“A study towards genetic diversity and genetic basis of prolificacy in important sheep breeds of India.”

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Submitted by

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Under the Guidance of

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STATEMENT BY THE CANDIDATE

As required by the University Ordinance 770, I wish to state that the work embodied in this thesis titled "**A study towards genetic diversity and genetic basis of prolificacy in important sheep breeds of India**" forms my own contribution to the research work carried out under the guidance of **Dr. Vidya S. Gupta** at the National Chemical Laboratory, Pune, India. This work has not been submitted for any other degree of this or any other University. Whenever references have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.

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AN INTER-INSTITUTIONAL COLLABORATIVE RESEARCH EFFORT

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Varsha C. Pardeshi

LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AI	Artificial insemination
AMOVA	Analysis of molecular variance
AnGR	Animal genetic resources
BMPR-IB	Bone morphogenetic protein receptor IB
bp, kb	base pair, kilobase pair
DAD-IS	Domestic animal diversity information system
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
FAO	Food and agriculture organization of the United Nations
h, min, s	hour, minute, second
HWE	Hardy weinberg equilibrium
IBD	Isolation by distance
ISGC	International sheep genomics consortium
Kg, gm, mg, µg, ng,	kilogram, gram, milligram, microgram, nanogram
LS	Litter size
M, mM, µM	mole, milimoles, micromoles
MAS	Marker assisted selection
MCMC	Markov chain monte carlo
mL, µL	milliliter, microlitre
mm	millimeter
MoDAD	Measurement of domestic animal diversity
MOET	Multiple ovulation and embryo transfer
mtDNA	Mitochondrial deoxyribonucleic acid
NARI	Nimbkar Agricultural Research Institute
nDNA	nuclear deoxyribonucleic acid
NJ	Neighbor joining
OR	Ovulation rate
PCoA	Principal coordinate analysis

PCR	Polymerase chain reaction
QTL	Quantitative trait locus
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
SSD	Sum of squared deviation
tRNA	Transfer ribonucleic acid
tRNA-Phe	Phenyl transfer ribonucleic acid
UTR	Untranslated region

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Chapter 1

Introduction
and
review of literature

1.1 Livestock domestication

Hominids and early man were hunters and gatherers for millions of years. Domestication of livestock by man introduced a major cultural revolution. Domestic livestock species have played an important role in the agricultural, social and economic aspects of human population and are vital to the food security of the world population. Domestication ensured a steady food supply as well as other products such as skin and manure. Among the livestock species, sheep and goat were the first domesticated animals as the food items (Clutton-Brock, 1999).

The domestication of livestock, although considered to be one of the most important developments in history (Diamond, 2002), was complex, gradual and is not well understood. Fundamental questions such as why, how, when and where remain largely unanswered. Unraveling the geographic pattern and history of the dispersal of livestock is essential in order to identify geographic areas with high diversity and hence are potential priority areas for conservation efforts. This requires extensive mapping of genetic diversity. The intersection between archaeology and genetics have helped to document the locations of livestock domestication (Zeder *et al.*, 2006), which started around 12,000- 14,000 years ago, with archaeology guiding genetic research and genetics providing support to some controversial archaeological theories or revealing possible new geographic origins for livestock species and their diversity. However, the genetic revolutions in the past few decades discovered that nearly all major livestock species are the result of multiple domestication events in distinct geographic areas (12 areas; Hanotte 2006; Fig. 1.1). Findings of these studies indicated that after the initial domestication events, the spread of farming into nearly all terrestrial habitats followed rapidly (Diamond & Bellwood, 2003; Fig. 1.1). In short, the process of domestication was much more complex than revealed by the archeological data.

1.1.1 Complexity of domestication

Although multiple domestication events were detected in nearly all major livestock species (Fig. 1.1), the recent studies revealed that the apparent independent livestock domestication events were not necessarily culturally independent. However, some independent domestication events representing the movement of a few domesticated individuals into a new area might have occurred, wherein the genetic signatures of the

introduced founders were subsequently submerged by local wild animals (Zeder *et al.,* 2006).

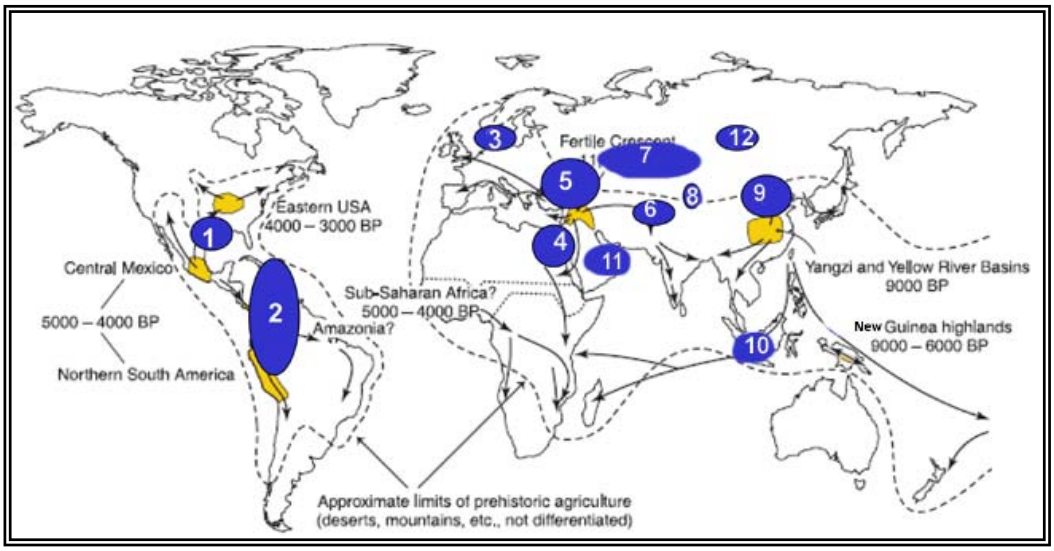


Figure 1.1: Major centers of livestock domestication – based on archaeological and molecular genetic information (Hanotte, 2007)
(1) Central America (turkey) (2) The Andean chain of South America (guinea pig, llama, alpaca) (3) Europe (pig, rabbit) (4) Northeast Africa (cattle, donkey) (5) Southwest Asia including the Fertile Crescent (cattle, pig, goat, sheep, Bactrian camel) (6) The Indus valley region (cattle, goat, chicken, river buffalo) (7) Eurasian steppe (horse) (8) The Himalayan plateau (yak) (9) East China (pig, swamp buffalo, chicken) (10) Southeast Asia (chicken, pig, Bali cattle) (11) North Asia (dromedary) (12) The southern part of the Arabian Peninsula (reindeer)

Alternatively, the ancient signatures of local domestication events might now be hidden by more recent arrivals of livestock from other centers of origin. Further detailed studies indicate that not only cross-breeding between livestock populations was common, but also the genetic introgression from wild populations occurred after the initial domestication events. When they occurred outside the species' geographic area of origin and after its initial dispersion, these wild introgressions might have resulted in localized livestock genetic populations with unique genetic backgrounds (Hanotte, 2007). The introgression might have been sometimes asymmetric with respect to the sexes. Also, there could be differences in domestication of males and females of a species because the herders usually slaughtered the males but kept the females to produce offspring or they kept few males for breeding in every generation.

Although each species has its own domestication history, a common finding was the "East-West duality" in domestication events suggesting that water buffalo (Tanaka *et al.*, 1996; Lau *et al.*, 1998), cattle (MacHugh *et al*., 1997), pig (Guiffra *et*

al., 2000) and sheep (Hiendleder *et al.*, 2002) were domesticated at least twice, independently at different sites. However, the East-West duality found in mtDNA was subjected to modification as a more detailed study suggested possible presence of the third domestication center possibly in the southwestern Asia (Bruford *et al*., 2003; Bruford & Townsend, 2006). The pig (Guiffra *et al*., 2000), dog (Savolainen *et al.,* 2002), goat (Luikart *et al*., 2001; Joshi *et al*., 2004; Naderi *et al.*, 2007) and sheep (Guo *et al*., 2005; Pedrosa *et al.*, 2005; Chen *et al.*, 2006; Tapio *et al.*, 2006a; Meadows *et al*., 2007) showed presence of multiple lineages indicating multiple domestication events. In addition, the above studies also revealed that the samples near the centers of domestication had high genetic diversity which decreased when one moved away from it (Freeman *et al.*, 2006).

1.1.2 Sheep domestication

Sheep (*Ovis aries* L.) are among the earliest livestock species to be domesticated. The sheep paleontology and behavioral evidence suggests that sheep have evolved from the goat-antelope, Rupicaprini represented by the Capriconis of Southeast Asia (Geist, 1971). During the late Pleistocene period, goat and sheep formed an interbreeding population (Payne, 1968). However, such a link is unlikely as suggested by the present day failure to interbreed (between sheep and goats) and large differences in the chromosome structure, blood antigens and proteins (Dain, 1970; Curtain, 1971; Lindley *et al.*, 1971).

The term sheep refers to species of the genus *Ovis,* although sometimes the term has been applied also to the genera *Ammotragus*, *Hemitragus* and *Pseudois*. The genus is highly polymorphic and the taxonomic status is open to dispute. Presently, six species of wild sheep are known [*O. ammon* (argali or giant sheep), *O. orientalis* (Asiatic mouflon), *O. canadensis* (bighorn sheep), *O. dalli* (Dall's sheep), *O. nivicola* (snow sheep) and *O. vignei* (urial)] (International Commission of Zoological Nomenclature 2003; Myers *et al.*, 2005; Fig. 1.2). Any of these might have acted as an ancestor to the present day domestic breeds (Ryder, 1983, 1984) as ovine populations could interbreed despite differences in chromosome numbers (Nadler *et al.*, 1973; Ryder, 1983). Domesticated sheep have 2n = 54 chromosomes, the same chromosome number as the European mouflon, the Asiatic mouflon, the Bighorn and the Dall sheep. The snow sheep of eastern Siberia has 52 chromosomes while the Argali has 56 and Urial has 58 (Ryder, 1984). It is unclear whether these

chromosomal differences represent the cause of speciation and domestication (Short, 1976).

