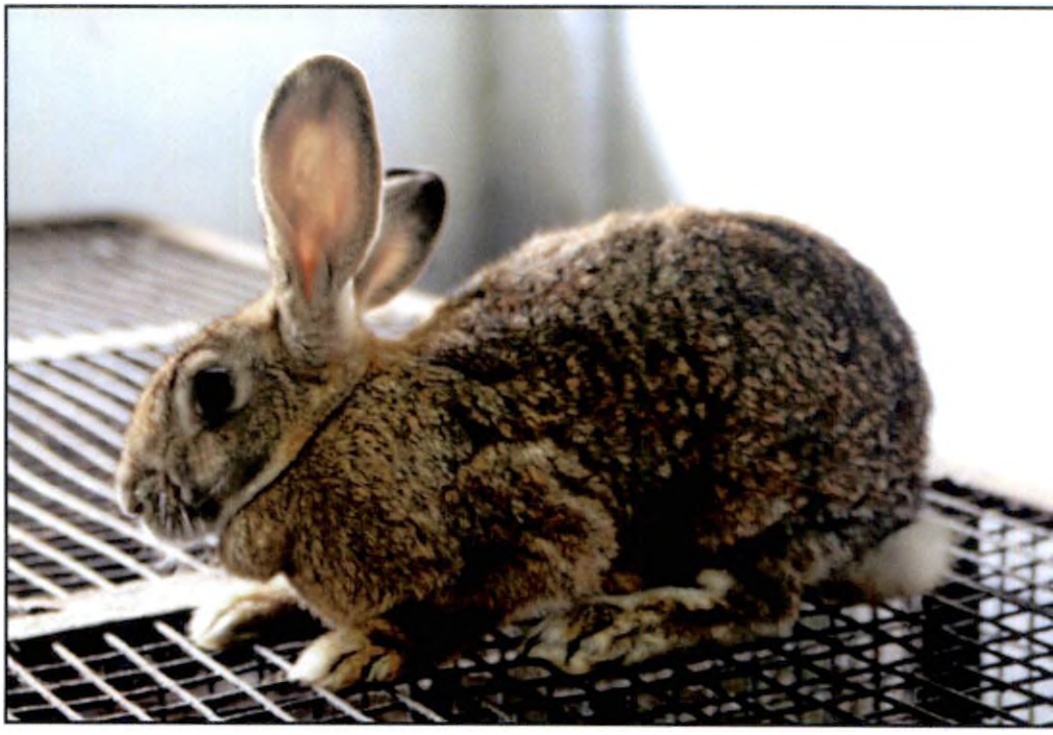

Fig: 10 Grey Giant

5. DISCUSSION

5.1 MICROSATELLITE PRIMERS

Microsatellites, the tandem repeat loci, can be generally considered to be hypervariable in length. This variability is a reflection of a general mechanism, most likely, slippage occurring during DNA repair or replication processes. At these loci, a mutation may alter the size of an allele by adding or deleting one or more repeats. Slippage mutations are sufficiently frequent to maintain a high degree of polymorphism within populations, but not frequent enough to occur in successive generations. The mutation rate is exceptionally high, implying a higher degree of polymorphism (Tautz, 1989).

Microsatellites have proved to be useful polymorphic markers for the analysis of genetic relationships. The usefulness of microsatellite markers for the estimation of genetic distances among closely related populations has been documented by Ciampolini *et al.* (1995).

Twelve microsatellite markers (INRA 005, INRA 063, TGLA 53, TGLA 63, ILSTS 030, HUJ 1177, BM 6121, EMX 1, LA 4, Sol 03, Sol 33 and Sol 44) were tested in the present study. Out of these, only three markers (Sol 03, Sol 33 and Sol 44) were found to be polymorphic. Six primers failed to produce PCR amplification. Among the markers which had successful amplification, three (INRA 005, HUJ 1177 and EMX 1) demonstrated no polymorphism. Pemberton *et al.*, (1995) and de Gortari *et al.*, (1997) observed that the homologous markers tend to be shorter in related species, are less polymorphic and have a higher fraction of null alleles .

5.1.1 Sol 03

In the present study, this marker well amplified with a total of 14 alleles in the pooled population, with a size range of 205 to 247 bp. The heterozygosity values at this locus in three breeds of rabbits ranged from 0.725 to 0.840. Rico *et al.* (1994) reported five alleles with a size range of 237 to 249 bp and obtained a heterozygosity of 0.800 for the same locus in European wild rabbit (*Oryctolagus cuniculus*).

