MITOGENOME ANALYSIS OF CATTLE USING HIGH DENSITY SNP ARRAY

THESIS SUBMITTED TO THE

ICAR - NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE

IN

ANIMAL GENETICS AND BREEDING

BY

PATEL HEMALKUMAR BALUBHAI

B.V.Sc & A.H

ANIMAL GENETICS AND BREEDING DIVISION

ICAR- NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

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Approved by:

( )
EXTERNAL EXAMINER

Dr. S. P. DIXIT 23/6/18
MAJOR ADVISOR
Principal Scientist
ICAR-NBAGR

Members of Advisory Committee

1. **Dr. INDRAJIT GANGULY**
   Senior Scientist, ICAR-NBAGR
2. **Dr. SANJEEV SINGH**
   Senior Scientist, ICAR-NBAGR
3. **Dr. MUKESH BHAKAT**
   Senior Scientist, LPM Division, ICAR-NDRI
4. **Dr. S. DE**
   Principal Scientist, ABTC Division, ICAR-NDRI

ANIMAL GENETICS AND BREEDING DIVISION
ICAR - National Dairy Research Institute
(Deemed University)
Karnal –132001, Haryana, India

Dr. S. P. DIXIT
Principal Scientist,
ICAR-NBAGR.

CERTIFICATE

This is to certify that the thesis entitled, **"MITOGENOME ANALYSIS OF CATTLE USING HIGH DENSITY SNP ARRAY"** submitted by **Dr. PATEL HEMALKUMAR BALUBHAI** in partial fulfilment of the requirement for award of the degree of **MASTER OF VETERINARY SCIENCE IN ANIMAL GENETICS AND BREEDING DIVISION** of the **ICAR - National Dairy Research Institute (Deemed University), Karnal,** Haryana, India, is a bonafide research work carried out under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 27/7/18

(Dr. S. P. DIXIT)
Major Advisor

Dedicate

To

Nature, Guruji

&

N.A.U.

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"-The single greatest cause of happiness is gratitude"

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Place: Karnal

Date: *(Patel Hemal Kumar)*

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LIST OF ABBREVIATIONS

AMOVA	:	Analysis of Molecular Variance
AnGR	:	Animal Genetic Resources
ATP	:	Adenosine triphosphate
bp	:	Base Pair
CR	:	Control Region
DBT	:	Department of Biotechnology
df	:	Degree of Freedom
D-loop	:	Displacement loop
DNA.	:	Deoxyribonucleic Acid
et al.	:	Co-workers
Fig.	:	Figure
F_{st}	:	Fixation Index
GnRH	:	Gonadotropin-releasing hormone
HWE	:	Hardy–Weinberg equilibrium
MAF	:	Minor Allele Frequency
MDS	:	Multidimensional Scaling
MEGA	:	Molecular Evolutionary Genetics Analysis
mtDNA	:	Mitochondrial Deoxy Ribonucleic Acid
NCBI	:	National Centre for Biological Information
NJ Network	:	Neighbour Joining Network
PCR	:	Polymerase Chain Reaction
QTL	:	Quantitative trait loci
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribo Nucleic Acid
SNP	:	Single Nucleotide Polymorphism

ABSTRACT

Indian cattle (*Bos indicus*) play a significant role in the economy of small and marginal farmers, as they are a potential source of milk, draught power and manure. Assessment of population structure and genetic diversity is a basic tool for genetic improvement. Current study was conducted to study genetic diversity and functionality of mitogenome in different variants. Diversity was observed on the basis of mitogenome of 132 Indian cattle, breeds covering differen geographical area and different utility milch (Sahiwal, Tharparkar, Gir), Dual (Ongole, Hariana) Draft (Kangayam) and miniature (Vechur) purposes. Analyses revealed 81 haplotype with the haploypic diversity range from 0.9333 ± 0.0477 to 0.9883 ± 0.0210, Nucleotide diversity was ranging from 0 to 0.0862+/- 0.0446. AMOVA shows 2.61% variation among population and 97.39% variation within population. F_{ST} value is significantly different from 0 for all pair wise combinations representing significant amount of Genetic differentiation between populations. F_{ST} estimates between the population shows that Sahiwal and Vechur are more genetically closer (-0.00477) while animals from Gir and Kangayam are genetically more differentiated (0.06601). Multi Dimention Scalling indicate milch breeds are in same lineage as compare to dual and draft. The analysis was also carried out to assess the functionality of mtDNA variants. Result revealed that ND1, ND2, ND3, ND5, COX1, COX2, CYTB, ATP6 are top genes which are affect population. These genes are take participate in Biological process (cellular process, immune system, metabolic process), molecular function (catalytic activity, transport activity) and in pathways(ATP synthesis, CCKR signaling, GnRH receptor, Inflammation mediation, Toll receptor signaling). All the above results indicated higher haplotype diversity of mitogenome within breeds (~93 % and above), The milch breeds shared the common ancestry and was different from the dual and draft breeds, The functional diversity, based on top loci with higher F_{st} values, was restricted to seven genes involved mainly in Transporter activity, Immunity, metbolism and ATP synthesis. In future, the identified functional variants may be studied in detail for their utilization in the breeding program

सार

भारतीय पशुओं (बोस इंडिकस) ने विशेष रूप से विकासशील देशों में छोटे और सीमांत किसानों की अर्थव्यवस्था में एक महत्वपूर्ण भूमिका निभाई है, जैसा कि वे दूध, सूखे बिजली और खाद का एक संभावित स्रोत हैं। जनसंख्या संरचना और आनुवंशिक विविधता का आकलन आनुवंशिक सुधार के लिए एक बुनियादी उपकरण है। वर्तमान अध्ययन अलग-अलग रूपों में आनुवंशिक विविधता और माइटोकॉन्ड्रिया के जीन की कार्यक्षमता का अध्ययन करने के लिएआयोजित की गयी। 132 भारतीय पशुओं (सहिवाल, थारपारकर, गिर, ऑंगल, हरियाणा, कंगायम, वेचुर) की नस्लों, विभिन्न भौगोलिक क्षेत्र और विभिन्न उपयोगिता के आधार पर विभिन्नता देखी गई। विश्लेषण से पता चला कि 81 हेप्लोटाइप की विभिन्नता 0.988 ± 0.021 से 0.9346 ± 0.040 है, न्यूक्लियोटाइड विविधता को 0 से 0.0862 ± 0.0446 के बीच गया था। AMOVA जनसंख्या के साथ 2.61% और जनसंख्या के बीच मे 97.39% विभिन्नता दिखाता है। F_{st} मूल्य शून्य से भिन्न पाया गया जो दर्शाता हैं कि आबादी के बीच एक महत्वपूर्ण अनुवांशिक विभेदन हैं। आबादी के बीच F_{st} आंकलन दर्शाता है की साहीवाल और वेंचर के बीच मे करीबी आनुवंशिकता (-0.००४७७)है, जबकि गिर और कंगायम आनुवंशिक रूप से अधिक भिन्न (0.०६६०१) हैं। बहु परिमाण पैमाने बताते हैं कि ड्यूल और ड्राफ्ट नस्ल की तुलना में मिल्च नस्ल एक अलग वंश की नस्ल हैं। माइटोकांड्रियल डीएनए की कार्यकी के लिए भी एक समीक्षा की गई। परिणाम दर्शातें हैं कि ND1, ND2, ND3, ND5 COX1, COX2 CYTB ATP6 जीन सर्वोच्च हैं और वे आबादी को प्रभावित करते हैं। ये सभी जीन जैविक क्रियाएँ (कोशकीय क्रियाकलापों, रक्षा तंत्र, उपापचयी क्रियाएँ) , आणविक कार्य (उत्प्रेरक एवं परिवहन क्रियाएँ) और पथिक क्रियाएँ (ATP उत्पादन, CCKR संकेत, GnRH ग्राहक, सूजन माध्यम, TLR संकेत) में सम्मिलित होते होती हैं। ऊपरी सभी परिणाम, नस्लों के बीच मे माईटो जीनोम की 93% अधिक हेप्लाटिपिक विविधता दर्शाते हैं। मिल्च नस्ल सामान्य वंशावली साझा करते हैं तथा ड्यूल और ड्राफ्ट नस्ल भिन्न हैं। F_{st} मूल्य की सर्वोच्च लोकाई के आधार पर कार्यात्मक विविधता सात जीन जो उपापचयी क्रियाओं, परिवहन क्रियाओं, ATP उत्पादन और रक्षात्मक क्रियाओं में सम्मिलित है, तक प्रतिबंधित है। भविष्य में पहचानी हुई क्रियात्मक भिन्नताएँ विस्तार से पढ़ी जा सकेंगी।

CHAPTER –1

INTRODUCTION

INTRODUCTION

Indian cattle (*Bos indicus*) play a significant role in the economy of small and marginal farmers, as they are a potential source of milk, drought power and manure. India has a wide range of climates, mainly affected by different altitudes. Following the variations in agro-ecological zones and climates, investigation has been done on the morphological characteristics of Indian cattle breeds and also on the genetic relationship between native cattle breeds based on blood protein typing and karyotyping, which revealed a relatively high genetic variability within the breeds. Nevertheless, genetic studies have indicated that morphological characteristics of livestock breeds may provide incomplete or misleading information on their evolutionary history.