As a large number of wild and possibly ancestral species and subspecies exist, many different theories regarding the origin of domestic sheep have been proposed. The nature of this wild ancestral population, the number of domestication events and its process still remain unknown. Based on morphological data, several Euroasian wild sheep were suggested as ancestors of domestic sheep (Hiendleder *et al*., 2002). The most important of these were the Argali (*Ovis ammon*), the Urial (*Ovis vignei*), the Mouflon (*Ovis musimon*) and the Bighorn (*Ovis canadensis*) sheep. It was believed that the urial was domesticated first, since the remains of Urial were found around the area (in the Aralo-Caspian basin) where domestication appeared to have begun (Ryder, 1984). These domestic forms subsequently spread throughout the Middle East and into Europe and gave rise to "wool" sheep whereas the "hair" sheep was believed to be originated from the Mouflon (Zeuner, 1963). Others believe that only a single wild species contributed to the gene pool of present day domestic sheep. As per the most accepted theory, domesticated sheep were derived from Mouflon *(O. musimon, O. orientalis)* stock which was brought to Europe and mixed with the urial (*O. vignei*) derivatives, but Argali (*O. ammon*) alleles were also introduced repeatedly into these lines (Hiendleder *et al.*, 1998a). Chromosome counts and blood protein analysis (Schmidtt & Ulbright, 1968; Ryder, 1984) supported this theory. However, later Urial and Argali were rejected as the ancestor based on mitochondrial DNA analysis (Hiendleder *et al*., 1998b; Wu *et al*., 2003). Furthermore, mitochondrial haplotype analysis of the domestic sheep showed two distinct clades, termed as 'A' and 'B', suggesting a biphyletic origin (Wood & Phua 1996; Hiendleder *et al*., 1998a; Hiendleder *et al*., 2002). Sequence analysis showed that clade 'B' haplotype was derived from the wild European Mouflon (*Ovis musimon*) and observed in a range of breeds from Europe, the Near East and New Zealand (Hiendleder *et al*., 2002). The maternal ancestor of clade 'A' haplotypes, found mostly in the sheep breeds of Asia and New Zealand, remains unknown. Recently, Chinese and Near East sheep population (representing Asian type sheep breeds) showed a new haplogroup called type 'C' indicating one more domestication event (Guo *et al*., 2005, Pedrosa *et al.*, 2005; Chen *et al*, 2006; Meadows *et al*, 2007) and two more 'D' and 'E' haplogroups were also inferred to occur in sheep from Near East (Meadows *et al.,* 2007; Fig. 1.3).

A

Ovis ammon
(Argali)

B

Ovis orientalis oritentalis
(Mouflon)

C

Ovis orientalis vignei
(Urial)

D

Ovis cannadensis
(Bighorn sheep)

E

Ovis dalli
(Dall sheep)

F

Ovis nivicola
(Snow sheep)

Figure 1.2: The main species of the genus *Ovis*
Source:

- http://animaldiversity.ummz.umich.edu/site/accounts/classification/Ovis.html
- http://www.arkive.org/species/GES/mammals/Ovis_orientalis/more_still_images.html manzanita@calacademy.org
- http://www.wildsheep.com/sheep/international.htm

The three additional lineages were found mainly in the animals from Near East which was suggested as the centre of sheep domestication based on the archaeological data, (Gupta, 2004), hence high genetic diversity and presence of multiple lineages was expected in the sheep breeds from this region. These findings further suggested that three independent domestication events might have occurred during sheep domestication. Out of the reported five lineages, only three lineages formed starburst clusters, indicating population expansion; however, distant haplotypes were reported in each group. In clade 'A', these outliers were suggested as group substructure with perhaps a more complicated ovine population history (Chen *et al*., 2006). Research including samples of the different Mouflon subspecies is necessary for a better understanding of the origin of domestic sheep. Also, investigation of local sheep from other Asian regions, proposed as domestication centers and sampling of more Asian animals would help in clarifying the situation.

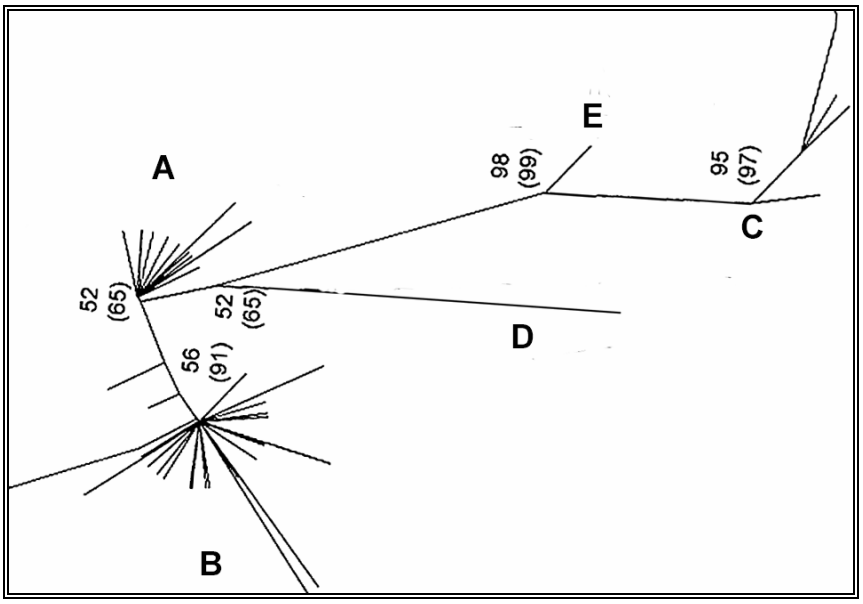


Figure 1.3: Unrooted neighbor joining tree constructed using the mtDNA sequences illustrating five lineages of domestic sheep (Source: Meadows et al., 2007)

1.2 Sheep genetic resources

Sheep has become a valuable source of livelihood for mankind since domestication (Zeuner, 1963; Schaller, 1977). Today, sheep is the most widely distributed domestic species globally and there are nearly 1400 sheep breeds of diverse phenotypes in varied ecological conditions (Fig. 1.4; Scherf, 2000).

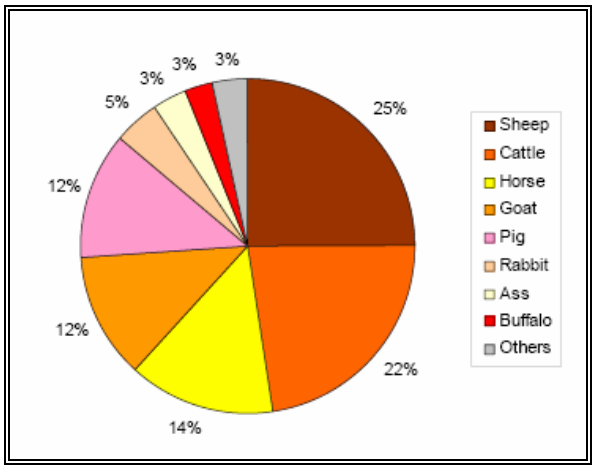


Figure 1.4: Distribution of the world's mammalian breeds by species (Source: Rischkowsky *et al.*, 2007)

Majority of these breeds are local (Rischkowsky *et al.*, 2007) and have modest production capability compared to the highly specialized breeds. However, they are an important genetic asset because, over time, they have developed unique combinations of adaptive traits to best respond to pressures of the local environment and yet retain significant genetic diversity**.** Such genetic diversity is required to meet the varied needs in diverse environments, to allow sustained genetic improvement and to facilitate rapid adaptation to changing breeding objectives. Maintenance of genetic resources in domestic animals is an urgent issue as one or two breeds become extinct each week (Simianer, 2005). Therefore, characterization of the native genetic resources before they are lost and development of proper conservation strategies are important. The Food and Agricultural Organization (FAO) of the United Nations has proposed a global program for the management of genetic resources using molecular methodology for breed characterization (Bjornstad & Roed, 2001). This strategy places a strong emphasis on the use of molecular markers to assist the conservation and assessment of endangered breeds and to determine the genetic status of available breeds.

In addition, it is also essential to collect data about various parameters, which would reflect upon the economic scenario of sheep rearing. For example, improving lambing percentage is one of the key ways to increase farm profitability as most of the domestic sheep breeds are reported to have only one or two lambs at each lambing. Recent developments in molecular biology and statistics have opened the possibility

of identifying and using genomic variation and major genes for the genetic improvement of livestock. Significant research efforts into quantitative trait loci (QTL) analysis are underway and a number of commercial sheep gene tests have already become available (Anderson & Georges, 2004).

1.3 Genetic variation in sheep

In order to plan breeding, recognizing crossbred individuals, quantifying kinship and rate of inbreeding and estimating other population genetic parameters, the morphological data and the pedigree records are the most suitable source of information (Caballero & Toro, 2002). However, surveys of cattle data show that such data provided limited information (provided only recent history of <20 generations) and also had about 3–20% incorrectly recorded parentages (Baumung & Sölkner, 2003; Gutiérrez *et al.*, 2003). The typical sheep breed pedigree data covered an even smaller number of generations because sheep are kept on small holdings without long-standing, coordinated breeding programs (Goyache *et al.*, 2003). Moreover, in domestic species, genetic diversity is partitioned between and within breeds, roughly in equal proportion. Therefore, in order to cover the considered time-scale (only partially) to compare different breeds and to get reliable estimates of population genetic parameters, more robust and neutral methods are essential. Molecular genetic methods fulfill all the above criteria and hence, are the most favored tools. A large number of studies, particularly during the 1970s, documented the characterization of blood group and allozyme systems (Baker & Manwell, 1980). However, the level of polymorphism observed in proteins was often low, reducing the general applicability of protein typing in diversity studies. DNA-based polymorphisms which are neutral in nature are now the markers of choice for molecular-based surveys of genetic diversity. Importantly, polymorphic DNA markers showing different patterns of Mendelian inheritance can be studied in nearly all major livestock species. Typically, they include D-loop and cytochrome *b* mitochondrial DNA (mtDNA) sequences (maternal inheritance), Y chromosome-specific single nucleotide polymorphisms (SNPs) and microsatellites (paternal inheritance). Various genetic markers provide different levels of genetic diversity information. Autosomal microsatellite loci are commonly used for population diversity estimations, differentiation of populations, calculation of genetic distances, estimation of genetic relationships and population

genetic admixture. MtDNA sequences are most popular for domestication studies whereas Y chromosome polymorphism is an easy and rapid way to detect and quantify male mediated admixture. However, extensive sequencing of the Y chromosome has revealed very little usable variation in the sheep Y chromosome (Meadows *et al.*, 2004). The information generated can be used in planning population management and to identify genetically important populations for conservation (Weitzman, 1992, 1993; Petit *et al.,* 1998; Eding *et al.,* 2002; Piyasatian & Kinghorn, 2003; Bennewitz & Meuwissen, 2005).

1.4 Mitochondrial DNA: A small molecule with unique biology

Mitochondria occupy a central position in the biology of cells and are crucial to life. It is the only animal organelle which has its own DNA and is found in the cell cytoplasm outside the cell nucleus. The mitochondrial genome is a closed circular, double-stranded DNA molecule about 16,600 base pairs long and is only about 0.00055% of the total human genome. The mitochondrial and nuclear genomes differ in many other ways, such as the ploidy, mode of inheritance, degree of recombination, number of introns, effective population size, mutation rate, repair mechanisms, etc. (Scheffler, 1999). The structure and gene organization of mtDNA is conserved in mammals and it contains 13 protein-coding genes, 22 transfer RNA genes and 2 ribosomal RNA genes (Fig. 1.5). The molecule is very tightly organized with no introns in the genes. Except for the control region which governs the transcription and replication, intergenic sequences are absent or limited to a few bases (Taanman, 1999). It shows maternal inheritance in mammals (Hutchison *et al.,* 1974; Hayashi *et al.*, 1978; Giles *et al.*, 1980); however, rare paternal inheritance and recombination among mitochondrial lineages has been suggested, but this remains controversial (Piganeau & Eyre-Walker, 2004; Piganeau *et al.*, 2004).