The allele 235 bp was more predominant in Newzealand White with a frequency of 0.250 while the alleles 223 bp and 229 bp predominated in Soviet Chinchilla (0.438) and Grey Giant (0.350), respectively.

The value for heterozygosity in Newzealand White was recorded as 0.840, in Soviet Chinchilla as 0.766, while in Grey Giant, the heterozygosity value stood at 0.775.

Several others who had worked at this locus has reported eight to seventeen alleles in rabbits with a size range of 225 to 249 bp (Surridge *et al.*,1997; Surridge *et al.*, 1999 and Burton *et al.*, 2002).

5.1.2 Sol 33

The maximum number of alleles (eight) for Sol 33 locus was observed in Grey Giant, followed by four each in Newzealand White and Soviet Chinchilla. The allele size ranged from 206 to 224 bp. A total of 16 different genotypes were recorded in the pooled population. The allele size of 216 bp occurred in highest frequency both in Newzealand White (0.480) and Soviet Chinchilla (0.410) for Sol 33 locus while two alleles 214 and 222 bp topped in Grey Giant (0.190).

Surridge *et al.* (1997) obtained 15 alleles with a size range of 189 to 219 bp in the European wild rabbit (*Oryctolagus cuniculus*) and they observed that this locus amplified well in other lagomorph species also.

The polymorphism at Sol 33 locus was exploited by Surridge *et al.*, (1999) and Burton *et al.*, (2002) to study the population genetic structure of rabbits and obtained nine to sixteen alleles in various studies.

The heterozygosity values recorded the maximum in Grey Giant (0.858), followed by Soviet Chinchilla (0.691) and Newzealand White (0.672). These values indicate the suitability of this marker for diversity studies.

5.1.3 Sol 44

The maximum number of alleles (four) for Sol 44 locus was observed in Grey Giant while three alleles each were recorded in Newzealand White and Soviet Chinchilla. The allele size ranged from 205 to 211 bp. The 211 bp allele was absent in Soviet Chinchilla and the allele 209 bp appeared to be the most frequent one with frequencies of 0.480, 0.500 and 0.375 in Newzealand White, Soviet Chinchilla and Grey Giant, respectively.

Surridge *et al.* (1997) detected a total of nine alleles with a size range of 178 to 208 bp in the European wild rabbit.

The maximum value for heterozygosity (0.728) was observed in Grey Giant while Soviet Chinchilla (0.567) recorded the lowest. In Newzealand White, the value for heterozygosity at Sol 44 locus was 0.586.

The number of alleles at different marker loci and their frequencies are indicators of genetic variability and also form the basis of all diversity indices for

estimation of genetic distances and construction of phylogenetic trees (Mukesh *et al.*, 2006).

The level of variation depicted by the number of alleles at each locus serves as a measure of genetic variability having direct impact on differentiation of breeds within a species (Arora and Bhatia, 2006).

Among the three breeds studied, the maximum number of alleles (eleven) was observed in Soviet Chinchilla for Sol 03 locus. As regards Sol 33 locus, a maximum of four alleles were detected in both Newzealand White and Soviet Chinchilla whereas Sol 44 locus revealed a maximum of four alleles in both Newzealand White and Grey Giant. The high mean number of alleles per loci is an indication of high genetic variability within breeds (Sodhi *et al.*, 2003). The mean number of alleles is dependent on sample size because of the presence of unique alleles which occur in low frequencies in the population and also because of the number of observed alleles which tend to increase with increase in population size (Kotze *et al.*, 2004).

The allele frequencies for all the microsatellite loci ranged from 0.020 to 0.500. The observed number of alleles per locus ranged from four to fourteen. The number of genotypes per locus ranged from five to twenty eight and the genotypic frequency ranged from 0.040 to 0.800.

The number of genotypes being high could be due to the number of alleles being high in the population. This may also indicate the existence of heterozygous genotypes in this population. The low allele frequency in this study could be explained by the fact that the number of alleles is high and the sample size being low (Selvi *et al.*, 2004).

Certain breed specific alleles were also detected in the population under study. Sol 03 locus revealed 219 and 237 bp in Soviet Chinchilla and 217 bp in

Grey Giant which was not observed in the other two breeds. Similarly, Sol 33 revealed 218, 222 and 224 bp in Grey Giant.

The occurrence of a few highly frequent alleles in the different populations could be suggestive of probable linkage of these loci to traits of economic importance and selection for those traits. In the present study, the allele 209 bp was found to be the most frequent one in Newzealand White, Soviet Chinchilla and Grey Giant. However, to obtain more consistent results and definite conclusions, a study is warranted incorporating a higher number of animals representing each breed.