Recent advancements in molecular biology have been developed to study the genetic variations of population in humans as well as livestock using DNA markers. However in developing countries generally the adoption of livestock technologies has been low due to the environment and poor resource base of farmers (Iniguez, 2011). Various technologies to study genetic diversity include PCR-RFLP, mini-satellites, micro-satellites, mitochondrial DNA analysis and SNP chips. mtDNA is only a small portion of the DNA in eukaryotic cell as most of DNA can be found in the nucleus. The mtDNA has proved to be valuable in the study of genetic diversity as it shows maternal inheritance and changes rapidly than single copy nuclear DNA in mammals(Brown *et al*., 1979).

Mitochondria are the principle energy source in all cells of eukaryotes. Animal mitochondrial DNA is small , extrachromosomal genome and self replicating. The copy number of bovine mt DNA molecules, ranges from 220 to 1720 per cell, and has applicability even in severely decomposed sample in which nuclear DNA has been already degraded (Xu *et al*., 2006). The animal mitochondrial DNA is circular molecule of 15-20kb in length. Size of mitogenome in *B. taurus* and *B. indicus* is 16,338 and 16,339 bp, respectively, and differs at 237 positions. With few exceptions all mitochondrial genomes contain the same 37 genes, all of which are involved in the production of energy and its storage in ATP. 22 genes among these are encoded by transfer RNAs. Remaining genes encode proteins (13 genes) involve in electron

transport and oxidative phosphorylation and ribosome RNA (2 genes) that translate the protein genes within the mitochondria. The only noncoding area of the mtDNA is the control region typically 1kb, involved in the regulation and initiation of mtDNA replication and transcription, In fact each mitochondrion has several copies of its own genome, and there are several hundred to several thousand mitochondria per cell. Mitochondrial DNA is highly polymorphic due to high rate of evolution (5-10 times of nuclear DNA) (Irwin *et al*., 1991). The mitochondrial chromosome displays exclusively maternal inheritance (Wallace, 1993). Mitogenome consist of 1) control region, 2)D-loop, 3) hypervariable region. Replication of mtDNA can occure in two different ways, both starting in the D-loop region.One way continues replication of the heavy strand through a substantial part of the circular molecule, and then replication of the light strand begins. Some study mode starts at a different origin within the D-loop region and uses coupled-strand replication with simultaneous synthesis of both strands, control region refers to the fact that this region contains the signals that control RNA and DNA synthesis. The polymorphism is mostly concentrated in hyper variable region of the D-loop. This region has a rate of nucleotide substitution 5-10 times higher than the nuclear DNA (Brown *et al*., 1979).So mtDNA can estimate relationship between both closely related and distantly related breed population. It permits an evaluation of relatedness among individuals in the population. This is an ideal tool for studying genetic diversity and population structure, because it has unique features of maternal inheritance, has a relatively fast rate of evolution and lack recombination. To describe the population genetic diversity and structure within and between the populations, there is need to investigate the patterns of mitochondrial D-loop sequence variation.

It is important to generate the information on genetic diversity and composition of population for adopting the correct methodology for genetic improvement of the breed. The different breeds in the adoptive herd at various part of the country. Thus, before the application of breeding strategies and planning for the genetic improvement and conservation of germplasm it is mandatory to assess the genetic diversity and population structure of the individuals comprising the herd. So preliminary study on genetic diversity and structure of different Indian breed is required to generate baseline information about important economic QTL traits.

Hence the present investigation will be undertaken with the following objectives:

(1) To analyse mitochondrial diversity and Lineage of diverse cattle breeds.

(2) To assess the functionality of the unique mtDNA variants among the milch, dual and draft purpose cattle breeds.

CHAPTER –2

REVIEW OF LITERATURE

2.1 MITOCHINDRIAL GENOME

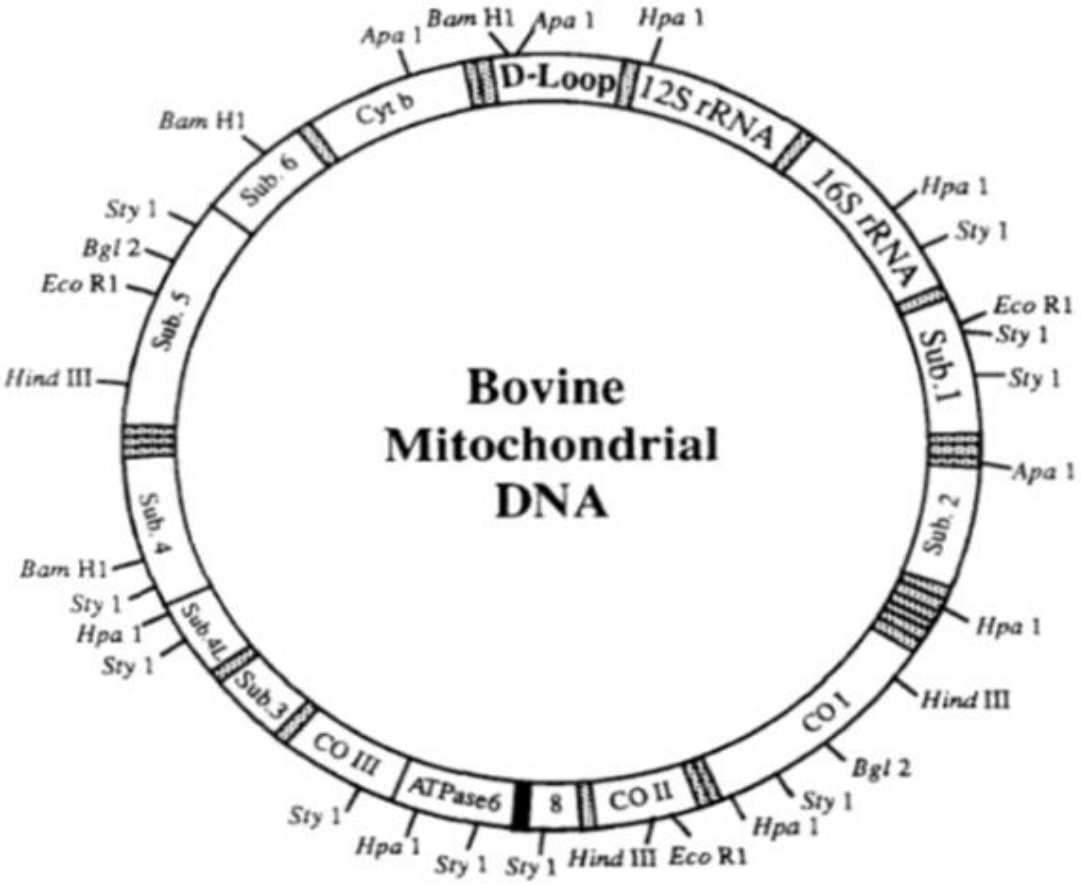

Figure 2.1: Structure of mtDNA

Mitochondrial DNA is only a small portion of the DNA in eukaryotic cell as most of DNA can be found in the nucleus. Anderson *et al.*, (1982) conducted study on complete mitochondrial genome of *Bos taurus* and *Bos Indicus* encompassing 16,338 and 16,339 nucleotides, respectively, and differed at 237 positions. It has a double linked annular structure which is covalent and close. The non-coding region is the control region. (Wang *et al.*, 2009) Its genes do not usually recombine (Upholt and Dawid., 1977).

Table. 2.1: Revolutionary work in complete Mitogenome of domestic animals

Sr. No.	Name Of Species	Complete Sequence (bp)	Scientists Involved In work
1	Human	16569	Anderson *et al.,* 1981
2	Mouse	16295	Bibb *et al.,* 1981
3	Cattle	16338	Anderson *et al.,* 1982
4	Chicken	16775	Desjardins *et al.,* 1990
5	Cat	17009	Lopez *et al.,* 1996
6	Sheep	16616	Hiendleder *et al.,* 1998
7	Dog	16782	Kim *et al.,* 1998
8	Pig	16613	Lin *et al.,* 1999
9	Japanese Quail	16697	Nishibori *et al.,* 2001
10	Goat	16640	Pietro *et al.,* 2003
11	Buffalo	16355	Parma *et al.,* 2004

Mitochondrion contains several copies (200 to 1720) of its own genome and is unique as it has remained autonomous outside the nuclear genome. The animal mitochondrial DNA is circular in nature with a length of 15-20 Kb containing around 37 genes all of which are mostly involved in production of energy. Out of above 37 genes 13 genes encode proteins which are involved in oxidative phosphorylation and electron transport chain. The other 22 genes encode for tRNA and two other genes for rRNA. mtDNA has a rate of nucleotide substitution 5-10 times higher than the nuclear DNA (Brown *et al.,* 1979) Mitochondrial DNA can estimate relationship between both closely related and distantly related breed population. This is an ideal tool for studying genetic diversity and population structure, because it has unique features of maternal inheritance, has a relatively fast rate of evolution and lack recombination.

2.2 DIVERSITY STUDIES IN CATTLE

The analysis of mitochondrial DNA (mtDNA) sequence variation has been widely used to study the origins of cattle and inter breed relationships (Bruford *et al.,* 2003), to see the diversification of modern cattle populations (Loftus *et al.,* 1994) and to

ascertain domestication and genetic background of cattle (Grigson, 1980; Loftus *et al.*, 1994).