In sheep, one crossbred ewe was reported to repeatedly produce offspring with mitochondria derived from the father (Zhao *et al.*, 2004). It is not known if the paternal mitochondria were present in the germ line cells. Slate & Phua (2003) did not detect signs of recombination in sheep mtDNA variation, which suggests that paternal inheritance is insignificant.

The mitochondrial DNA (mtDNA) has been used extensively in the last three decades as a tool for inferring the evolutionary and demographic past of both,

populations and species, because of the following three main characteristics: 1) it is sufficiently evolutionarily conserved allowing the identification of the wild taxon or population from which the species descends, 2) it is variable and structured enough across the geographical range of the species hence useful to identify approximate locality of domestication and 3) it evolves at a rapid but constant rate; this feature allows the origin of a particular polymorphism to be dated.

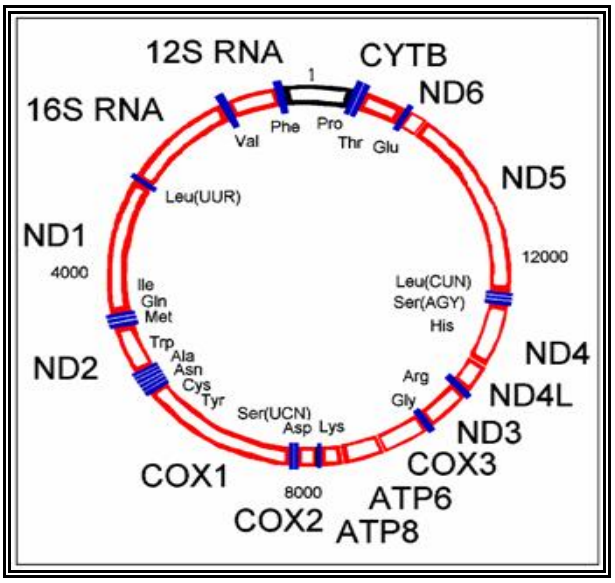


Figure 1.5: A simplified view of mtDNA
(Source: http://www.mitomap.org/) The circular strand of mtDNA is shown with nucleotide base 1 at the 12 o'clock position. By convention, the numbering of the nucleotide bases goes in an anti-clockwise direction. The coding areas for the genes are shown *in red,* with their corresponding labels outside. The tRNA's are shown *in blue* with the three-letter abbreviations for the amino acids inside.

The first studies considering mtDNA variation employed restriction fragment length polymorphisms (RFLP) (Avise *et al*., 1979a, b; Brown & Wright, 1979). These studies set the stage for further work and were instrumental in developing mtDNA as a molecular tool. Using the RFLP technique, Brown *et al*. (1979) demonstrated that the rate of mtDNA sequence evolution in higher primates was high, about 2% per million years, a statistic that has been repeatedly employed in the literature. The focus started to shift from RFLP to sequence analysis when Kocher *et al.* (1989) published highly conserved primers that could be used to amplify the DNA from a wide range of taxa. This and several properties such as the lack of recombination, maternal mode of inheritance and high mutation rate made mtDNA the marker of choice to study the phylogenetic relationships and recent demographic processes.

1.4.1 Differential evolution of mtDNA regions: Mutation rate bias

The advent of routine DNA sequencing allowed detecting DNA variation at the highest resolution. These studies revealed that mammalian mtDNA evolved about 5 to 10 times more rapidly than single-copy nuclear DNA (nDNA) at an overall nucleotide substitution rate of 2% per million years (Brown *et al*., 1979; Wilson *et al*., 1985). The base composition varies among mitochondrial regions and also between species. For example, in cattle 70% and in sheep 64% of bases in 5' hypervariable section of control region are A or T, whereas in the central conserved domain of control region the proportion of A and T bases is approximately equal to the proportion of C and G bases (Sbisa *et al*., 1997). Moreover, various functional regions of mtDNA evolve at different rates and there is strong heterogeneneity of mutation rates in the hypervariable region (Penny *et al.*, 1995; Malyarchuk *et al.*, 2002) and also in other parts of the molecule (Ballard, 2000). Such mutational biases may influence the evolution of mtDNA and may have potential to cause problems in phylogenetic and phylogeographical inferences.

Several studies have shown that the most rapidly evolving part of the mitochondrial genome is the non-coding control region which contains the displacement loop (D-loop; Upholt & Dawid, 1977; Walberg & Clayton, 1981; Chang & Clayton, 1984). The control region can be divided into two peripheral highly variable segments (hypervariable regions I and II or ETAS and CSB domains) and a central conserved region (Fig. 1.6). The sequences of the hypervariable region I have been widely used in molecular evolutionary studies. The average substitution rate for this domain is three quarters of the mitochondrial synonymous rate (Pesole *et al.*, 1999). The substitution rate of some sites may be over four times the average rate for this domain (Excoffier & Yang, 1999) and there are a large number of variable sites present which make this short segment a very informative region in intra-specific studies. For example, in humans for a single highly variable section of the control region over 500 distinct haplotypes were identified (Handt *et al*., 1998), whereas 331 haplotypes were identified from 406 individuals in domestic goats (*Capra hircus*) (Luikart *et al*., 2001). The more conservative central region of the D-loop also exhibits extended nucleotide similarities between species, and diverges no more than the mitochondrial protein-coding genes (Brown *et al*., 1986) and hence can be used to estimate more distant relationships. Overall, the D-loop region shows extraordinary

levels of variation within species, allowing to track geographic patterns of diversity and evolution (Avise, 2000), dispersal, gene flow, demographic expansion, genetic drift and hybridization (Bruford *et al.*, 2003).

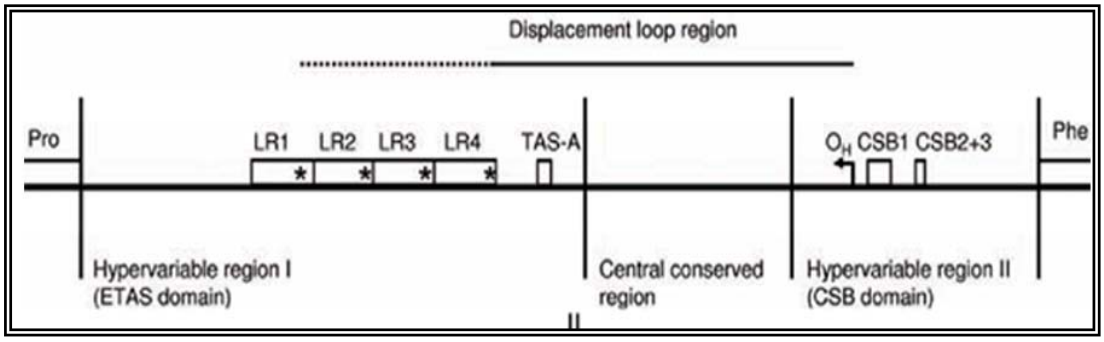


Figure 1.6: Schematic representation of the sheep mitochondrial control region bound by the tRNA genes for proline (Pro) and phenylalanine (Phe)
Long tandem repeats (LR1, 2, 3 and 4) with putative termination signals (asterisk), the binding site for a protein regulating termination and transcription (TAS-A element), the origin of heavy strand replication (OH), and conserved sequence blocks (CSB1 and CSB2+3) are presented. The line above indicates the three stranded displacement loop region between OH and termination sites (source: Tapio *et al.*, 2006b)

Most importantly, high rate of evolutionary change and ability to detect differentiation between domestic lineages make the control region a powerful tool for establishing genetic diversity and phylogenetic structure within a species and consequently the marker of choice for livestock studies.

Among the various coding regions of mtDNA, the cytochrome *b* gene has been used in numerous studies of phylogenetic relationships within mammals, and it is the gene for which the most sequence information from different mammalian species is available (Irwin *et al*., 1991; Meyer, 1994; Johns & Avise, 1998). This gene has proven to be especially important in livestock studies (Kadwell *et al*., 2001; Luikart *et al*., 2001) because its tempo and mode of evolution is well understood and is thought to be relatively constant and similar among large-bodied terrestrial mammals (Irwin *et al*., 1991). For example, in sheep (*Ovis aries*) and goats (*Caprahircus*), cytochrome *b* evolves at a rate between 3.8 and 5.4% per million years, depending on the interpretation of the fossil record (Luikart *et al.,* 2001). Consequently, cytochrome *b*, rather than the control region, is often used in conjunction with the fossil record to 'calibrate' a 'molecular clock' among mitochondrial DNA lineages in livestock. Also, the sequence variability of

cytochrome *b* is most useful for the comparison of species in the same genus or the same family. The results obtained in many of the phylogenetic studies using this gene led to the proposition of new classification schemes that better reflected the phylogenetic relationships among the species studied (Arnason *et al*., 1995; Lara *et al.*, 1996; Faulkes *et al*., 1997; LeDuc *et al.*, 1999; Matthee & Robinson, 1999). In some studies, the new classifications were proposed to preserve monophyletic taxa, but in many others, they were also intended to maintain comparable levels of divergence in groups with the same taxonomic rank in such a way that the new classifications conveyed more useful comparative information (Avise & Johns, 1999). Furthermore, cytochrome *b* phylogenies can also help in the genus assignment of newly described species (Giao *et al*., 1998).

1.5 Autosomal markers

Autosomal markers are located in autosomal chromosomes and diploid individuals have two copies of these marker loci, one copy inherited from each of the parents. The allelic status of both the copies is known for co-dominant markers. Different kinds of autosomal molecular markers are known, such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites and single nucleotide polymorphisms (SNPs). Each has its advantages and disadvantages and, in the future, other systems are also likely to be developed. Among the DNA markers, microsatellite alleles show co-dominant inheritance, making them relatively easy to score. They are inherited in Mendelian fashion and alleles at a specific locus differ in the number of repeats. Microsatellite loci are highly abundant and almost uniformly distributed throughout the genome (Ortí *et al*., 1997; Schlötterer, 1998), commonly occurring in noncoding regions, often called the "junk" DNA and the variation is mostly neutral. They are often hypervariable within populations and show much higher mutation rates (ranging from 10^{-2} to 10^{-5} per generation) than other nuclear regions (Edwards *et al*., 1992; Weissenbach *et al*., 1992; Weber & Wong, 1993) but the rates vary between loci and organisms (Ellegren, 2004). The mutation rate for sheep dinucleotide microsatellites has been estimated to be 1.4×10^{-4} mutations per gamete per locus (Crawford & Cuthbertson, 1996). Such high mutation rate of

microsatellite is useful for studying the evolution over short time spans (hundreds or thousands of years).