5.2 HETEROZYGOSITY AND POLYMORPHIC INFORMATION CONTENT

An important characteristic of a genetic marker is its heterozygosity, that is, the probability that a random individual is heterozygous for that marker (Ott, 1992). Heterozygosity reflects the genetic diversity of a population and thus, to a certain extent, also reflects the state of inbreeding.

The values for heterozygosity for Sol 03 locus ranged from 0.775 to 0.840 while that of Sol 33 locus ranged from 0.672 to 0.858 among the three breeds studied. With regard to Sol 44 locus, the heterozygosity values ranged from 0.567 to 0.728.

The mean heterozygosity values for the three markers used in the present study ranged from 0.627 to 0.794. Takezaki and Nei (1996) showed that, having an average heterozygosity between 0.3 and 0.8 in the population is useful for measuring genetic variation.

All the three breeds demonstrated high heterozygosity values, which were in accordance with the expectations for the microsatellite loci. Similar values for heterozygosity were reported in rabbits by Rico *et al.* (1994).

PIC is a measure of the informativeness of the marker. Botstein *et al.* (1980) developed the PIC value for the measurement of a marker's informativeness in linkage analysis.

PIC values were high for all loci studied in all the breeds and ranged from 0.560 to 0.790. A fairly high PIC values (>0.5) suggest the suitability of microsatellites as markers of choice for studying genetic distance in rabbits.

5.3 GENETIC DISTANCE

Genetic distance is the basis for genetic diversity research. Genetic distance derived from microsatellite can reflect the time of diversity as well as the genetics and variation among breeds (Tu *et al.*, 2006).

The genetic distance measures the time that had elapsed since the populations were genetically equivalent. It indicates the time of divergence of the breeds from each other.

The genetic distance can be estimated from the difference in the allele frequency using several methods. Vijh *et al.* (2004) proved that the Nei's genetic distance was the most appropriate.

In the present study, allele frequencies at each locus for each breed were used for computing the genetic distance between each pair of breeds. The Nei's genetic distance between breeds ranged from 0.2022 to 0.6942. The genetic distance between Newzealand White and Soviet Chinchilla was found to be 0.2022, between Newzealand White and Grey Giant 0.6130 and between Soviet Chinchilla and Grey Giant 0.6942. The results showed that the genetic distance between Soviet Chinchilla and Grey Giant was the highest.

5.4 DENDROGRAM

The dendrogram is a phylogenetic tree to show the relationship among different population. In the present study, the dendrogram constructed from the genetic distance data using POPGENE program grouped Newzealand White and Soviet Chinchilla in one cluster. Grey Giant was found to be the most widely separated breed.

The clustering based on UPGMA and Nei's genetic distance are the best methods for analysing genetic diversity among the different breeds (Tu *et al.*, 2006). The present study too supports this view.

It was found that the set of microsatellite markers tested in this study could be used satisfactorily for molecular characterisation and genetic diversity studies in rabbits. However, using more number of markers and increasing the sample size would allow a better differentiation between populations.

The study characterised the three breeds of rabbit, viz. Newzealand White, Soviet Chinchilla and Grey Giant, using microsatellite markers. The allele and genotype frequencies and the genetic distance obtained revealed that Grey Giant is widely separated from the other two breeds. Newzealand White and Soviet Chinchilla are more closely related. This information can be utilized in formulating an appropriate breeding programme since the cross between more genetically distant animals produce young ones with better hybrid vigor. The present study indicates the scope for the production of crossbreds with high vigor by crossing the genetically distant Grey Giant with either of the other two breeds, viz. Newzealand white or Soviet Chinchilla for the benefit of rabbit farmers.

Summary

6. SUMMARY

The present study aimed at characterisation of the three breeds of rabbit, viz. Newzealand White, Soviet Chinchilla and Grey Giant using microsatellite markers. The phylogenetic relationship existing among these breeds has also been investigated by estimating the allele and genotype frequencies and genetic distance.

DNA isolated from 25 animals of each breed by standard Phenol-Chloroform extraction procedure formed the material for the study. Out of the 12 microsatellite markers (INRA 005, INRA 063, TGLA 53, TGLA 63, ILSTS 063, HUJ 1177, BM 6121, EMX 1, LA 4, Sol 03, Sol 33 and Sol 44) tested, Sol 03, Sol 33 and Sol 44 were found to be polymorphic while INRA 005, HUJ 1177 and EMX 1 proved to be monomorphic.