Lai *et al.* (2006) determined the origin and genetic diversity of Chinese cattle by analysing the complete mtDNA D-loop sequences of 84 cattle from 14 breeds of southeast and west China along with the available cattle sequences. The results showed that the Chinese cattle samples converged into two main groups, which corresponding to *Bos taurus* and *Bos indicus*. Evidence for two independent domestications of cattle was also investigated by D G Bradley *et al.*, (1996); in which origin and taxonomic status of domesticated cattle were controversial. Zebu and taurine breeds are differentiated primarily by the presence or absence of a hump and have been recognised as separate species *(Bos indicus* and *Bos taurus).*However the most widely held view is that both types of cattle derive from a single domestication event 8000-10000 years ago.

Mitochondrial DNA sequences have been examined from representatives of six European (taurine) breeds, three Indian (Zebu) breeds, and four African (three zebus, one taurine) breeds. However, the sequences fell into two very distinct geographical lineages that do not correspond with the taurine-zebu dichotomy. All European and African breeds were in one lineage, and all Indian breeds in other. There was little indication of breeds clustering within either lineage. Application of a molecular clock suggests that the two major mtDNA divergence atleast 200,000 years ago. This relatively large divergence is interpreted most simply as evidence for two separate domestications events; presumably of different subspecies like *Bos primigenius*. The clustering of all African Zebu mtDNA sequences within the taurine lineage is attributed to ancestral crossbreeding with the earlier *Bos taurus* inhabitants of the continent.

Study about the molecular evolutionary relationship between the mtDNA of cattle, yak species and other species based on the substitution rates specific for each mammalian mtDNA functional component (Pesole *et al.,* 1999), the time since divergence was found to be around 4.38-5.32million years between cattle/Zebu and yak.

The investigation of the sequence data of mitochondrial DNA control region revealed that taurine and zebu cattle diverged 200,000-1,000,000 years before (Loftus *et al.*,1994). Mammalian mt-DNA shows several special features such as absence of introns,maternal inheritance, the existence of single copy orthologous genes, lack of recombination events and high mutation rate (Irwin *et al.*,1991;Pesole *et al.*,1999).

Sequence comparisons of mtDNA have been widely used to evaluate genetic diversity and phylogeny performance among individuals and populations of cattle (Loftus *et al.*,1994; Bradley *et al.*,1996)

Lie *et al.* (2006), worked on complete mitochondrial D-loop sequences (910 bp), in 82 cattle from 4 breeds in Guizhou province. The results revealed 31 mitochondrial haplotypes, 65 polymorphic sites, covering 7.14% of the entire length of the sequence. The nucleotide diversity and haplotype diversity estimated from mtDNA D-loop region in 4 cattle breeds in Guizhou varied from 2.16% to 2.61% and 0.695 to 0.909 respectively showing abundant mitochondrial genetic diversity exists in Guizhou cattle breeds.

Wang *et al.* (2009) studied Chinese Leiqiong cattle and analysed complete mtDNA cyt b genes and three haplotypes of 18 individuals were identified from 2 polymorphic sites with length of 1140bp.The average haplotype diversity and nucleotide diversity were 0.0741 and 0.0012 showing less genetic diversity in leiqiong cattle. A neighbour joining tree was constructed and revealed that leiqiong cattle only originated from *Bos indicus* and had no direct relationship with *Bos taurus,Bos grunniens* and *Bos javanicus* cattle.

Cai *et al.* (2007) tried to clarify the genetic diversity of indigenous cattle breeds of china, they carried out phylogenetic analysis of representatives of those breeds by determining mitochondrial gene polymorphism.Cyt b gene sequences (1140 bp) were determined for a total of 36 individuals from 18 different breeds and these sequences were clustered into two distinct genetic lineages of taurine and zebu. Analysis of polymorphism showed declining south to north gradient of female zebu introgression and geographical hybrid zone of *Bos taurus* and *Bos indicus* in china.

Shangang *et al*. (2007) studied complete mtDNA D-loop region from 123 cattle of 12 Chinese breeds and two individuals of Germany yellow cattle breed. The 13 cattle breeds were divided into two main groups-*Bos taurus* and *Bos indicus*.Apei-jiaza cattle breed of Tibet, which was similar to that of yak was at a higher level than other cattle breeds providing enough evidence of introgression of genes from yak.

Genetic diversity of 277 nucleotides in the mitochondrial DNA control region of crossbreed beef cattle as well as in Nellore samples (*Bos indicus*) were studied by Henkes *et al.,(*2005).more than fifty mutations were found in Brangus-Ibage comprising

18 haplotypes and more than sixty nucleotide changes in Nellore. The data indicated sequence identities of 99.6 and 92.1% between the *Bos taurus* reference sequence and Brangus-Ibage and Nellore respectively. The comparison of data with sequence data for 612 individuals recovered from GenBank showed a total of 205 haplotypes defined by 99 polymorphic sites. Most of the variability was due to differentiation within breeds.

The studies of Mannen *et al*.(1998) on complete mtDNA D-loop sequences from 32 Japanese black cattle and the analysis of these data in conjugation with previously published sequences from African, European and Indian Subjects on the origins and diversity of North east Asian domesticated cattle was unclear. The earliest domesticated cattle in the region were *Bos taurus* and may be domesticated from local cattle breeds in the wild. In phylogenetic analysis taurine sequences form a dense tree with a centre consisting of intermingled European and Japanese sequences with one group of Japanese and another of all African sequences, each forming distinct clusters at extremes of phylogeny. This topology and calibrated level of sequences, each forming distinct clusters at extremes of phylogeny, suggests that the clusters may represent three different strains of ancestral aurochs, adopted at geographically and temporarily separate stages of domestication process.

Sharma *et al.,* (2015) analyzed 170 mitochondrial D-loop sequence from 11 Indain cattle breeds. The result revealed 60 haplotype found with average haplotypic diversity of 0.9024 and average nucleotide diversity was 0.02688.Two major cluster of haplotype were found. Result shows that south Indian breed Ongole was distinct from north/central Indian breeds.

Correia *et al.,* (2017) analyzed total 40 samples to check mitochondrial DNA D-loop (521-bp) based diversity of four breed of main Portuguese Lidia bovine populations and clarify their genetic relationships with Spanish Lidia lineages. The mtDNA diversity recorded was similar to that observed in Lidia cattle. Haplotype T3 was the most common (62.5%), followed by the African T1 haplotype (25%); very low frequencies were recorded for haplotypes T2 (2.5%) The results support the existence of two major ancestral lines for the Lidia breed: European and African, similar to other Mediterranean breeds.

Rong Li *et al.,* (2018) analyzed a 910-bp fragment of mitochondrial DNA (mtDNA) D-loop region sequences of 280 individuals from 6 Yunnan native cattle

breeds, of which 251 sequences were newly determined. There were 93 variable sites that defined 117 haplotypes among all sequences. Phylogenetic analysis of all haplotypes revealed that Yunnan native cattle had two distinct mtDNA lineages - taurine and zebu. The taurine sequences fell into four haplogroups T1a, T2, T3 and T4, whereas the zebu sequences grouped into two haplogroups I1 and I2. results revealed patterns of gradient changes in frequencies of taurine and zebu mtDNA lineages across different geographic regions of Yunnan.

Bhuiyan *et al.,* (2007) analyzed mtDNA displacement loop (D-loop) sequences of 48 samples along with 22 previously published sequences from Bos indicus and Bos taurus breeds. 25 haplotypes were identified in Red Chittagong cattle that were defined by 44 polymorphic sites and nucleotide diversity was 0.0055±0.0026. The phylogenetic studies showed Red chittahong cattle clustered with Bos indicus lineage with two distinct haplogroups representing high genetic variability of this breed.

2.3 DIVERSITY STUDIES IN OTHER DOMESTIC ANIMALS

Hoda *et al.,* (2014) analyzed 77 mtDNA D-loop sequences from six different Albanian goat breeds. The result revealed 67 different haplotypes, with haplotype diversity ranging from 0.864 to 1 and nucleotide diversity values ranging from 0.016 to 0.106 and analysis indicated that 98.7% of the variation was found within the goat breeds and only 1.3% among them.

Silva *et al.,* 2017 conducted study aimed at genetic characterisation of 5 major goat populations of Srilanka including four indigenous populations and one stabilized crossbred. Genetic diversity was evaluated using microsatellite markers of mtDNA D-loop variation. The allelic diversity and observed and expected Heterozygosity were moderate but less than Eurasian and Indian goat breeds.

Naqvi *et al., 2*017 undertook study to analyze the genetic diversity of 5 economically important goat breeds of Pakistan. The estimated inbreeding coefficient was low. Overall the population was less diverse than Eurasian goat breeds but didn't exhibit loss of diversity. The mitochondrial DNA control region sequences showed a total of 60 distinct haplotypes belonging to two major maternal lineages A and B1 with frequency of 76.9% and 23.1% respectively.