The use of microsatellites in livestock animal studies started in the beginning of 90s (MacHugh *et al.*, 1994) and FAO conducted studies to standardize the microsatellite loci to be used in analyzing the genetic variation within and among breeds. The list of the microastellite loci suggested by FAO can be found on its webpage:http://dad.fao.org/en/refer/library/guidelin/marker_without_link.pdf.
However, not much data based on these has yet been accumulated. There are mainly three uses of microsatellites in livestock animals: 1) to measure the genetic variation within and among breeds (Diez-Tascón *et al*., 2000), 2) to determine the "genetic admixture" experienced in the samples (MacHugh *et al.*, 1997) and 3) to assign the individuals to the breeds according to their genetic resemblance (Cornuet *et al.*, 1999). A survey of diversity studies revealed microsatellites to be the main marker type in sheep; used in 90% of the research projects (Baumung *et al.*, 2004) and there are over 1,200 mapped microsatellite markers available in sheep (Maddox *et al.*, 2004). Microsatellite based studies were also reported to give results matching documented breed histories in sheep (Buchanan *et al.*, 1994). To date, the published sheep microsatellite studies have typically examined relationships among breeds within a country (Arranz *et al.*, 1998; Stahlberger-Saitbekova *et al.*, 2001; Àlvarez *et al.*, 2004) or relationships between populations of a breed (Diez-Tascón *et al.*, 2000; Pariset *et al.*, 2003). Results from large-scale geographical studies are becoming available and some preliminary results have been published (Lenstra & the ECONOGENE Consortium 2005). Many on genetic diversity of many Indian sheep breeds *viz.*, Garole (Sodhi *et al*., 2003), Muzzafarnagri (Arora & Bhatia, 2004), Nali and Chokla (Sodhi *et al*., 2006), Hassan (Sharma *et al*., 2006) and Bellary (Kumar *et al*., 2007b) have been carried out using microsatellite marker set recommended by the FAO.

1.6 Genetic improvement of livestock using molecular markers

The genetic improvement of animals is a continuous and complex process. Various reproductive technological developments such as artificial insemination (AI) and multiple ovulation followed by embryo transfer (MOET) have already had a major impact on livestock improvement in developed countries. These technologies

speed up genetic progress, reduce the risk of disease transmission and expand the number of animals that can be bred from a superior parent; however, the later technology is largely restricted to the developed countries (Land, 1988). Alternatively, breed differences are an important source of genetic variation and crossbreeding can be an effective way of improvement in the efficiency of livestock production; however, it is a slow process (Dickerson, 1969). Moreover, in classical genetic improvement programs, selection is carried out based on observable phenotypes of the candidates and/or their relatives but without knowing which genes are actually being selected. Most of the traits considered in animal genetic improvement programs are quantitative and the underlying genes have small effects on the phenotype observed e.g. milk yield and growth rate. Substantial advances have been made over the past decades through the application of molecular genetics in the identification of loci and chromosomal regions that affect the traits of importance in livestock production (Anderson, 2001). The identification of mutations responsible for the variation of several interesting monogenic phenotypic traits as well as complex traits (Blott *et al*., 2002; Grisart *et al*., 2002; Winter *et al*., 2002; Van Laere *et al*., 2003), has already illustrated the potential of domestic animals for uncovering genes that underlie phenotypic diversity. This has also enabled opportunities to enhance genetic improvement programs in livestock by direct selection of genes or genomic regions that affect economic traits through marker-assisted selection and gene introgression (Dekkers & Hospital, 2002).

1.6.1 Sheep genetic improvement for meat production

Because of high human population growth rates and rapidly diminishing grazing land, there are now greater pressures to improve production efficiency of small ruminants in the tropics. The global food model of the International Food Policy Research Institute predicts that consumption of meat in developing countries will grow by 2.8% per year until 2020 (Delgado *et al.*, 1999). Sheep are an important small ruminant species for the arid and semi-arid areas of many developing countries such as India. For exponential growth of sheep industry in terms of meat, the objective of increased prolificacy or fecundity i.e. increased litter size per animal through prolificacy genes needs utmost attention. Several studies demonstrated that the variations in litter size or ovulation rate in different breeds of sheep can be associated with the segregation of several major genes, collectively named as fecundity (*Fec*) genes (Davis, 2005). This

promoted the research to discover the genes or mutations with large effect on ovulation rate and thus litter size, and a number of breeds with high fecundity have been discovered *viz*., Booroola (Piper & Bindon, 1982a), Thoka (Jónmundsson & Adalsteinsson, 1985), Javanese Thin Tailed (Bradford *et al*., 1986), Belclare and Cambridge (Hanrahan *et al*., 1991), Olkuka (Martyniuk & Radomsa, 1991), Inverdale (Davis *et al*., 1991b, c), Hanna (Galloway *et al.*, 2000), Woodlands (Davis *et al*., 2001b), Garole (Davis *et al.*, 2002), Lacaune (Bodin *et al*., 2002), Han and Hu (Wang *et al.*, 2003; Table 1.1). At present, the major genes for prolificacy in sheep can be classified into three categories: (1) genes with the known mode of inheritance but the mutation has not yet been identified, (2) putative genes where there is evidence of apparent genetic segregation but there are insufficient records to ascertain the mode of inheritance and (3) genes with known mutations. Such natural mutations affecting the reproductive fitness represent a valuable reserve to determine the key points in the biochemical pathways controlling the development of ovarian follicles.

1.6.2 The BMP receptor-IB (ALK6) or FecB gene

The first major gene for prolificacy identified in sheep was the Booroola (*FecB*) gene in Australia (Piper *et al.*, 1985). It shows simple Mendelian autosomal inheritance. Since its discovery, it has been introgressed into many breeds across the world (Davis *et al.*, 1991a) and its effect on ovulation rate (OR) and litter size (LS) are well documented (Table 1.2). As it is clear from the table, the increase in OR with one copy of the gene ranged from 0.92 (Piper & Bindon, 1982a) to 1.65 (Davis *et al.*, 1982; Piper *et al*.,1985; Bradford *et al.*, 1986; Gootwine *et al.*, 1995; Southey *et al*., 2002). The increase in LS with one copy of *FecB* ranged from 0.45 live lambs (Gootwine *et al.*, 2003) to 0.9 lambs (Davis *et al.*, 1982; Piper *et al.*, 1985; Bradford *et al.*, 1986; Teyssier *et al.*, 2003). Some studies (Davis *et al.*, 1982; Piper *et al.*, 1985; Inounu *et al.*, 1993) estimated an additional increase of 0.4-0.6 in litter size with a second copy of the gene. More recent studies estimated this increase to be 0.18 lambs (Present study, Nimbkar *et al.*, 2003a) and 0.04 live lambs (Gootwine *et al.*, 2003), while Flanigan (2004) estimated the scanned number of foetuses in homozygous ($FecB^{B}/FecB^{B}$) ewes to be less (by 0.4) than heterozygous ($FecB^{B}/FecB^{+}$) ewes. Overall, the gene is additive for ovulation rate and partially dominant for litter size.

Table 1.1: The summary of the putative and known prolificacy genes in sheep breeds

Category	Location/Gene	Breed	Increase in		Remark	Reference
			Ovulation rate	Litter size		
Gene with known mode of inheritance	X-linked imprinted gene (woodlands, *FecX2W*)	Coopworth	0.4	0.25	Maternally inherited	Jonmundosson & Adalstinsson, 1985; Davis *et al*., 2001b
	Thoka gene	Iceland sheep	1.2	0.7	Mechanism similar to *FecB*	
Putative genes	NA	Polish Olkuska	1.0	0.6	Endangered breed	Martyniuk & Radomska, 1991; Malher & Le Chere, 1998; Davis *et al*., 2003;
		Belle-Ile	1-7	1-8	Endangered breed	
		Romney, Perendale &	NA	NA		
		Border Leicester x Romney sheep	NA	NA		
Prolificacy genes with known mutations	*BMPR-1B*	Booroola	1.65	1.0		Bradford *et al.*, 1991; Galloway, 2000, 2004; Hanrahan *et al*., 2004; Wilson *et al*., 2001; wang *et al.*, 2003; Davis *et al.*, 1991b,c 2002, 2006
		Garole	1	0.7		
		Han	NA	NA		
		Javanese	1.3	0.9		
		Thin Tailed Hu	NA	NA		
	BMP15 ($FecX^I$)	Inverdale	0.8-2.4	0.6	Sterility in homozygous animals	
	($FecX^H$)	Hanna	1.0	0.6		
	($FecX^B$)	Belclare	1.0	NA		
	$FecX^G$	Belclare & Cambridge	0.7	NA		
	GDF9 ($FecG^H$)	Belclare & Cambridge	1.4	NA		
	$FecL^L$	Lacaune	5.8	0.7		

NA: Information not available.

Introgression of the Booroola gene into non-dairy Awassi flocks was found to have a positive net value in most cases (Spharim & Gootwine, 1997) but in dairy Awassi flocks, the project was found to be marginally profitable only when meat price was high (Gootwine *et al.*, 2001). However, there is a high risk of mortality of lambs and ewes associated with multiple births (Davis *et al*. 1991a; Meyer *et al*., 1994a; Southey *et al*., 2002). The decreased survival of lambs born to heterozygous ewes has been attributed to the larger LS and to the larger OR with subsequent mortality of one or more embryos (Meyer *et al.*, 1994a).

1.6.3 Search for the FecB mutation

The search for a marker linked to the *FecB* mutation started in 1982. The first successful DNA marker for the *FecB*/Booroola gene was developed in 1993 using the two linked microsatellite markers, OarAE101 and OarHH55 (Montgomery *et al*., 1993). Subsequent studies showed that the locus was situated in the region of ovine chromosome 6 and showed synteny to human chromosome 4q22-23 (Montgomery *et al*., 1993, 1994), which also contains the bone morphogenetic protein receptor (*BMPR-IB*) gene (Wilson *et al*., 2001). The *BMPR-IB* is a member of the transforming growth factor superfamily β (TGF-β) and the members of this superfamily are multifunctional proteins that regulate growth and differentiation of many cell types. Therefore, a mutation in *BMPR-IB* could have physiological importance in triggering the phenotype consistently observed in the Booroola animals as it maps within the critical region for the locus and segregates with the *FecB* phenotype. In 2001, three independent laboratories simultaneously identified the non-conservative point mutation (Q249R) in the intracellular kinase-signalling domain of the *BMPR-IB* gene responsible for the Booroola phenotype (Mulsant *et al.,* 2001; Souza *et al.,* 2001; Wilson *et al.,* 2001). The mutation, Q249R, is the A→G transition at position 746 (GenBank Acc. No. AF357007), substituting the glutamine present in the wild type sequence with an arginine.