PCR conditions were optimized separately for each primer. Each reaction was carried out in 10 µl volume containing 1µl of 10X PCR reaction buffer, 200 µ*M* dNTP, 5 p*M* each of forward and reverse primer and 0.3U of *Thermus aquaticus* DNA polymerase. A $MgCl_2$ concentration of 1.5 m*M* for Sol 03 and 1.25 m*M* for Sol 33 and Sol 44 was found to be satisfactory. The annealing temperature was optimized at 60.9°C for Sol 03, 60.5 °C for Sol 33 and 67.7 °C for Sol 44. The forward primer of each primer pair was endlabelled with γ^{32}P-ATP.

Denaturing polyacrylamide gel electrophoresis was used to fractionate the amplified products and bands were visualized by autoradiography. The size of alleles at each locus was assessed by comparing with the sequence of M13 single stranded DNA size standard.

A total of eight, eleven and seven alleles were observed for Sol 03 locus in Newzealand White, Soviet Chinchilla and Grey Giant respectively. The allele of 217 bp was found exclusively in Grey Giant. The allele of 235 bp occurred in highest frequency in Newzealand White. The values for heterozygosity and PIC in Newzealand White at the Sol 03 locus were recorded as 0.840 and 0.836, in Soviet Chinchilla as 0.766 and 0.764, while in Grey Giant, the heterozygosity and PIC values stood at 0.775 and 0.765, respectively.

With regard to Sol 33 locus, eight alleles in Grey Giant and four alleles each in Newzealand White and Soviet Chinchilla were detected. Eight genotypes and three alleles were found to be specific in Grey Giant. At the Sol 33 locus, the maximum values for heterozygosity (0.858) and PIC (0.854) were observed in Grey Giant while Newzealand White (0.672 and 0.667, respectively) recorded the lowest. In Soviet Chinchilla, values for heterozygosity and PIC were 0.691 and 0.680 respectively.

The third microsatellite marker Sol 44 gave rise to four alleles in Grey Giant and three alleles each in Newzealand White and Soviet Chinchilla. The heterozygosity and PIC values were highest in Grey Giant (0.728 and 0.702) while lowest values (0.567 and 0.477) were recorded in Soviet Chinchilla. In Newzealand White, the values were 0.586 and 0.502, respectively.

Allele frequencies at each locus for each breed were used to calculate genetic distances among the three breeds. Nei's genetic distance was worked out using the POPGENE version 1.31 program. The genetic distance between Soviet Chinchilla and Grey Giant was found to be 0.6942 while that between Newzealand White and Grey Giant was 0.6130. The genetic distance between Newzealand White and Soviet Chinchilla was found to be 0.2022. The dendrogram constructed using the POPGENE program grouped Newzealand White and Soviet Chinchilla in one cluster. These results revealed that Soviet Chinchilla and Newzealand White are more closely related when compared to

Grey Giant. The cross between more genetically distant animals produces young ones with better hybrid vigor. This information gathered herein can contribute to database based on which the breeding programme shall be formulated The present study indicates the scope for the production of crossbreds with high vigor by crossing the genetically distant Grey Giant with either of the other two breeds, viz. Newzealand white or Soviet Chinchilla for the benefit of rabbit farmers.

The study also indicated that the set of microsatellite markers tested could be used satisfactorily for molecular characterization and genetic diversity studies in rabbits. The results reveal the scope for further attempts using more markers in larger sample sizes so as to generate clearer picture as regards differentiation among closely related populations.

References

REFERENCES

Andersson, A., Thulin, C. and Tegelstrom, H. 1999. Applicability of rabbit microsatellite primers for studies of hybridization between an introduced and a native hare species. *Hereditas* 130: 309-315

Arora, R. and Bhatia, S. 2006. Genetic diversity of Magra sheep from India using microsatellite analysis. *Asian- Aust. J. Anim. Sci.* 19: 938-942

Arora, R., Lakhchaura, B.D., Prasad, R.B., Tantia, M.S., Sharma, U. and Vijh, R.K. 2003. Evaluation of heterologous microsatellite markers for genetic polymorphism and genetic distancing in indigenous buffalo populations. *Indian J. Anim. Sci.* 73:1247-1255

Bassam, B.J., Caetano-Anolles, G. and Gresshoff, P.M. 1991. Fast and sensitive silver staining of DNA in polyacrilamide gels. *Anal. Biochem.* 196: 80-83

Biggin, M.D., Gibson, T.J. and Hong, F. 1983. Buffer gradient gels and ^{35} S label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA.* 80: 3963-3965

Blin, N. and Stafford, D.W. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res.* 3: 2303-2308

Blott, S.C., Williams, J.L. and Haley, C.S. 1999. Discriminating among cattle breeds using genetic markers. *Heredity* 82: 613-619