Ming *et al.,* 2017 conducted study on camel population of 113 individuals representing 11 domestic breeds by examining 809 bp MtDNA and found 15 different

haplotypes and the phylogenetic analysis suggests domestic and wild Bactrian camels have two distinct lineages and the analysis of domestic Bactrian camels from different geographical locations had no significant genetic divergence in China, Russia and Mongolia

2.4 LIMITATIONS OF MITOCHONDRIAL DNA (MTDNA) MARKER IN LIVESTOCK DIVERSITY

Mitochondrial DNA has been used as a molecular clock, as it can be used for estimation of time of origin of breed, its divergence and phylogeography. It is mostly because of accumulation of neutral mutations occurring at approximately constant evolutionary rate within the mtDNA. The mtDNA divergence level therefore roughly reflects the divergence in the population(Howell *et al.,* 2003;Galtier *et al.,* 2009). mtDNA marker has been widely utilized in population studies particularly based on maternal lineages (Grechko,2002;Kim *et al.,* 2003) among the breeds of several species like buffalo (Kumar *et al.,* 2007), goat (Joshi *et al*,2004), pig (Larson *et al.,* 2005), horse (Cozzi *et al.,* 2004) and cattle (Bradley *et al.,* 1996; Cai *et al.,* 2007).

Although mtDNA is widely used for diversity and phylogeographic studies, in recent years issues arise due to difference of mitochondria and nuclear genome at ploidy level, mode of inheritance, degree of recombination, effective population size, mutation rate, repair mechanisms, etc (Scheffler,1999).It is also concluded by certain workers that inferring about biology of whole organism from a small fraction of the genome, such as mitochondria, is inappropriate as this single molecule alone may not always be sufficient to infer about the entire organism (William *et al.,* 2004).Further, allozymes were replaced by mtDNA in population history and diversity studies with the hypothesis that mtDNA diversity would reflect effective population size more accurately than allozymes (Foltz,2003). Bazin *et al.,*2006 found that diversity of a given species does not reflect its average population size. Furthermore, mtDNA is unable to detect the male mediated gene flow during evolution or during diversity analysis. So for an overall diversity analysis, the approach of mtDNA need to be supplemented by other markers such as autosomal microsatellite markers and Y-chromosome DNA based information.

2.5 ROLE OF MITOCHONDRIAL DNA IN PERFORMANCE TRAITS:

There is abundant evidence for a high degree of polymorphism in mtDNA from both RFLP studies (Watanabe *et al.,* 1985; Brown *et al.,* 1989; Koehler *et al.,* 1991;

Ron *et al.,* 1993) and nucleotide sequence analysis (Ron *et al.,* 1993). In livestock species, mtDNA variability has been studied in connection with maternally inherited physiological parameters and it has been suggested that bovine mtDNA may affect milk production as well as some other productive traits (Schutz *et al.,* 1992; Schutz *et al.,* 1994; Mannen *et al.,* 2003; Henkes *et al.,* 2004,).Schutz *et al.*(1994) reported the association of mitochondrial DNA with milk production and reproduction traits in Holstein cow. Ribeiro *et al.* (2009) found out that cytoplasmic lineages accounted for 1.6%, 1.5% and 1.2% of the total phenotypic variance of milk production, 305 day milk yield and lactation length. It was also found that the mtDNA variability was also significant for age at 1st calving and was necessary in the formation of contemporary groups for this feature in Brazilian Gir cattle. The significant association between single nucleotide polymorphisms (SNPs) of ATPase 8/6 genes and milk production traits in Chinese Holstein cows was reported. This provides more genetic evidences accounting for the cytoplasmic contribution to production performance in farm animals (Qin *et al.,* 2012). In farm animals, the cytoplasmic effect was also suggested to account for a significant source of phenotypic variation for production traits and the direct association between mtDNA polymorphisms and production traits was also reported in cattle pig and other species(Sutarna *et al.,* 2002; Mannen *et al.,* 2003; Zhang *et al.,* 2008).

CHAPTER –3

MATERIALS & METHOD

MATERIAL AND METHODS

The data used for the present study was generated under the project titled sponsored by Department of Biotechnology (DBT).

3.1 Experimental animals/ Resource Populations

Total 132 individuals DNA samples of seven diverse Indian native cattle breeds (Gir, Sahiwal, Tharparkar, Vechur, Hariana, Kangayam and Ongole) distributed over various geographical regions as well as covering different utility purposes (Milch/Dual/Draught/) were included in the present study.

Table 3.1 : Experimental animals

BREED	No. of samples	Utility	Home track
Sahiwal	19	Milch	Punjab and Rajasthan
Tharparkar	17	Milch	Rajasthan
Gir	16	Milch	Gujrat
Ongole	24	Dual	Andhra Pradesh
Hariana	18	Dual	Haryana, Uttar Pradesh and Rajasthan
Kangayam	18	Draft	Tamil Nadu
Vechur	20	Miniature	Kerala
Total	132		

3.2 Mitochondrial DNA isolation

Mitochondrial DNA was isolated from whole blood using commercially available DNA isolation kit, following the prescribed protocol as per the manufacturer's instructions.

3.3 SNP Genotyping

The isolated DNA then was outsourced for genotyping of the mitochondrial genome using Illumina Bovine HD chip by SANDOOR LIFE SCIENCES Pvt Ltd, Hyderabad.

3.4 Data analysis

3.4.1 Quality Control

The SNP genotype data were analyzed by using Genome Studio software (Illumina). The data were filtered by using different parameters like SNP call rate (≤ 95%), and minor allele frequency (≤ 0.05) using PLINK version 1.07 (Purcell *et al*., 2007).

3.4.2 Analysis of genetic diversity

After pruning the data using above mentioned quality control parameters, various software's were used for SNP data analysis as per the need. GenAIEx version 6.503 (Peakall *et al*., 2006) was used to find out the different haplotypes and their frequencies in different populations of the cattle under study. Arlequin version 3.5.2 (Excoffier *et al*., 2010) was used to estimate haplotype diversity (XD), Nucleotide Diversity (Pi) and pairwise differences (K value) between all the possible sets for each population.

3.4.3 AMOVA Analysis

Along with the frequency of molecular markers even the mutational differences between different genes can be obtained for molecular data. Analysis of Molecular Variance (AMOVA) which estimates the population differentiation directly from molecular data is more useful than using just Mendelian frequencies. Analysis of Molecular Variance (AMOVA) was carried out using programme Arlequin version 3.5.2 (Excoffier, 2010). AMOVA was utilized further to quantify the extent of population differentiation and the distribution of genetic variation in the sample population.

3.4.4 GLOBAL FIXATION INDEX (F_{ST})

The fixation is a measure of population differentiation due to genetic structure. It is frequently estimated from genetic polymorphism data such as single nucleotide polymorphism (SNP). F_{st} at a given locus is based on the variance of allele frequencies between populations and on the probability of identical by descent. The values range from 0 to 1. A zero value implies a complete pan-mixia that is two populations are interbreeding freely. A value of one implies that all genetic variation is explained and the

two populations do not share any genetic diversity. F_{st} in the present study was calculated by using Arlequin version 3.5.2 (Excoffier, 2010). Based on population pairwise F_{st} is used to construct multi dimension scaling (MDS) with the help of Software SPSS 16.0

3.5 Assessing the functionality of Mitogenomic Variants

3.5.1 Identification of Genes

The aim of study was to assess the functionality of identified variant. Top 20 markers with higher F_{st} values were selected and their positions on mitogenome were ascertained using the bovine HD chip SNP marker ID. Further the position of the SNP marker was checked for the presence of gene using the NCBI genome data viewer online tool.

3.5.2 Checking functionality of genes

After obtaining the list of genes showing the higher F_{st} values, they were subjected to different types of analyses viz. functional analysis, molecular analysis and pathway analysis using the PANTHER web based software to ascertain the functionality of the called variants**.**

CHAPTER – 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Total, 132 samples from 7 Indian cattle breeds covering different utility and geographic area were included in the study, With the aim to screen the haplogroup, haplotypes and thus the genetic diversity of different cattle breeds of India. The results obtained during the course of present study are presented with discussion under various heads.

4.1 *Quality Control*

Samples that had more than 10% missing genotypes were excluded. SNPs with call rate (CR) (≤ 95%), minor allele frequency (MAF) (≤ 0.05), and HWE (P ≤ 0.001) were also excluded. After these all filtration a total of 343 SNP were left.

4.2 Allelic Patterns across Populations

Table no. 4.1 Allelic Patterns across population

Breed	Na	Ne	I	h	uh
Hariana	12	9.529	2.370	0.895	0.948
Ongole	18	14.400	2.788	0.931	0.971
Kangayam	12	8.526	2.322	0.883	0.935
Tharparkar	15	12.565	2.639	0.920	0.978
Sahiwal	17	15.696	2.799	0.936	0.988
Vechur	16	14.286	2.718	0.930	0.979
Gir	11	8.00	2.253	0.875	0.933