The mechanism of action of the mutated gene has not yet been fully understood, however, it results in ovulation of a higher number of follicles with smaller diameter and fewer granulosa cells than that of the wild type, thus speeding the differentiation of ovulatory follicles (Mulsant *et al*., 2001). In $FecB^{B+}$ carrier ewes, Q249R substitution would impair the inhibitory effect of *BMPR-IB* on granulosa cell steroidogenesis, leading to their advanced differentiation and an advanced maturation of follicles (Mulsant *et al*., 2001). Souza *et al*. (2001) also found another point mutation at position 1113 (C→A); however, this mutation did not change the coding amino acid. Additionally, two more substitutions in the 3' untranslated region (UTR) were also observed (Lehman *et al*., 2003) without any effect on the prolificacy. However, it is known that the mutations in the 3' or 5' UTR region play an important role in the regulation and expression of the gene. Hence, further studies need to be carried out to evaluate the effects of these mutations.

1.6.4 Effect of the FecB gene in various prolific sheep breeds

The expression of the *FecB* gene was found to alter in different breeds. The *FecJ* gene was the major gene segregating in Javanese thin Tailed sheep (JTT) and had a smaller additive effect on LS and OR than that of the *FecB* gene (Roberts, 2000). The effect of one copy of *FecJ* on OR in the Javanese sheep was about 0.8 (Bradford *et al*., 1991), which was only half of that reported for the *FecB* by Piper *et al*. (1985) in other breeds. The mean litter sizes of the homozygous carriers of the *FecB* (2.59; Piper & Bindon, 1996) and *FecJ* (2.83; Bradford *et al*., 1991) were comparable, although the difference between the corresponding ORs of Booroola (5.65) and JTT (2.92) was large. Davis *et al*. (2002) subsequently found that the *FecJ* gene had the same mutation as *FecB* and its effects were lower in JTT breed. The Chinese Hu sheep was reported to have a mean LS of about 2.1 (individual litters range from 1 to 8) (Feng *et al*., 1996; Yue, 1996) and 2.61 (Wang *et al*., 2003) and had the ability to lamb twice per year. The Small Tailed Han sheep from China are also highly prolific, averaging 2.47 lambs born per ewe lambing (Feng *et al*., 1996, Wang *et al*., 2003). Although, the Garole breed is proposed as the ancestral sheep breed for the *FecB* gene mutation (Davis *et al*., 2002), it has a lower mean LS (2.27; Ghalsasi & Nimbkar, 1993 and 2.30; Davis *et al*., 2002). Many homozygous carriers in the Garole breed showed maximum LS of twins (Davis *et al*., 2002). However, some of the heterozygous ewes produced even triplets and/or quadruplets (Kumar *et al*., 2006), but heterozygous Javanese sheep did not produce triplets (Davis *et al*., 2002) indicating that there is no consistent LS criterion for determining the status of the *FecB* mutation in Garole ewes. The observed differences in the fecundity of the prolific breeds carrying the *FecB* mutation probably could be because of environmental conditions, ewe parity, selection, maternal nutrition, breed background and these are discussed below.

(a) Environmental conditions

The varied expression of the *FecB* gene in different breeds could be due to the differential expression of the gene in different environments or could also be due to the genotype and the environment interaction. Although the effect of the *FecB* gene in Booroola Merino showed nearly similar increase in OR (1.54) and LS (0.6) in

Table 1.2 Mean ovulation rate (OR) and litter size (LS) of *FecB* carrier (heterozygous and homozygous) and non-carrier ewes of various breeds produced by crossing with the Booroola Merino (number of ewes in brackets)

Breed/cross	Country	FecB+/FecB+		$FecB^B$/FecB+		$FecB^B$/$FecB^B$		Method of genotype detection	Reference
		OR	LS	OR	LS	OR	LS		
Merino non- carrier, F1 carrier	Australia	1.06 (179)	1.13±0.04 (178)	1.98 (197)	1.70±0.04 (191)	-	-	pedigree	Piper & Bindon, 1982b
Pure Booroola Merino	Australia	-	1.33±0.09[1] (166)	-	2.09±0.09[1] (139)	-	1.72±0.13[1] (51)	DNA test	Flanigan, 2004
Booroola x Merino F1	New Zealand	1.54±0.02 (133)	1.38±0.02 (133)	2.78±0.03 (150)	2.07±0.03 (150)	-	-	OR	Davis *et al*., 1982
¼ Merino ¾ Booroola	New Zealand	1.50±0.04 (38)	1.34 (38)	2.78±0.04 (61)	2.24 (61)	4.59±0.15 (20)	2.71 (20)	OR	Davis *et al*., 1982
¾ Rambouillet ¼ Booroola	U.S.A.	1.53[2]		2.71[2]		-	-	Microsatellite markers	Schulze *et al*., 2003
Merinos D'Arles (7/8 or 15/16)	France	-	1.21 (269)	-	2.10 (417)	-	-	OR	Teyssier *et al*., 2003
Booroola-Assaf Backcrosses	Israel	-	1.75±0.05 (78)	-	2.44±0.04 (248)	-	2.59±0.08 (52)	DNA test	Gootwine *et al*., 2003
Booroola-Assaf Backcrosses	Israel	-	1.65[3]±0.04 (78)	-	2.10[3]±0.03 (248)	-	2.14[3]±0.07 (52)	DNA test	Gootwine *et al*., 2003
Non-carrier Assaf	Israel	1.72±0.10 (97)	-	2.92±0.10 (70)	-	-	-	OR	Gootwine *et al*., 1993
Carrier F1 Awassi backcrosses	Israel	1.37±0.1[4] (44)	1.22[5] (99)	2.67±0.1[4] (42)	1.88[5](149)	-	-	[4]Pedigree and OR [5]microsatellite	[4]Gootwine *et al*., 1995; [5]2001

[1]Scanned foetal number at 50-90 days of pregnancy [2]First parity [3]Live litter size (Source: Nimbkar, 2006)

different environments (Davis *et al*., 1991a), in case of Garole and Hu sheep breeds, the gene expression varied as per location. The reported mean LS of the Garole from hot and humid costal region of Sunderban of West Bengal, the breed's native tract, was 2.27 (Ghalsasi & Nimbkar, 1993), which reduced to 1.74 and 1.68–1.87 in the semi-arid climate of the Deccan Plateau of Maharashtra (Nimbkar *et al*., 1998) and Rajasthan (Sharma *et al*., 1999, 2001), respectively. The Hu is the predominant breed in Suzhou, Shanghai and Dongshan regions of China, where it is well adapted to the hot and humid environment. Litter size (2.12) at first lambing from the Hu at Natural Source Conservative Region was found to be significantly higher than that from the other two regions sampled (Shanghai, 1.78 and Suzhou, 1.90; $p < 0.05$) (Guan *et al*., 2006). These findings point that either, the *FecB* gene itself or some other gene(s)/ QTL modulating the *FecB* gene expression is present in the Garole and the Hu sheep, which are in turn being modulated by the environmental factors.

(b) Ewe parity

Bathei (1994) studied the Iranian fat tailed Mehraban breed of sheep and reported that the ewe productivity increases as parity proceeds. Similar observations were reported for the Booroola Merino sired ewes and Small Tailed Han sheep (Young & Dickerson, 1991). However, in case of Garole x Malpura ewes, it was observed that the ewe's productivity increased up to third parity and decreased thereafter (one copy of the *FecB* mutation increased LS by 0.93, 0.78, 1.2 and 0.8 in the first, second, third and fourth parities, respectively; Kumar *et al*., 2006). These observations indicate the possibility of the role of sexual maturity regulatory genes in modulation of OR and/or LS with parity.

(c) Breed effect

To date, the *FecB* gene has been introduced into a range of different sheep breeds in several countries in Africa, Asia, America, Europe, Oceania etc., including Merino of different strains and prolific breeds such as the Finnsheep and Romnov (Davis *et al*., 1991a; Thimonier *et al*., 1991). The assessment of performance of the Booroola-crossed flocks in different countries showed that the carriers of the prolificacy mutation had higher ewe productivity. Shulze *et al*., (2003) reported that the introgression of the *FecB* allele in Rambuillet sheep increased OR by 1.18 for the B+ ewes, whereas Southey *et al*. (2002) reported an increase in OR of 1.54. In the back-

crosses of the Booroola Merino and Mérinos d'Arles from France with different genotypic classes, the differences between B+ and ++ ewes for OR ranged from 0.92 to 1.72 with an average difference of 1.2 (Bodin *et al*., 1991). The mean LS of Booroola crossed Awassi and Booroola crossed Assaf was increased by 0.66 lambs born per ewe lambing (Gootwine *et al*., 2001). Similarly, the results from New Zealand using either the Romney (Davis & Hinch, 1985; Davis *et al*., 1991a) or Coopworth (Piper *et al*., 1988) or Merinos d'Arles from France (Bodin *et al*., 1991; Elsen *et al*., 1994) as the recipient ewes produced 0.6 more ova and 0.3 more lambs. Teyssier *et al*. (1998) reported an increase of 0.9 lambs born/ewe lambing due to the presence of the *FecB* allele in Merino d'Arles-cross ewes. These effects of the *FecB* allele on prolificacy were slightly lower than those estimated by Piper *et al*. (1985), but were similar to those from the worldwide summary reported by Davis *et al*. (1991a) of several local breeds compared to BM-local breed crossbreds (mostly ½ & ¼ BM). Chinese Merino prolific meat strain is also reported to have lower OR (2.83) than the Booroola Merino. In the Garole crossbred animals, introduction of one copy of the *FecB* allele showed an increase of 0.7 (Present study, Nimbkar *et al.*, 2003a; Kumar *et al*., 2007a) in the mean LS which was lower than that estimated for Booroola crosses (0.9-1.2 lambs per ewe). In the Garole x Malpura ewes, some heterozygous (B+) individuals produced single lamb even after 2–4 parturitions (Kumar *et al*., 2007). Similarly the JTT sheep from Indonesia, Hu and Small Tailed Han sheep from China also showed lower mean LS (2.5, 2.09, 2.47, respectively) compared to the Booroola Merino (Davis *et al*., 2002; Liu *et al*., 2003; Wang *et al*., 2003). The above findings suggest that the reduction in fecundity or LS might have been affected by the background genotypes of the recipient breed. The genes of the recipient breed and/or the prolificacy environment in the recipient breed might play an important role in *FecB* gene expression.