Botstein, D., White, R.L., Skolnick, M. and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32: 314-331

Burton, C., Krebs, C.J. and Taylor, E.B. 2002. Population genetic structure of the cyclic snowshoe hare (*Lepus americanus*) in Southwestern Yukon, Canada. *Mol. Ecol.* 11: 1689-1701

Canon, J., Alexandrino, P., Bessa, I., Carleos, C., Carretero, Y., Dunner, S., Ferrand, N., Garcia, D., Jordana, J., Laloe, D., Pereira, A., Sanchez, A. and Moazami-Goudarzi, K. 2001. Genetic diversity measures of local European beef cattle breeds for conservation purposes. *Genet. Sel. Evol.* 33: 311-332

Cervini, M., Henrique-Silva, F., Mortari, N. and Matheucci, E. 2006. Genetic variability of 10 microsatellite markers in the characterization of Brazilian Nellore cattle (*Bos indicus*). *Genet. Mol. Biol.* 29: 486-490

Chakraborty, R., Kimmel, M., Stivers, D.N., Davison, L.J. and Deka, R. 1997. Relative mutation rates at di-, tri-, and tetranucleotide microsatellite loci. *Proc. Natl. Acad. Sci. USA.* 94: 1041-1046

Chantry- Darmon, C., Urien, C., Hayes, H., Bertaud, M., Chandi-Taourit, S., Chardon, P., Vaiman, D. and Rogel-Gaillard, C. 2005. Construction of a cytogenetically anchored microsatellite map in rabbit. *Mamm. Genome* 16: 442-459

Chenyambuga, S.W., Hanotte, O., Hirbo, J., Watts, P.C., Kemp, S.J., Kifaro, G.C., Gwakisa, P.S., Peterson, P.H. and Rege, J.E.O. 2004. Genetic characterization of indigenous goats of sub-saharan Africa using microsatellite DNA markers. *Asian-Aust. J. Anim. Sci.* 17: 445-452

Cho, G.J. 2005. Microsatellite polymorphism and genetic relationship in dog breeds in Korea. *Asian- Aust. J. Anim. Sci.* 18: 1071-1074

Cho, G.J. and Cho, B.W. 2004. Microsatellite DNA typing using 16 markers for parental identification of the Korean Native Horse. *Asian-Aust. J. Anim. Sci.* 17: 750-754

Ciampolini, R., Moazami-Goudarzi, K., Vaiman, D., Dillmann, C., Mazzanti, E., Foulley, J., Leveziel, H. and Cianci, D. 1995. Individual multilocus genotypes using microsatellite polymorphisms to permit the analysis of the genetic variability within and between Italian beef cattle breeds. *J. Anim. Sci.* 73: 3259-3268

de Gortari, M.J., Freking, B.A., Kappes, S.M., Leymaster, K.A., Crawford, A.M., Stone, R.T. and Beattle, C.W. 1997. Extensive genomic conservation of cattle microsatellite heterozygosity in Sheep. *Anim. Genet.*28: 274-290

Dorji, T., Hanotte, O., Arbenz, M., Rege, O. and Roder, W. 2003. Genetic diversity of indigenous cattle populations in Bhutan: Implications for conservation. *Asian-Aust. J. Anim. Sci.* 16: 946-951

Ehrlich, H.A., Gelfand, D. and Sninsky, J.J. 1991. Recent advances in the Polymerase Chain Reaction. *Science* 252: 1643-1651

Flagstad, O., Olsaker, I. and Roed, K.H. 1999. The use of heterologous primers for analyzing microsatellite variation in hartebeest *Alcelaphus buselaphus. Hereditas* 130: 337-340

Grimberg, J., Nawoschik, S., Belluscio, L., McKee, R., Turck, A. and Eisenberg, A.1989. A simple and efficient non-organic procedure for the isolation of genomic DNA from blood. *Nucleic Acids Res.* 17: 8390

Hai- Guo, J., Yu-min, Z. and Guo-li, Z. 2005. Analysis of microsatellite DNA polymorphisms in five China native cattle breeds and application to population genetic studies. *Asian- Aust. J. Anim. Sci.* 18: 1696-1700

Hite, J.M., Eckert, K.A. and Cheng, K.C. 1996. Factors affecting fidelity of DNA synthesis during PCR amplification of $d(C\text{-}A)_n.d(G\text{-}T)_n$ microsatellite repeats. *Nucleic Acids Res.* 24: 2429-2434

Holland, P.M., Abramson, R.D., Watson, R. and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA.* 88: 7276-7280