Where,

Na = No. of Different Alleles

Ne = No. of Effective Alleles

I = Shannon's Information Index

h=Diversity

uh= Unbiased Diversity

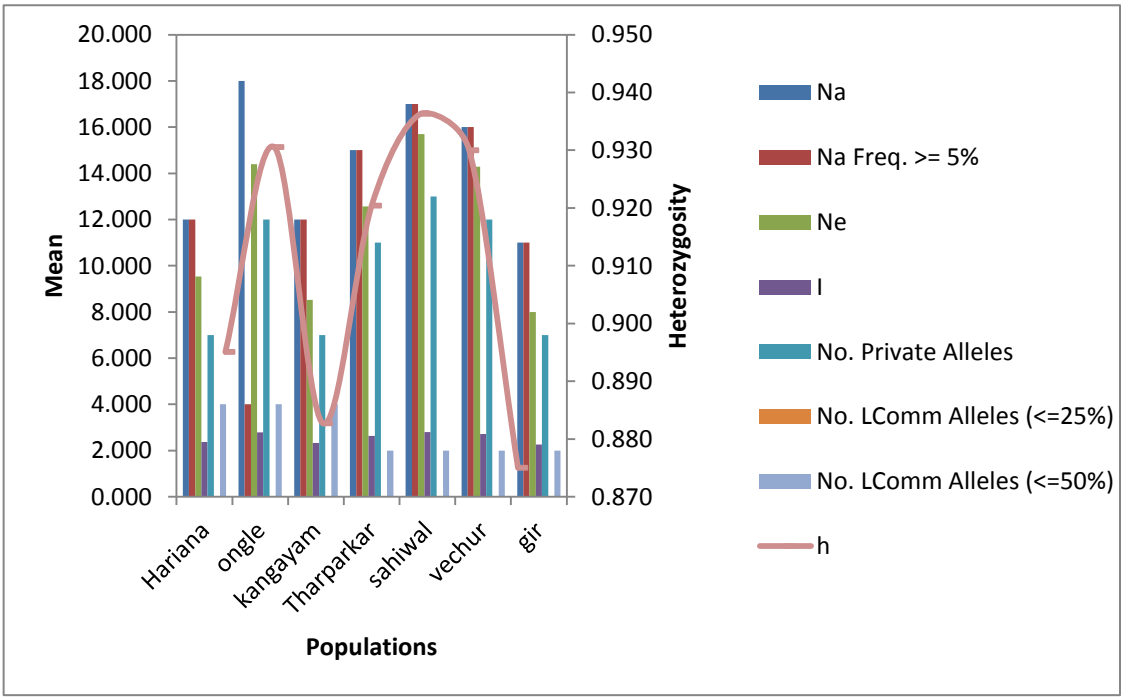

Figure no. 4.1 Allelic Patterns across Populations

Total 132 individuals of 7 breeds were analyzed by GenAIEx ver 6.503 (Peakall *et al*., 2006), Result revealed number of different allele(Na) ranging from 11 to 18 and effective allele(Ne) ranging from 8 to 15.696. The Shannon information index was ranging from 2.253 to 2.799 . Unbiases Diversity is above 93% for all the Breeds (as Table no.4.1) which indicate high amount of diversity.

4.3 Analysis of Genetic Diversity

4.3.1 Haplotype and Nucleotide Diversity

Total 81 haplotype were find out of 132 individuals. Identical sequence consider as same haplotype. Haplotypic frequency breed wise shown in Table No. 4.3. Within population wise demography indices, the number of haplotype(H) were 12 (Hariana), 18(Ongole), 12(Kangayam), 15(Tharparkar), 17(Sahiwal), 16(Vechur), 11(Gir) wherea as Haplotypic Diversity(HD), Nucleotide Diversity (Pi), Average nucleotide Differences(k) mention in Table no. 4.2.

Table 4.2 : Genetic Diversity indices

Breed	No. of sample	No. of haplotype	Nucleotide Diversity (pi)	Avg. Nucleotide Differences(k)	Haplotype diversity
Hariana	18	12	0.0004 ± 0.0006	0.2222 ± 0.2758	0.9477 ± 0.333
Ongole	24	18	0.0862 ± 0.0446	14.5724 ± 6.7623	0.9710 ± 0.0208
Kangayam	18	12	0.0004 ± 0.0006	0.2222 ± 0.2758	0.9346 ± 0.0409
Tharparkar	17	15	0.0050 ± 0.0030	3.2352 ± 1.7545	0.9779 ± 0.0313
Sahiwal	19	17	0.0038 ± 0.0028	1.2280 ± 0.8153	0.9883 ± 0.0210
Vechur	20	16	0.0031 ± 0.0027	0.7421 ± 0.5730	0.9789 ± 0.0214
Gir	16	11	0.0000 ± 0.0000	0.0000 ± 0.0000	0.9333 ± 0.0477
Total	132	101	0.0141 ± 0.0081	2.888 ±1.488	0.962 ± 0.309

Nucleotide diversity (Pi) was found to be in between 0.000 ± 0.000 to 0.0862 ± 0.0446 with overall Nucleotide diversity of 0.01413 ± 0.0077 (Table 4.2). Overall average number of nucleotide differences (K) among the populations was highest in Ongole(14.5724 ± 6.7623) and lowest in Gir 0.000 ± 0.000. The overall avg. number of nucleotide differences was 2.888 ±1.488.(Table no. 4.2).Haplotype diversity highest observed in Sahiwal (0.9883 ± 0.0210) and lowest is in Gir (0.9333 ± 0.0477) with overall haplotypic diversity was 0.962±0.309. The mitogenome based diversity indices indicated presence of substantial genetic diversity and differentiation within the all breeds. This constitutes the good diverse population to be considered as a base population for genetic improvement of this breed in India.