(d) Other gene(s) or QTL

Davis *et al*. (2006) observed that one non-carrier Han ewe had consistently large litters (four sets of triplets, one set of quadruplets and one set of quintuplets) suggesting that other genes besides those reported till date might also be present in the Han sheep, causing high prolificacy. Recent studies in Small Tailed Han sheep of the ovine melatonin receptor 1a gene (*MTNR-1A*) located on ovine chromosome 26 showed an association between a polymorphism at nucleotide position 604 of exon 2

and prolificacy (Chu *et al*., 2003). Three types of genotypes were observed in Small Tailed Han sheep, AA (290 bp, 290 bp), AB (290 bp, 267 bp/23 bp) and BB (267 bp/23 bp, 267 bp/23 bp). The AA genotype had mean LS that was 1.06 and 0.94 higher than the AB and BB averages, respectively. The effect in adult ewes (second parity) was large. Furthermore, Chu *et al*. (2007) reported another prolificacy mutation *BMP-15* ($FecX^G$) in Small Tailed Han sheep. Ewes carrying mutations in both the *BMPR-IB* and *BMP-15* genes had greater LS than those with either mutation alone. The interaction between the *FecB* and $FecX^G$ mutations appears to be multiplicative in those animals, which were heterozygous for both the Booroola and Inverdale mutations and had ORs greater than the increase expected for an additive effect alone (Davis *et al*., 1999). However, the effect of the *BMPR-IB* mutation was observed to be greater than that of the *BMP-15* gene mutation on LS.

Besides these, other factors such as sheep husbandry practices and flock management (Wang *et al*., 1998; Geng *et al*., 2002), nutrition (Roberts, 2000) and uterine capacity could also play an important role (Meyer & Piper, 1992; Meyer *et al*., 1994b, Gootwine 2005)

1.7 Garole, a source of the FecB mutation

An early Australian Booroola Merino flock was reported to include the prolific Bengal sheep (Turner, 1982), which prompted Dr. Helen Newton Turner (1982) to suggest that the Garole sheep could be the source of the *FecB* mutation in highly prolific Booroola Merinos. Later, Davis *et al.* (2002) detected the *FecB* mutation in the Garole and the Indonesian Javanese sheep, thereby confirming that the *FecB* gene in the Garole is the same as that reported in Australian Booroola Merinos. As the same mutation was also found in the Javanese sheep, it was hypothesized that the presence of *FecB* in Booroola Merino and Javanese sheep could probably be traced back to the Garole sheep of India (Davis *et al.,* 2002, 2006). Later, the *FecB* mutation was also reported in the Chinese Hu and Small Tailed Han sheep breeds (Davis *et al*., 2006). Recent research confirmed that the *FecB* mutation is fixed in populations of the Garole and the Hu sheep but is segregating in the Javanese, the Booroola Merino and the Han breeds (Davis *et al*., 2002, 2006). It is suggested that the mutation in the Garole and the Hu sheep breeds could be due to two separate mutation events or these breeds could have a common ancestor. The Garole is a micro-sheep (11-14 kg adult

weight) with short-tail and light brown fleece. The females of this breed are polled and males are horned. On the contrary, the Hu breed from Jiangsu and Zhejiang provinces of China, is large (32–44 kg) having a small fat-tail with a triangular fat deposit near the base. The fleece is white and both the sexes are polled. However, despite the marked phenotypic differences between the Garole and the Hu sheep, both breeds are reported to have some individuals with an earless phenotype (Mason 1980b; Bose *et al*., 1999), suggesting that these breeds might be distantly related. The earless phenotype is also present in Javanese Garut sheep (Mason, 1980a), reported to have the *FecB* gene segregating in the population (Davis *et al*., 2002). It is thus quite possible that transportation of the animals carrying the *FecB* gene might have occurred through the ancient silk route (from Kolkata to Shanghai) resulting in the introduction of the gene in China. However, Gong *et al.* (2002) reported that both the Hu and the Han breeds descended from the Mongolian sheep. Records suggest that the traders brought Mongolian sheep to Zhejiang, Jiangsu, Hebei, Henan and Shandong provinces as early as the 5th century AD (Feng *et al*., 1996) and the Hu (Zhejiang and Jiangsu) and the Han (Hebei, Henan and Shandong) sheep are presently concentrated in these provinces. On the other hand, narration by George Bogle (Markham, 1875) & Dorji *et al*., (2003) reported the presence of sheep resembling the Garole (prolific breed) in the Tibet Bhutan border region. The sheep were brought by the Tibetan traders and were traded in the plains of Bengal in the pre-colonial era. Hence, the original source of the Garole carrying the *FecB* mutation remains debatable.

1.8 Indian Sheep

India is a vast country with rich biodiversity and diverse ecological conditions. It has a vast genetic resource of livestock. Sheep constitutes an important species of livestock and plays an important role in the rural agricultural economy of arid and semi-arid zones in India. Sheep meat is the main source of animal protein to the ever-growing human population available at comparatively cheaper cost. The sheep population in India was estimated to be about 62 million (FAO, 2004) ranking 4th in the world and accounting for about 4.57% of the world sheep population. The sheep diversity in India is reflected by the 42 breeds (Acharya, 1982) distributed in various agro-climatic zones of the country. These breeds have mainly evolved through natural

selection for adaptation to specific agro-ecological conditions and very little effort has been made to develop these breeds through artificial selection to meet any socio-economic need. The Indian sheep are thin tailed, medium to coarse wool type in North temperate and North western regions and hairy type in southern peninsular and eastern regions. They are endowed with unique attributes such as superior adaptability to harsh climate, long migration, resistance to tropical diseases, ability to subsist on inferior and scarce feed resources and shortage of drinking water. However, indiscriminate crossbreeding, uncontrolled intermixing and geographical reorganization have put to risk this potentially important ovine genetic material. Therefore, conservation of indigenous ovine germplasm has been recognized as an increasing national concern. Moreover, the ancestor of Indian sheep breeds is still unknown. Initially the wild varieties of sheep *(Ovis orientalis vignei)* from the mountains in Afghanistan to Armenia were proposed as the ancestors of the domesticated sheep of India (Acharya, 1982), however, the mtDNA data ruled out the *Ovis orientalis vignei* as the maternal ancestor of domestic sheep (Hiendleder *et al*., 2002; Wu *et al*., 2003). Taking these factors in account, there is an urgent need for an extensive study of Indian sheep breeds to understand their diversity, population structure, origin, divergence and past migration patterns.

1.8.1 Indian sheep breeds under study

Although there are 42 breeds of sheep in India, only a few sheep breeds contribute largely to the Indian sheep economy. Therefore, in this study thesis four Indian sheep breeds that are economically important and/or have unique characters and belong to different geographic locations (Fig. 1.4) were studied.

The Deccani in the southern-central peninsular region of the India is numerically the most important sheep breed and might be the largest contributor to the meat production in the country (Khan *et al.*, 2002). This breed is a medium sized leggy breed that varies widely in color. The ewes weigh 20-25 kg and rams 35-40 kg on an average. It is estimated that 57% Deccani sheep are black in color, 18% white and 15% have mixed color. There are five strains reported for this breed; Sangola, Madgyal, Kolhapuri, Lonand and Sangamneri (Jangnure, 1992). The Sangola strain has mainly a white or black spotted face (Fig. 1.7a), the wool is finer and has a comparatively longer staple and the percentage of black wooled sheep is high. The main areas where these sheep are found are Sangola, Malshiras, Pandharpur and

adjoining areas of Sholapur and Sangli districts. The Madgyal strain has mainly a white and brown spotted face with Roman nose (Fig. 1.7b). The Kolhapuri strain is akin to Bellery sheep of Karnataka state (Fig. 1.7c) and the animals are leggy, long eared, and narrow-chested, with a long fine face and Roman nose. The Lonand strain is mainly brown and brown spotted (Fig. 1.7d) and the percentage of black-woolen sheep is very low in this strain. These sheep are found in Khanapur taluka of Sangli district and Daund, Indapur and Purandar talukes of Pune district of Maharashtra state. The wool is rough and comparatively short stapled. The Sangamneri strain is found in Sangamner taluka of Ahmednagar district (Fig. 1.7e) and it has a brown or tan face with brown or tan-spotted fleece. The wool is rough and of a comparatively long staple.

The Bannur (also called 'Bandur' or 'Mandya'), is a hairy sheep from Karnataka State (Fig. 1.7f) with relatively medium size (35 kg), white color, but in some cases the face is light brown, and this color may extend to the neck. Ears are long, leafy and drooping. A large percentage of animals carry wattle with slightly Roman nose. Both sexes are polled and the coat is extremely coarse and hairy. This breed has the best body conformation for meat production in India (Acharya, 1982) and it is well adapted to a hot and dry climate.

The Madras Red is from the southern part of India (Fig. 1.7g) and it is mainly reared for meat; however, the sheep skin is also an important product and used for making leather articles. This breed is found at Kanchipuram, Virudhachalam and Vellore districts of Tamil Nadu state. It is mostly red in color; however, in some animals white spots over head and abdomen are seen. Curled horns are seen in males while females have no horns. Average body weight in male is about 35 kg while it is 23 kg in females.

The Garole, as explained earlier, is a micro-sheep from Sunderban region of West Bengal state of India (Fig. 1.7h). It is the only prolific sheep breed that has been found so far in India with an average adult weight of 15 kg (Ghalsasi & Nimbkar, 1993; Fahmy & Mason, 1996). Their color is generally white; however, some animals of black or brown color are also observed. The males are generally horned and females are polled. They have three distinct types of ears: small, medium and long. An ability to breed throughout the year and to graze in knee-deep water, resistance to foot rot and a strong mothering instinct are some of the special features of this breed. It is mainly reared by small holders for meat. Twin and triplet births are common

(Sharma *et al.*, 2004). Usually around 10-15 ewes are kept. The breeding ram is shared between two houses and the new breeding ram is purchased from a near by area. Hence it is predicted that this breed might show high inbreeding and subpopulations.