Jackson, D.P., Lewis, F.A., Taylor, G.R., Boylston, A.W. and Quirke, P. 1990. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. *J. Clin. Pathol.* 43: 499-504

Korstanje, R., Gillissen, G.F., den Bieman, M., Versteeg, S.A., van Oost, B., Fox, R.R., van Lith, H.A. and van Zutphen, L.F.M. 2001. Mapping of rabbit chromosome 1 markers generated from a microsatellite- enriched chromosome- specific library. *Anim. Genet.* 32: 308-312

Korstanje, R., Gillissen, G.F., Kodde, L.F., den Bieman, M., Lankhorst, A., van Zutphen, L.F.M. and van Lith, H.A. 2001. Mapping of microsatellite loci and association of aorta atherosclerosis with LG VI markers in the rabbit. *Physiol. Genomics* 6: 11-18

Kotze, A., Grobler, J.P., Swart, H. and Nemaangani, A. 2004. A genetic profile of the Kalahari Red goat breed from southern Africa. *S. Afr. J. Anim. Sci.* 34: 10-12

Li, M.H., Nogovitsina, E., Ivanova, Z., Erhardt, G., Vilkki, J., Popov, R., Ammosov, I., Kiselyova, T. and Kantanen, J. 2005. Genetic contribution of indigenous Yakutian cattle to two hybrid populations revealed by microsatellite variation. *Asian-Aust. J. Anim. Sci.* 18: 613-619

Li, C., Wang, Z., Liu, B., Yang, S., Zhu, Z., Fan, B., Yu, M., Zhao, S. and Li, K. 2004. Evaluation of the genetic relationship among ten Chinese indigenous Pig breeds with twenty six microsatellite markers. *Asian-Aust. J. Anim. Sci.* 17: 441-444

Li, S.J., Yang, S.H., Zhao, S.H., Fan, H., Yu, M., Wang, H.S., Li, M.H., Liu, B., Xiong, T.A. and Li, K. 2004. Genetic diversity analyses of 10 indigenous Chinese pig populations based on 20 microsatellites. *J. Anim. Sci.* 82: 368-374

Litt, M. and Luty, J.A. 1989. A hypervariable microsatellite revealed by in-vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* 44: 397-401

MacHugh, D.E., Shriver, M.D., Loftus, R.T., Cunningham, P. and Bradley, D.G. 1997. Microsatellite DNA variation and the evolution, domestication and phylogeography of Taurine and Zebu cattle (*Bos Taurus* and *Bos indicus*). *Genetics* 146: 1071-1086

Martinez, A.M., Carrera, M.P., Acosta, J.M., Rodriguez-Gallardo, P.P., Cabello, A., Camacho, E. and Delgado, J.V. 2004. Genetic characterization of the Blancha Andaluza goat based on microsatellite markers. *S. Afr. J. Anim. Sci.* 34: 17-19

Maudet, C., Luikart, G. and Taberlet, P. 2002. Genetic diversity and assignment tests among seven French cattle breeds based on microsatellite DNA analysis. *J. Anim. Sci.* 80: 942-950

Maxam, A.M. and Gilbert, W. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA* 74: 560-564

Miller, S.A., Dykes, D.D. and Polesky, H.F. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16: 1215

Montaldo, H.H. and Meza-Herrera, C.A. 1998. Use of molecular markers and major genes in the genetic improvement of livestock. *J.Biotechnol.* 1: 1-7

Mukesh, M., Sodhi, M., Mishra, B.P., Vijh, P.K., Tantia, M.S. and Bhatia, S. 2006. Assessment of population structure and microsatellite based genetic variability in Sahiwal cattle. *Indian J. Anim. Sci.* 74: 1051-1055

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583-590

Notter, D.R. 1999. The importance of genetic diversity in livestock populations of the future. *J. Anim. Sci.* 77: 61-69

Ott, J. 1992. Strategies for characterizing highly polymorphic markers in human gene mapping. *Am. J. Hum. Genet.* 51: 283-290

Pang, S.W.Y., Ritland, C., Carlson, J.E. and Cheng, K.M. 1999. Japanese quail microsatellite loci amplified with chicken specific primers. *Anim. Genet.* 30: 195-199

Pemberton, J.M., Slate, J., Bancroft, D.R. and Barrett, J.A. 1995. Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Mol. Ecol.* 4: 249-252

Queney, G., Vachot, A.M., Brun, J.M., Dennebuoy, N., Mulsant, P. and Monnerot, M. 2002. Different levels of human intervention in domestic rabbits: Effects on genetic diversity. *J. Hered.* 93: 205-208

Rico, C., Rico, I., Webb, N., Smith, S., Bell, D. and Hewitt, G. 1994. Four polymorphic microsatellite loci for the European wild rabbit, *Oryctolagus cuniculus. Anim. Genet.* 25: 367

Saitbekova, N., Gaillard, C., Obexer- Ruff, G. and Dolf, G. 1999. Genetic diversity in Swiss goat breeds based on microsatellite analysis. *Anim. Genet.* 30: 36-41

Sambrook, J., Fritsh, E.T. and Maniatis, T. 1989. *Molecular cloning: A laboratory manual.* Second edition. Cold spring Harbor Press, New York, 1886 p.