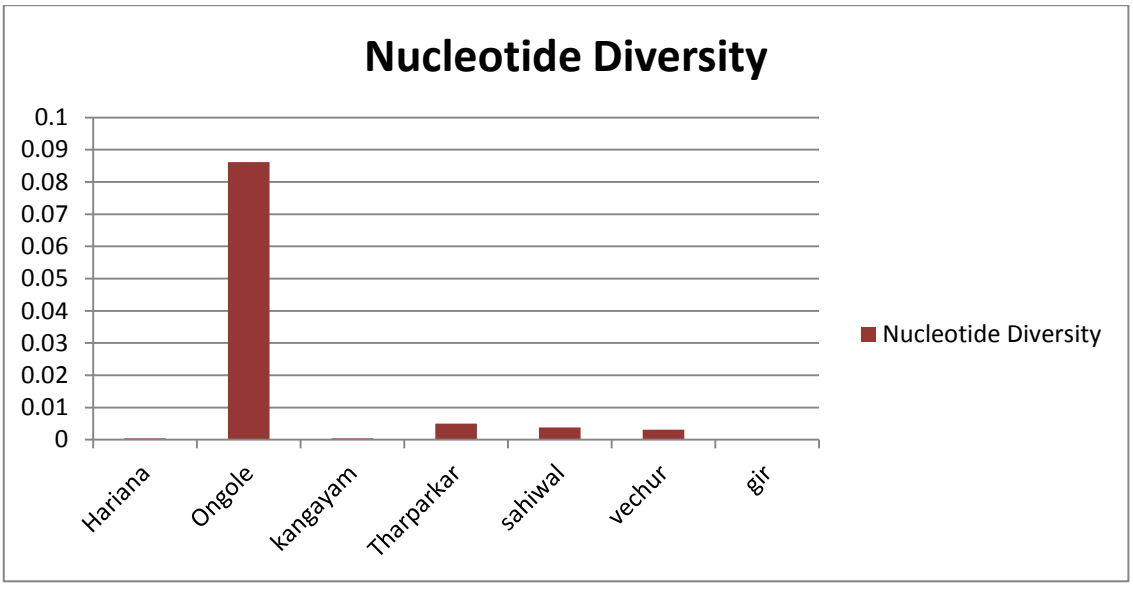

Figure. 4.2 : Nucleotide Diversity

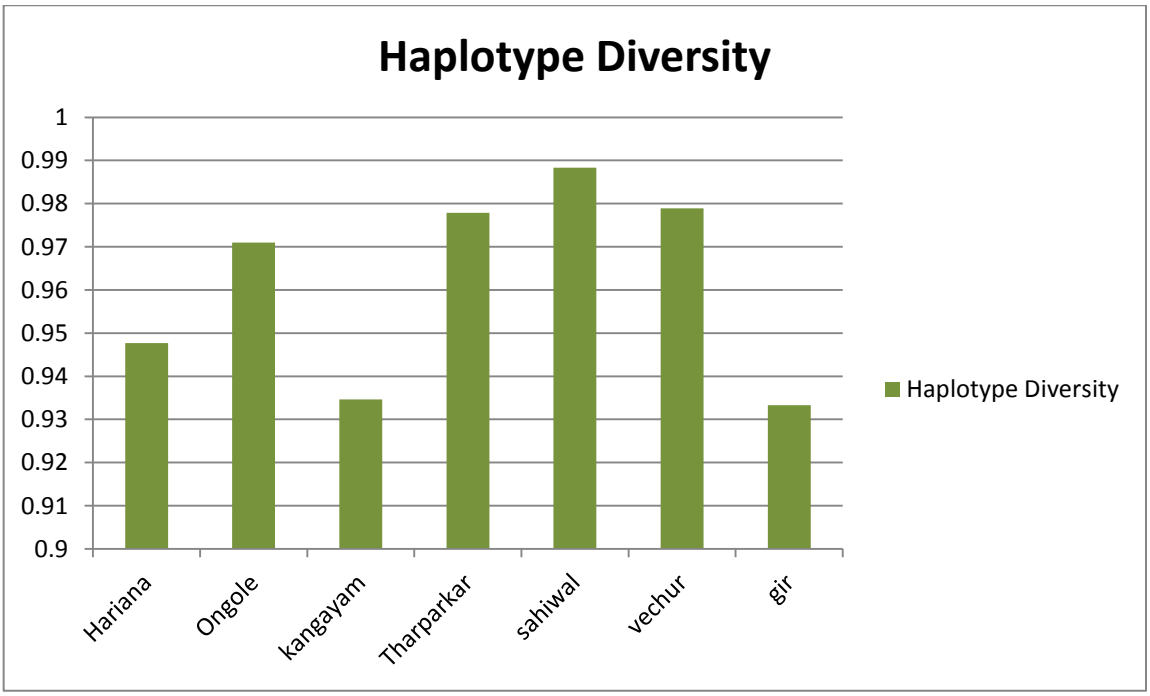

Figure 4.3 : Haplotype Diversity

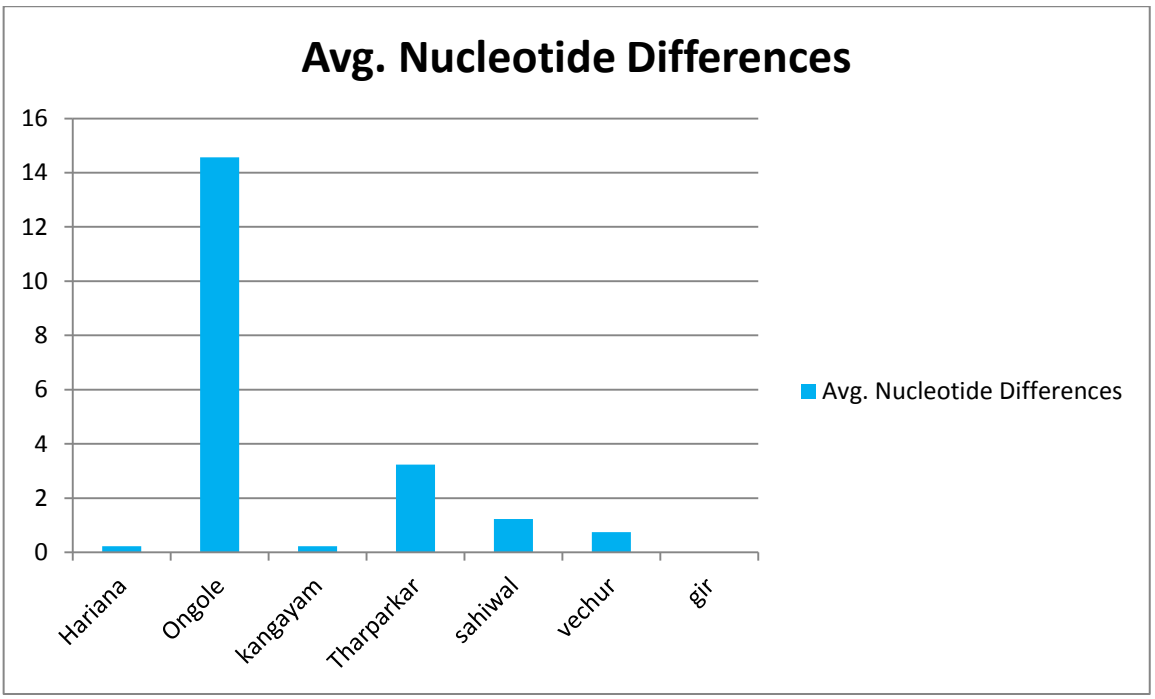

Figure 4.4 : Avg. Nucleotide Differences.

Table No : 4.3 Haploid Allele Frequencies by Population

BREED	Hariana	Ongole	Kangayam	Tharparkar	Sahiwal	Vechur	Gir
Sample Size	18	24	18	17	19	20	16
1						0.050	
2		0.042					
3		0.042					
4		0.042					
5		0.042					
6		0.042					
7		0.083					
8		0.042					
9	0.111	0.083					
10		0.042					
11	0.056	0.042	0.111				
12			0.222				
13	0.056	0.125	0.167				
14		0.042					
15		0.042					
16	0.056	0.042					
17		0.042					
18		0.125	0.056				
19		0.042	0.056				
20		0.042					
21					0.053		
22					0.053		
23					0.053		
24					0.053		
25					0.053		

26						0.050	
27						0.050	
28					0.053		
29			0.056				
30						0.050	
31						0.050	
32						0.050	
33					0.053		
34				0.059			
35							0.063
36				0.059			
37							0.063
38				0.059			0.063
39					0.053		
40							0.063
41							0.063
42							0.063
43							0.063
44	0.056						
45	0.111						
46			0.056				
47	0.056						
48						0.050	
49					0.053		
50					0.053		
51					0.053		
52				0.059			
53						0.050	
54	0.056						

55	0.056						
56				0.059			
57					0.105		
58				0.059	0.053	0.100	
59				0.059	0.053	0.100	0.250
60				0.059			
61						0.050	
62	0.056						
63	0.167						
64			0.056				
65						0.050	
66				0.059			
67				0.059			
68				0.176	0.105	0.050	0.125
69							0.125
70			0.056				
71						0.050	
72					0.053	0.100	0.063
73			0.056				
74			0.056				
75	0.167		0.056				
76						0.100	
77				0.059			
78				0.059			
79				0.059			
80				0.059			
81					0.053		

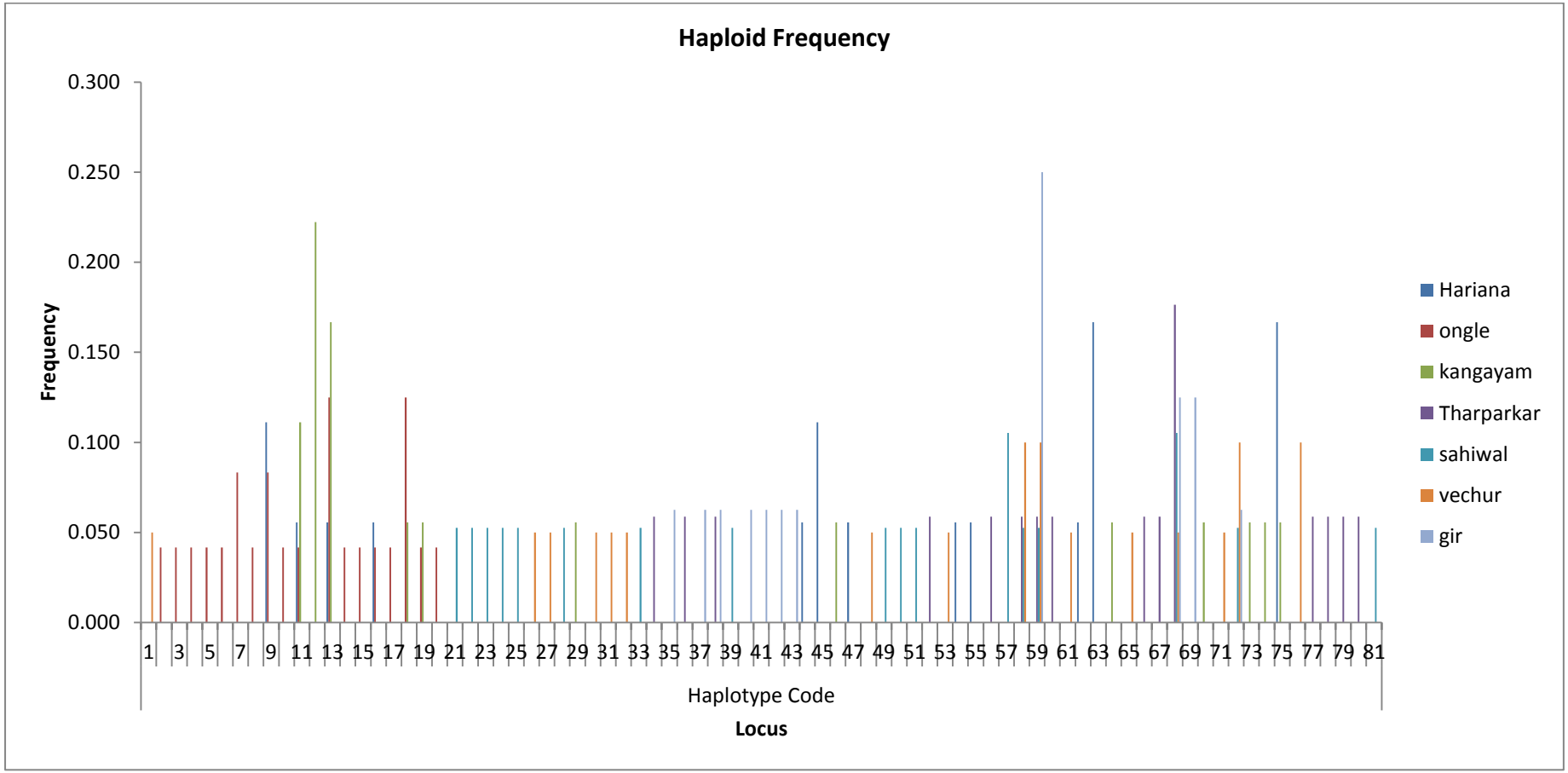

Figure 4.5 Haploid Frequency by Population

4.3.2 Analysis of molecular variance(AMOVA)

To understand the partitioning of the levels of genetic diversity of the 7 different populations of Indian cattle. The analysis of molecular variance (AMOVA) was conducted. The results of the AMOVA (Table 4.4) revealed that 97.39 % of total genetic diversity existed among the individuals within the populations and only 2.61% of total genetic diversity accounted for differences among populations.