1.9 Need to analyze the Indian sheep genetic resources

The Indian sheep breeds are classified either on the basis of their major products e.g. 1) apparel wool, 2) carpet wool, 3) meat and carpet wool and 4) meat type, or on the basis of agro-ecological regions such as 1) North temperate region, 2) North western arid and semi-arid region, 3) Southern peninsular region and 4) Eastern region (Acharya, 1982). Due to this, some of the breeds from adjoining areas have been identified as separate breeds although they might be genetically similar to each other. For example, Madras Red in Tamil-Nadu, Red Nellore in Andhra Pradesh and Ganjan sheep in Orissa, although identified as separate breeds, appear to be genetically similar (Singh & Bohra, 2000). The phenotypic differences among the breeds might be due to selection for specific characters or adaptation to specific agro-climatic conditions. Moreover, there are more than one strains in some of the breeds e.g. Deccani has five strains (Jangnure, 1992), Patanwadi has three strains (Dave, 1977; Mason, 1981) etc. Therefore, it is necessary to verify the genetic basis of these phenotypic differences. In addition, a sizable population of sheep is nondescript due to indiscriminate breeding and intermixing of breeds. There are no breeding societies and agencies to register animals of particular breeds, to maintain flock books and ensure purity of the breed. For many decades these indigenous animal genetic resources were perceived as unproductive and underestimated compared to other domestic species. This prompted the need to develop new breeds or upgrade local breeds which is mostly done by crossing with exotic breeds. As a result of this and various other factors, the number of indigenous breeds has declined rapidly during the

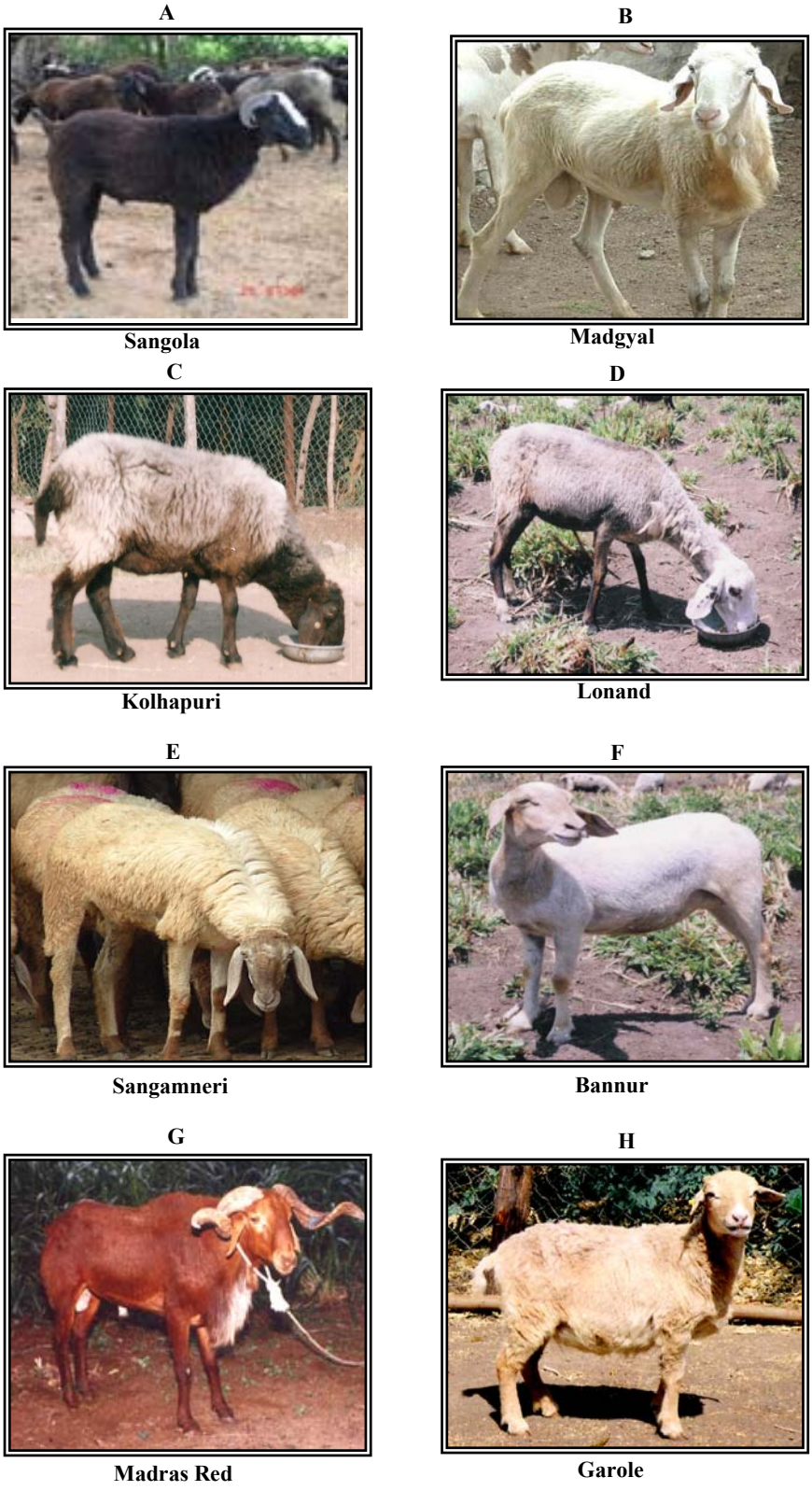


Figure 1.7: The Indian sheep breeds

twentieth century and caused dilution and even to virtual extinction of some indigenous genetic resources. The existing Indian sheep diversity, in the form of numerous indigenous breeds, is the outcome of differential socio-cultural regimes, structures and habitats. The adaptive variation has been utilized by breeders in selecting for specific economic characteristics or by natural selection to adapt the animal to specific environments. Consequently, the local breeds may have unproven potential to increase production without loss of the local adaptations (Hall & Bradley, 1995). Thus, the indigenous breeds provide genetic diversity, ensure stability and are building blocks for future breed development. Therefore, understanding the structure and the origin of these genetic resources will help in planning the conservation actions more efficiently. However, till date no serious consideration has been given to understand and characterize Indian sheep.

1.9.1 Indian sheep genetic improvement scenario

The Indian Council of Agricultural Research established a Network Project on sheep improvement for many Indian breeds at several centers in India. Under this project, crossbreeding of the Deccani breed with exotic breeds was carried out in 1967 (Amble *et al.*, 1967) and from 1977 to 1991 (Fernandes & Deshmukh, 1986; Jagtap *et al.*, 1988, 1989). Dorset x Deccani crosses had higher body weight up to nine months, daily weight gain and greasy fleece weight than Merino x Deccani or purebred Deccani lambs (Jagtap *et al.*, 1988). Thorat *et al*. (1990) found that both Dorset-Deccani and Merino-Deccani half breds had significantly higher greasy fleece yields than the Deccani but there was no significant difference between the two crossbred groups. In 1992, a program for the improvement of Deccani sheep (dual purpose; meat and wool, breed) was started at the Mahatma Phule Agricultural University at Rahuri in Maharashtra (Mahatma Phule Agricultural University, 2004). The program also aims to supply surplus rams and ram lambs to farmers. However, the impact of these programs on the sheep population at large has not been documented. There are literature reports of several crossbreeding experiments with different Indian sheep breeds and the exotic breeds Dorset and Suffolk. Crossbreds (F1 and quarterbreds) were reported to have higher body weights and daily weight gains (Kaila *et al.*, 1989; Ulanganathan *et al.*, 1989; Prasad *et al.*, 1991a), greasy fleece yield (Lal *et al.*, 2000), dressed weight and dressing percentage (Prasad *et al.*, 1991b) and higher feed conversion efficiency (Prakash *et al.*, 1990). Dorset-Nellore crosses were found to

mature and conceive at an earlier age than Nellore (Gupta *et al.*, 1987). Taneja (1974) reported very low fertility and poor lamb and adult survival rates for Rambouillet sheep introduced into an arid zone in India. Also, F1 Rambouillet x Chokla ewes were less fertile than pure Chokla ewes, although survival of F1 lambs was better than that of either parent breed. However, these crossbreeding trials remained isolated experiments, not built upon further, were mostly of academic interest only and crossbreeding with exotic breeds had not helped to improve the apparel quality of Indian wool (Kurup, 1994). Examples of other breeding programs being carried out at the Central Sheep and Wool Research Institute (CSWRI) are as follows:

1. Improvement of Marwari sheep for carpet wool production through selection: The objective is to increase annual greasy fleece yield to 2 kg with an average fibre diameter of 30-40 micron and around 50% modulation.
2. Improving Malpura sheep for mutton production through selection and crossbreeding with Garole. These are being carried out in the elite flock of Malpura sheep developed through intense selection.

Three new sheep strains were produced at CSWRI in the 1990s (CSWRI, 1992);

1. The Avikalin for carpet wool and mutton production (evolved by stabilizing a crossbred population of Rambouillet x Malpura at 50% inheritance),
2. The Bharat Merino for fine wool and mutton production (evolved by crossing Rambouillet and Russian Merino with the indigenous breeds Malpura, Chokla, Nali and Jaisalmeri and exotic inheritance stabilized at 75%)
3. The fine wool Gaddi synthetic for the sub-temperate Himalayan region.

Selection programs for further improvement in these strains are ongoing. However, sustainable livestock improvement would not be possible for most tropical areas of the world without utilizing the evolutionary adaptation of the indigenous livestock breeds (Baker & Rege, 1994).

1.10 Outline of the study

The present study aimed at characterization of the genetic variation in economically and numerically important Indian sheep breeds and understanding their origin. Initially, the study was restricted to characterize only the five strains of the Deccani sheep (Lonand, Kolhapuri, Sangamneri. Madgyal and Sangola) using molecular markers. Subsequently the investigations were extended to include other economically

important sheep breeds from different geographical regions of India *viz.,* Garole, Bannur and Madras Red.

The low fertility in sheep is the most important constraint of the Indian meat industry in deriving benefits. Hence, there is a need to improve the reproductive performance of the Deccani breed as it is the numerically second most important sheep breed in India used mainly for lamb and meat production. One of the approaches to increase meat production is to introduce the prolificacy gene into non-prolific sheep breeds having desired traits. Therefore, prolificacy gene screening was carried out in the above mentioned breeds and an introgression study of the prolificacy gene from Garole into Deccani was studied. The effect and expression of the gene in the crossbred ewes was studied using molecular marker and ovulation rate and litter size analysis.

The study consists of five chapters as follows:

Chapter 1: Introduction and review of literature (current chapter)

Chapter 2: Molecular genetic diversity study of geographically and economically important Indian sheep breeds using nuclear markers

Genotyping of four Indian breeds *viz.,* Garole, Deccani (represented by five subtypes, Lonand, Madgyal, Sangamneri, Sangola and Kolhapuri), Bannur and Madras Red using 31 microsatellite markers and genetic diversity analysis using various population genetics softwares

Chapter 3: Phylogenetic relationships and population expansion study using mtDNA

Sequencing of the cytochrome *b* gene and D-loop region of mtDNA from three breeds (Bannur, Garole and Deccani) and analysis of the sequence data using various softwares

Chapter 4: Identification and analysis of prolificacy gene in a few Indian sheep breeds and its introgression into the Deccani breed

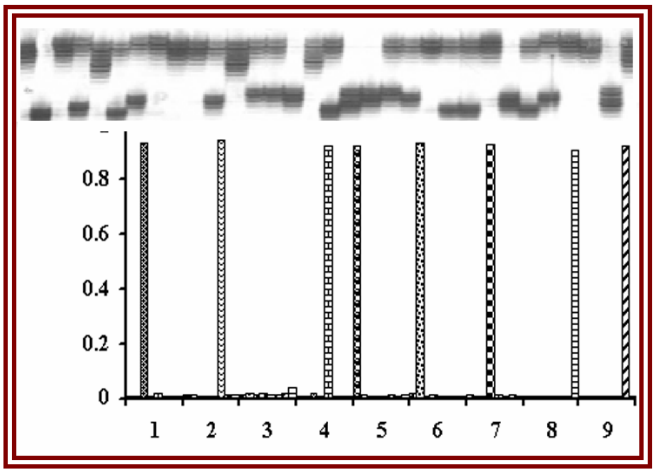
a) Sceening the four Indian sheep breeds *viz*., Garole, Bannur, Deccani and Madras Red for prolificacy gene mutation using PCR-RFLP method
b) Introgression study of the prolificacy gene from Garole into Deccani
c) Dampening effect of the prolificacy gene