Sanger, F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74: 5463-5467

Selvi, P.K., Panandam, J.M., Yusoff, K. and Tan, S.G. 2004. Molecular characterization of the Mafriwal dairy cattle of Malaysia using microsatellite markers. *Asian-Aust. J. Anim. Sci.* 17: 1366-1368

Sodhi, M., Mukesh, M., Arora, R., Tantia, M.S. and Bhatia, S. 2003. Genetic characterisation of Garole sheep using microsatellite markers. *J. Dairy Sci.* 56: 167-173

Sukla, S., Yadav, B.R. and Bhattacharya, T.K. 2006. Characterization of Indian Riverine buffaloes using microsatellite markers. *Asian-Aust. J. Anim. Sci.* 19: 1556-1560

Surridge, A.K., Bell, D.J., Hewitt, G.M. and Rico, G.M. 1997. Polymorphic microsatellite loci in the European rabbit (*Oryctolagus cuniculus*) are also amplified in other lagomorph species. *Anim. Genet.* 28: 302-305

Surridge, A.K., Bell, D.J., Ibrahim, K.M. and Hewitt, G.M. 1999a. Population structure and genetic variation of European wild rabbits (*Oryctolagus cuniculus*) in East Anglia. *Heredity* 82: 479-487

Surridge, A.K., Ibrahim, K.M., Bell, D.J., Webb, N.J., Rico, C. and Hewitt, G.M. 1999b. Fine-scale genetic structuring in a natural population of European wild rabbits (*Oryctolagus cuniculus*). *Mol. Ecol.* 8: 299-307

Takezaki, N. and Nei, M. 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* 144: 389-399

Tantia, M.S., Behl, R., Sheoran, N., Singh, R. and Vijh, R.K. 2004. Microsatellite data analysis for conservation of two goat breeds. *Indian J. Anim. Sci.* 74: 761-767

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.* 17: 6463-6471

Triwitayakorn, K., Moolmuang, B., Sraphet, S., Panyim, S., Na-Chiangmai, A. and Smith, D.R. 2006. Analysis of genetic diversity of the Thai Swamp Buffalo (*Bubalus bubalis)* using cattle microsatellite DNA markers. *Asian-Aust. J. Anim. Sci.* 19: 617-621

Tu, Y.J., Chen, K.W., Zhang, S.J., Tang, Q.P., Gao, Y.S. and Yang, N. 2006. Genetic diversity of 14 indigenous Grey Goose breeds in China based on microsatellite markers. *Asian- Aust. J. Anim. Sci.* 19: 1-6

Vijh, R.K., Pandey, A.K., Mishra, B., Choudhary, P., Tantia, M.S. and Ahlawat, S.P.S. 2004. Estimating genetic distances in indigenous poultry germplasm using infinite allele model. *Indian J. Anim. Sci.* 74: 534-542

Weber, J.L. and May, P.E. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* 44: 388-396

Wiener, P., Burton, D. and Williams, J.L. 2004. Breed relationships and definition in British cattle: a genetic analysis. *Heredity* 93: 597-602

Yeh, F.C., Boyle, T., Rongcai, Y., Ye, Z. and Xian, J.M. 1999. POPGENE Version 3.1.

Zhu, Y.F., Zhang, J.B., Ren, W.Z. and Wang, Y.Z. 2004. Genetic variation within and among five rabbit populations using microsatellite markers. *Proceedings of the 8th World Rabbit Congress, September 7-10, 2004,* World Rabbit Science Association, Mexico, pp. 181-185

Zijlstra, C., de Haan, N.A., Korstanje, K., Rogel- Gaillard, C., Piumi, F., van Lith, H.A., van Zutphen, L.F. and Bosma, A.A. 2002. Fourteen chromosomal localizations and an update of the cytogenetic map of the rabbit. *Cytogenet. Genome Res.* 97: 191-199

Annexures

ANNEXURE – 1

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

Acrylamide (40 %)

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

Agarose (1 %)

Weighed 0.6 g of agarose powder and mixed with 60 ml of 1 X TAE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

Ammonium persulphate (10 %)

Ammonium persulphate	100 mg
Water to	1 ml

Denaturing Polyacrylamide Gel

0.5 X TBE Gelmix	60 ml
TEMED	0.125 ml
Ammonium persulphate (10 %)	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 upto 100 ml. Stored at room temperature after filtration and autoclaving.