Table 4.4 Analysis of molecular variance(AMOVA)

Source of Variation	df	Sum of Square	Variance components	Percentage of variation
Among Population	6	4.345	0.01291	2.61
Within population	125	60.185	0.48148	97.39
total	131	64.530	0.49439	

4.3.3 Global Fixation Index (F_{st})

Fst values were significantly different from 0 for all pair wise combinations representing significant amount of Genetic differentiation between population.FST value ranged from -0.00477to 0.06601.pair-wise genetic differentiation between populations are represented in Table 4.5. F_{st} value was highest between population of Gir and Kangayam with 0.06601 and lowest between population of Sahiwal and Vechur with a value of -0.00477.Here minus value we was consider as zero because range of F_{st} value is 0 to 1 only. Where zero is indicating same genetic material individuals have, and 1 indicate completely genetically unrelated. These F_{st} estimates between the population shows that Gir and Kangayam more genetically differentiated while Sahiwal and Vechur are genetically closer.

Table 4.5 : Global Fixation Index(F_{st})

Breed	Hariana	Ongole	Kangayam	Tharparkar	Sahiwal	Vechur	Gir
Hariana							
Ongole	0.02002						
Kangayam	0.035	0.0126					
Tharparkar	0.03724	0.0256	0.04381				
Sahiwal	0.03191	0.02046	0.03842	0.00452			
Vechur	0.02574	0.00853	0.02683	0.01284	-0.00477		
Gir	0.0594	0.04725	0.06601	0.01902	0.00956	0.03138	

As mentioned in Table no. 4.6 positive sign indicate significant F_{st} value at P value of 0.05 while negative sign indicate non significant F_{st} value. The below mentioned matrix table 4.6 shows the relative significant or non significant values of F_{st}..

Table 4.6 : Significance of F_{st} value

Breed	Hariana	Ongole	Kangayam	Tharparkar	Sahiwal	Vechur	Gir
Hariana		+	-	+	+	+	+
Ongole	+		-	+	+	-	+
Kangayam	-	-		+	+	+	+
Tharparkar	+	+	+		-	-	-
Sahiwal	+	+	+	-		-	-
Vechur	+	-	+	-	-		+
Gir	+	+	+	-	-	+	

Based on F_{st} value MDS(multidimension Scalling) form which shows cluster formation milch breeds that indicate same lineage of origin of milch cattle, whereas Kangayam and Hariana breeds are far away from all breeds which indicate independant lineage. Ongole was showing intermediate lineage in between milch and draft Indian breeds.

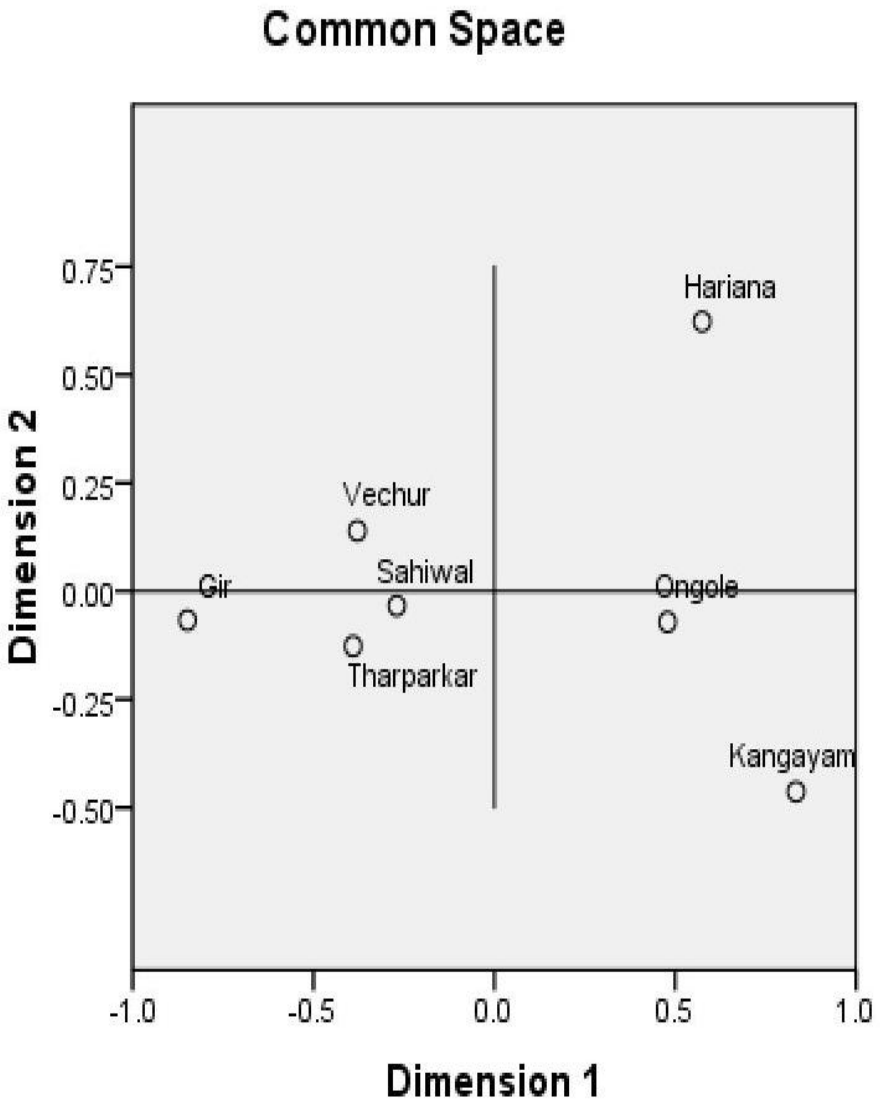

Figure 4.6 : Multidimention Scaling

Sharma et al., (2015) observed 170 Indain cattle of 11 Breeds. The result Revealed 60 haplotype with haplotype diversity of 0.9024, nucleotide diversity of 0.02688 and average nucleotide difference as 6.07407.where as in our study revealed 81 haplotype with haplotype diversity of 0.962, nucleotide diversity of 0.0141 and average nucleotide difference as 2.888.

4.4 Assessment of Functionality of Mitogenomic Variants

4.4.1 Identification of Allele

Table 4.7 : List of Identified Genes.

Allele selected based on F_{st} value	HD chip No.	Locus on mitogenome	Gene
254	BovineHD3200000301	12502	ND5
340	BovineHD3200000404	16176	NO GENE
160	BovineHD3200000190	7775	COX2
166	BovineHD3200000196	7975	COX2
185	BovineHD3200000220	8926	ATP6
57	BovineHD3200000067	3263	ND1
131	BovineHD3200000155	6687	COX1
210	BovineHD3200000250	9940	ND3
339	BovineHD3200000403	16158	NO GENE
1	BovineHD3200000002	190	NO GENE
250	BovineHD3200000297	12039	NO GENE
312	BovineHD3200000366	14730	CYTB
41	BovineHD3200000051	2558	NO GENE
168	BovineHD3200000198	8038	COX2
333	BovineHD3200000393	15666	CYTB
176	BovineHD3200000207	8541	ATP6
31	BovineHD3200000038	1817	NO GENE
90	BovineHD3200000106	4644	ND2

Top 20 alleles were selected based on locus wise F_{st} value. Position of that allele on mitogenome , gene corruspondus to that positon mention in Table no. 4.7.

4.4.2 Functionality of Gene

Mitochondrial Genes are involved in many activities like Molecular Functions(catalytic activity, Transport activity), Biological process (Cellular process, Immunological Process, Metabolic Process), Cellular component, Protein class(Enzyme modulator, oxidoreductase) and pathways (ATP synthesis, CCKR signaling, GnRH receptor, Inflammation mediated, toll receptor signalling). Among these function some important functions & involvement of gene mention in Table no. 4.8, Figure 4.6, Figure 4.7 Figure 4.8. In Table no.4.8 "+" indicate involvement of genes in that process.

Table 4.8 : Functions of Identified Genes

Gene	Functions				
	Catalytic activity	Transporter Activity	immune	metbolic	ATP synthesis
ND1	++	-	-	-	-
ND2	-	-	-	-	-
ND3	+	-	-	-	-
ND5	++	-	-	-	-
COX1	+++	++	+	++	++
COX2	++	+	+	+	-
CTYB	-	-	-	-	-
ATP6	-				+

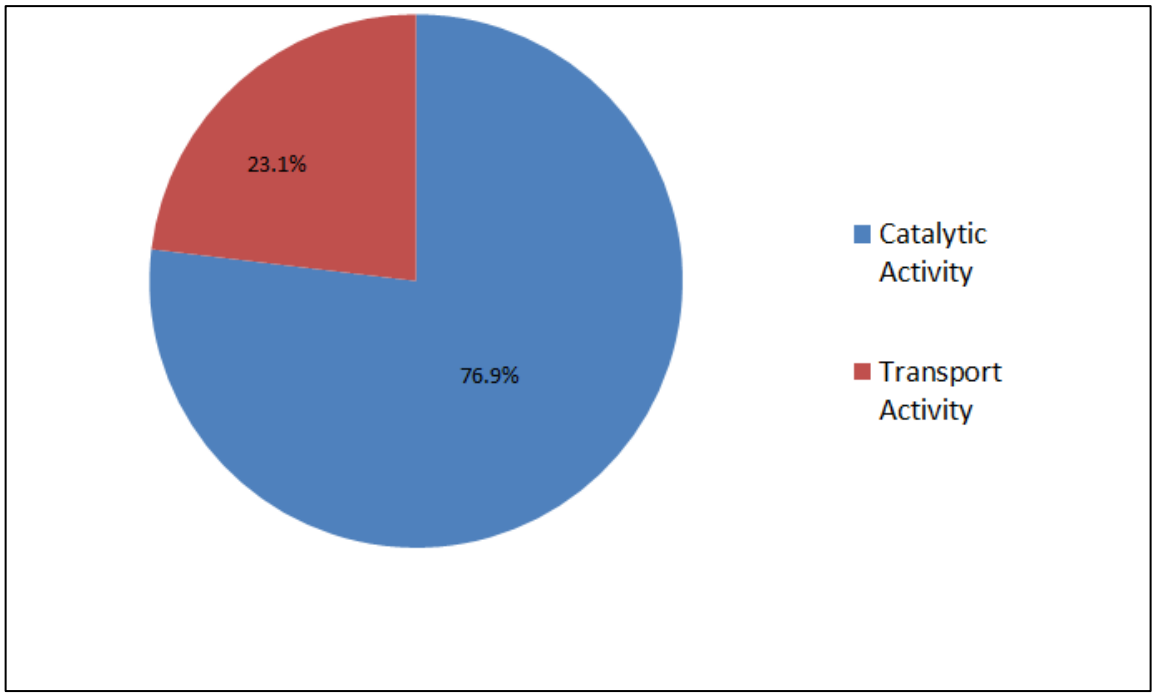

Figure 4.7 Genes involve in molecular Function

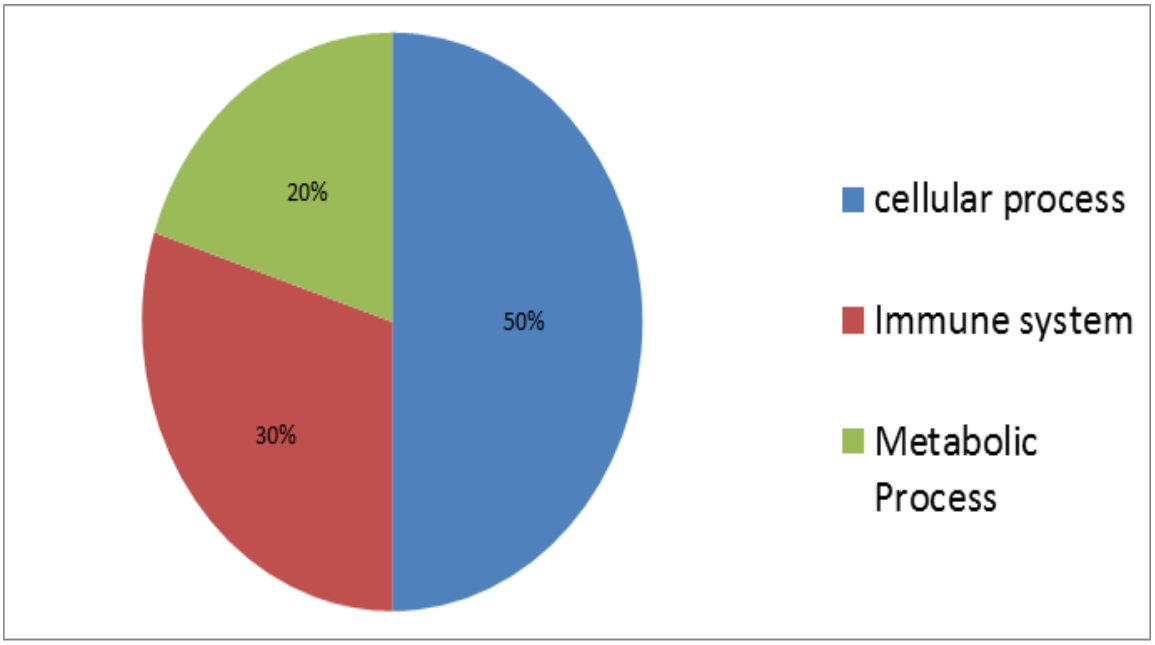

Figure 4.8 Genes involve in Biological process.

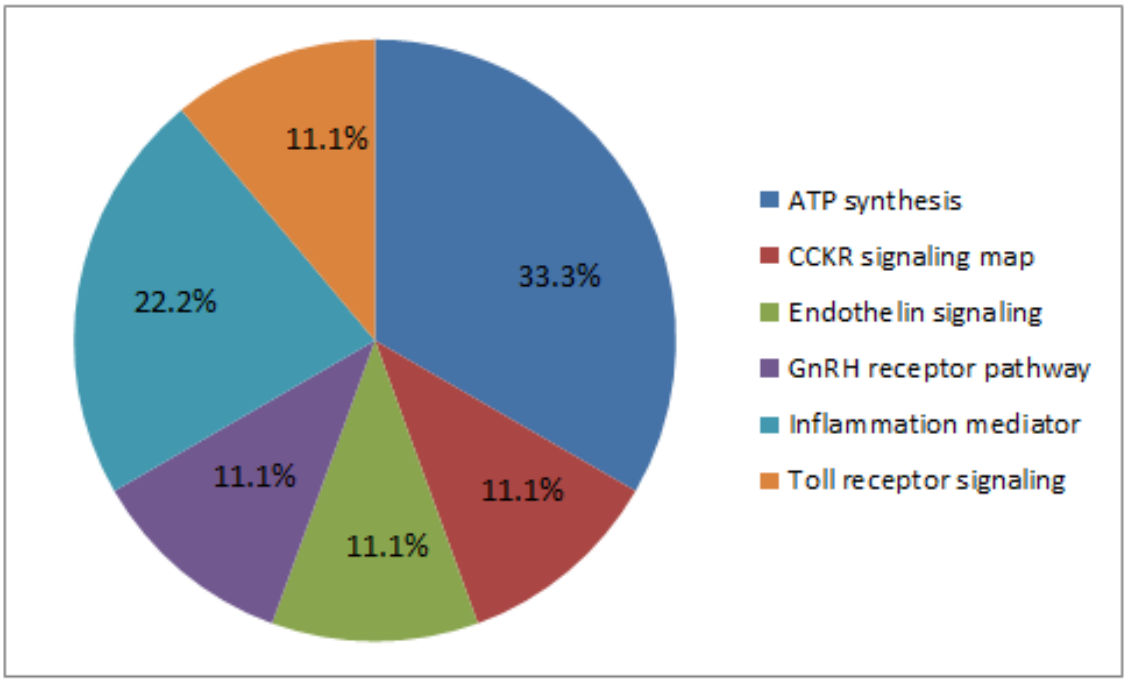

Figure 4.9 Genes involve in Pathways

CHAPTER –5

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSION

Indian cattle (*Bos indicus*) play a significant role in the economy of small and marginal farmers, especially in developing countries, as they are a potential source of milk, draught power and manure. Our country possesses a large diversity of cattle breeds and several works have been conducted to study inter breed diversity. Indian cattle breeds known for its heat tolerance and immune to disease. Total 132 DNA samples of different *Bos indicus* of different utility and geographic area were selected for study. SNP genotyping was done by Illumina 777K HD chip. 343 SNP were left after filtration of data by using plink version 1.07. Filtration was done on the basis of gene score, SNP call rate, minor allele frequency. Filtered data was analysed to know the genetic diversity by estimating haplotype diversity, nucleotide diversity, Global fixation index, Molecular diversity Index and AMOVA. A total of 81 haplotypes from a total of 132 animals were identified. The haplotype Diversity was range from 0.9333 ± 0.0477 to 0.9883 ± 0.0210, and overall haplotype diversity was 0.962±0.009 which shows that the population is diverse. Nucleotide diversity ranged from 0.000± 0.000 to 0.0862 ± 0.0446 with overall diversity of 0.01413±0.0077 across all 81 sequences and the average no. of nucleotide difference varied from 0.000± 0.000 to 14.5724 ± 6.7623). F_{st} value is significantly different from 0 for all pair wise combinations representing significant amount of Genetic differentiation between populations. F_{st} estimates between the population shows that Gir and Kangayam are more genetically differentiated (0.06601) while animals from Sahiwal and Vechur are genetically more closer (-0.0047). All these parameters show that significant amount of genetic variation exists between and within these populations.Amova analysis shows 97.39 % variation with in population and 2.61% variation

among population.MDS based on F_{st} value showing that Sahiwal, Tharparkar, Vachur has similar lineage of origin. Gir somehow similar to that lineage. But Kangayam is far away from all animals. It shows independent origin of Kangayam breed. Ongole and Hariana are inbetween draft and milch breed.

To assess the functionality of unique mtDNA variants, top 20 alleles were selected based on locus wise F_{st} value. Those allel were used to identify the position of gene on mitochondrial DNA based on MAP file. Gene were identified with the help of NCBI data bank. Functionality of identified genes were checked on Panther ontology

Conclusions

- The results indicated higher haplotype diversity of mitogenome within breeds (~93 % and above)
- The milch breeds shared the common ancestry and was different from the dual and draft breeds
- The functional diversity, based on top loci with higher F_{st} values, was restricted to seven genes involved mainly in Catalytic Activity, Transporter Activity, Immunity, Metabolism and ATP synthesis
- In future, the identified functional variants may be studied in detail for their utilization in the breeding program.

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