Ethidium Bromide (10 mg/ml)

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

Formamide dye / Stop buffer

Deionised formamide	98 %
Xylene cyanol	0.025 %
Bromophenol blue	0.025 %
0.5 *M* EDTA	10m*M*

Gel loading buffer

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

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

Phenol (Saturated, pH 7.8)

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

RBC lysis buffer

Ammonium chloride	150 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

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

Sodium chloride (5 M)

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

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

Sodium chloride	75 mM	4.383 g
EDTA	35 mM	9.306 g

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

Sodium dodecyl sulphate (SDS) 20 %

SDS	20 g
Distilled water make up to	100 ml

Stirred, filtered and stored at room temperature.

Tris Acetate EDTA (TAE) buffer (50X)

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

Autoclaved and stored at room temperature.

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

Tris base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g

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

TBE Gel mix (0.5 X)

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

Tris EDTA (TE) buffer (pH 8.0)

Tris base	10 mM	1.2114 g
EDTA	0.1 mM	0.3722 g

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

Tris 1M (pH 8.0)

Tris base	121.14 g

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

ANNEXURE – II

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

(A) CHEMICALS

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	-	Merck
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
RAPD	Random Amplified Polymorphic DNA
DNA	Deoxy Nucleic Acid
PAGE	Polyacrylamide Gel Electrophoresis
PIC	Polymorphic Information Content
EDTA	Ethylene Diamine Tetraacetic Acid
DTT	Dithiothreitol
TEMED	N, N, N′, N′ Tetramethylethylenediamine
APS	Ammonium Persulphate
PNK	Polynucleotide Kinase
He	Heterozygosity
µl	microlitres
µg	microgram
mg	milligram
mM.	millimolar
cm	centimeter
nm	nanometer
mCi	millicurie
Kb	Kilo basepair
Rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
dNTP.	Deoxy Nucleotide Triphosphate

ddATP.	Dideoxy Adenosine Triphosphate
ddCTP	Dideoxy Cytosine Triphosphate.
ddGTP.	Dideoxy Guanosine Triphosphate
ddTTP.	Dideoxy Thymidine Triphosphate

GENETIC DIVERGENCE IN RABBITS USED FOR BREEDING IN KERALA

NISHA VALSAN

Abstract of the 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

2007

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

ABSTRACT

The genetic divergence among three breeds of rabbit, viz. Newzealand White, Soviet Chinchilla and Grey Giant was studied using microsatellite markers.

A set of twelve microsatellite markers were tested, out of which three markers (Sol 03, Sol 33 and Sol 44) were selected based on their polymorphism. The PCR products were separated by denaturing polyacrylamide gel electrophoresis and autoradiographed.

The Sol 03 locus was found to be the most polymorphic with fourteen alleles in the pooled population. The values for heterozygosity and PIC in Newzealand White at the Sol 03 locus were recorded as 0.840 and 0.836, in Soviet Chinchilla as 0.766 and 0.764, while in Grey Giant, the heterozygosity and PIC values stood at 0.775 and 0.765, respectively.

Eight alleles were detected at the Sol 33 locus. The maximum values for heterozygosity (0.858) and PIC (0.854) were observed in Grey Giant while Newzealand White (0.672 and 0.667, respectively) recorded the lowest. In Soviet Chinchilla, values for heterozygosity and PIC were 0.691 and 0.680 respectively. with mean heterozygosity and PIC values of 0.740 and 0.764.

Sol 44 locus revealed four alleles. The highest values for heterozygosity (0.728) and PIC (0.702) at the Sol 44 locus were recorded in Grey Giant, while the lowest (0.567 and 0.477) in Soviet Chinchilla. The heterozygosity and PIC values were 0.586 and 0.502, respectively in Newzealand White.

The genetic distance was calculated based on Nei's formula, and the highest value was noticed between Soviet Chinchilla and Grey Giant (0.6942) while the lowest between Newzealand White and Soviet Chinchilla (0.2022).

The dendrogram constructed using POPGENE program grouped Newzealand White and Soviet Chinchilla in one cluster indicating their closer relationship. Grey Giant was found to be the most widely separated breed.