Chapter 5: Summary and future approaches

Bibliography

Chapter 2

Molecular genetic diversity study of geographically and economically important Indian sheep breeds using nuclear markers



Contents of this chapter have been communicated to Animal Genetics

2.1 Introduction

The sheep (*Ovis aries* L.) plays a key role in the rural pastoral economy of arid and semi-arid zones of India. However, it is underutilized compared to other domestic livestock animals. Furthermore, the productivity of sheep is low due to poor exploitation of the genetic potential of indigenous animals, insufficient health cover, supply of feed and fodder and inadequate efforts to improve the existing sheep breeds (Acharya, 1982). The Indian sheep are reared in traditional sustainable conditions and are subjected to reduced levels of artificial selection. Sheep production systems in India differ from those in countries such as Australia, United States and the Europe. Typically, flocks are owned by illiterate nomads and flock sizes are sufficiently / relatively small (20-100), with an average flock size of ~ 40 so that the shepherds can readily identify all the animals within their flocks. The flocks are communally grazed on stubbles and straw in fields and very few sheep owners have their own pastures. A typical flock consists of animals of all age groups, and generally three rams are maintained per hundred ewes. Despite the communal grazing, mating is strictly controlled by applying various methods in different regions such as tying a cotton string around the prepuce and the neck of the testes of rams during the breeding season (Taneja, 1978). The rams are used for three to four years before culling and are generally not used when they are older than 6 years. The decision to cull a ram is often based on an observation of increased mortality within the flock, which is presumably a consequence of inbreeding. Replacement rams are purchased from remote market places rather than nearby villages.

Over 42 sheep breeds are found in various agro-climatic zones of the country (Acharya, 1982). However, the definition of a breed is subjective and may not reflect the true underlying genetic structure. Sometimes, closely related populations may be defined as separate breeds. Therefore, accurate evaluation and characterization of these breeds is an important task for sheep breeders and geneticist for both conservation and exploitation of the sheep genetic resources. Analysis of genetic variability within and among the breeds forms an initial step towards breed characterization and molecular markers, such as DNA markers, which are neutral in behavior, are widely used for this purpose. Microsatellites are currently the most popular markers of choice in livestock genetic characterization studies (Sunnucks, 2001). They are highly polymorphic in nature, show co-dominant behavior and are

dense in distribution. They are good predictor of overall genetic diversity and useful to understand recent evolutionary dynamics. Such studies along with phenotypic and demographic data can be used to design conservation and management strategies (Mortiz, 1994).

The present study was undertaken with an objective to compare the results of molecular data with traditional breed classification. Total 31 microsatellite markers were used to understand the population structure and genetic relationships among the four economically and numerically important Indian sheep breeds *viz*., Garole, Deccani (represented by the Lonand, Sangamneri, Kolhapuri, Sangola and Madgyal strains), Bannur and Madras Red. A recently developed genetic clustering approach was applied to the microsatellite data from these breeds. A comparative study was carried out between the accepted traditional breed definition and inferred population structure based on the molecular data. It is expected that the level of genetic variability present within and among the breeds and the prevalent population structure will help to determine an appropriate conservation strategy.

2.2 Material and methods

2.2.1 Sample collection

Blood samples from the four breeds were collected from major representative areas of each breed population (Fig. 2.1). Sampling of closely related animals was avoided to ensure representative samples in the absence of the detailed pedigree information. To alleviate sampling concerns, males unrelated at the level of grandparents and females from different litters and parities were sampled. For each breed, a maximum of 2-3 individuals per flock were sampled. The sample sizes, number of flocks sampled per breed and the summary statistics are presented in Table 2.1.

2.2.2 DNA isolation and PCR amplification using microsatellite markers

Genomic DNA was isolated from white blood cells using whole blood (Miller *et al.*, 1998) or FTA paper as per the manufacturer's instructions (Whatman Biosciences, UK). Thirty-one microsatellite markers (Table 2.2) exhibiting high allelic diversity were selected for this study. Twenty markers recommended in the "Measurement of Domestic Animal Diversity (MoDAD)" project of FAO (1996) were used. In addition, four unpublished markers (LPLP2, D9M1722, CANP3S4 and POLBF17;

details available at (*http://rubens.its.unimelb.edu.au/~jillm/jill.htm*) and six published markers MAF48, MAF33 and OarFCB11 (Maddox *et al.*, 1996; De Gortari *et al.*, 1998), KD101 (Davies & Maddox, 1997), SPAG7 (Moore *et al.*, 1997) and MMP9 (Maddox, 2001) were also used.

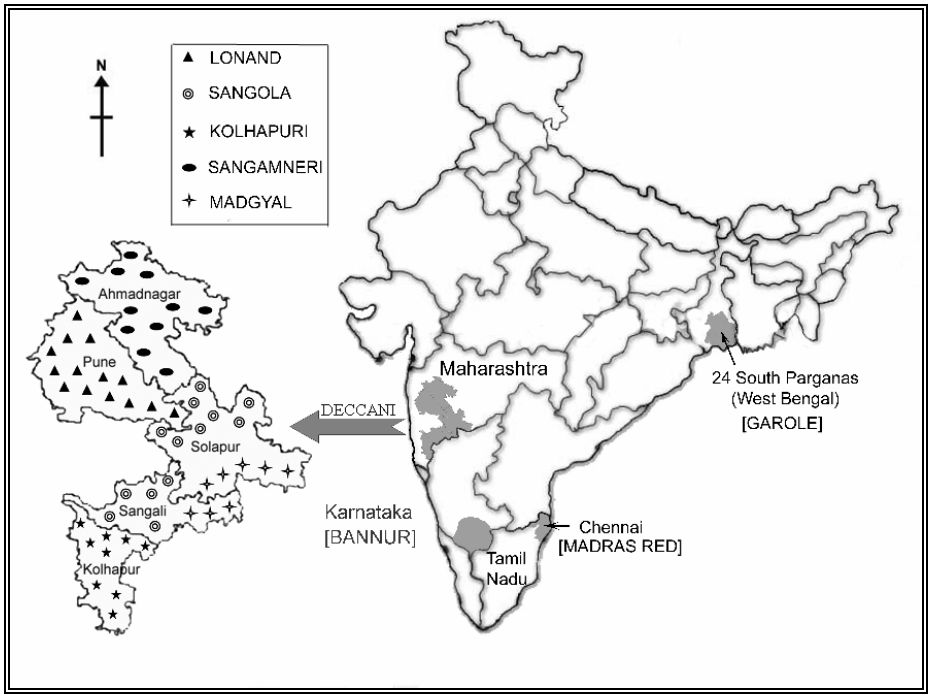


Figure 2.1: Geographical distribution of the four Indian sheep breeds

Thirty two nanograms of DNA or a single 1.2 mm FTA paper punch per sample per reaction was amplified in an 8 µL reaction volume containing 1X PCR buffer (10 mM Tris-HCl; pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% w/v gelatin), 100 µM of each of three unlabelled dNTPs (Amersham Biosciences, USA), 0.04 µCi, ^{33}P dATP (BRIT, India), 5 ng of forward and reverse primers, 0.44 µg of TaqStart antibody (Clontech, USA) and 0.2 U of AmpliTaq DNA polymerase (Perkin Elmer, USA) on a PTC 200 thermal cycler (MJ Research, USA). The PCR cycling profile consisted of initial denaturation (95ºC, 2.5 min) followed by 30 cycles of denaturation (95ºC, 10 s), annealing (at the respective temperature of each primer pair [Table 2.2], 30 seconds) and extension (72 ºC, 30 s) with a final extension of 72ºC for 2.5 min. The amplified PCR products were separated on 6% denaturing sequencing gel, exposed to X-ray film (Konica, India) for at least 24 hours and scored.

2.3.3 Statistical analysis

The allele scoring data were analyzed using POPGENE version 1.31 (Yeh *et al*., 1999) to calculate diversity parameters such as observed (*Ho*) and expected (*He*) heterozygosity and gene diversity for each microsatellite marker. The tests for deviations from Hardy–Weinberg Equilibrium (HWE) at each locus for each breed were performed using GENEPOP version 3.1 (Raymond & Rousset, 1995). The probability (*p*) values were corrected for multiple comparisons by applying a sequential Bonferroni correction (Hochberg, 1988). The population structure of the breeds was assessed using the F-statistics parameters *F* (F_{IT}, total inbreeding estimate), θ (F_{ST}, measurement of population differentiation) and *f* (F_{IS}, within-population inbreeding estimate) as proposed by Weir & Cockerham (1984). These parameters were computed using FSTAT version 2.9.3.2 (Goudet, 2002). The level of significance ($p < 0.05$) was determined from permutation tests with the sequential Bonferroni procedures. F_{ST} values among all possible breed pairs were calculated. Allelic richness based on minimum sample size ($n = 27$) was also estimated using FSTAT to eliminate the sample bias. The Principal Coordinate Analysis (PCoA) was performed on the Nei's genetic distance matrix using GenAleX version 6 (Peakall & Smouse, 2006) and the scatter was plotted on the first two coordinates. Breed differentiation was further investigated using Bayesian clustering approach as implemented in STRUCTURE version 2.0 (Pritchard *et al*., 2000). This program generates clusters of individuals based on their multilocus genotypes. The admixture model with a burn-in period of 1,00,000 iterations and 1,00,000 Markov Chain Monte Carlo (MCMC) repetitions was used to calculate the probable number of genetic clusters (*K*). Isolation by distance was tested by plotting Nei's standard genetic distances against the geographical distances. The significance of correlations was determined using the Mantel test (Mantel, 1967) as implemented in IBD version 2.1 (Jensen *et al*., 2005). Geographical distances among pairs of areas were calculated from linear distances between mean latitude and longitude positions of samples from each area and plotted against the Nei's genetic distances.

2.3 Results

2.3.1 Analysis of microsatellite loci in four Indian sheep breeds

Genotypic data from 31 microsatellite markers were used to assess the genetic diversity present in 319 animals representing four Indian sheep breeds. Representative pictures for each population are shown in Figure 2.2a – 2.2d. All the loci were polymorphic and amplified 229 alleles in total at an average of 7.39 alleles per marker. The allelic richness varied from 4.96 (Sangola) to 5.70 (Bannur) (Table 2.1), whereas the allele frequencies ranged from 4.16 - 5.12. Their allele distributions did not differ noticeably among the breeds and no significant difference in the predominant alleles was evident among the populations. Private alleles were present in all the populations (e.g., few alleles at some loci could be found in only one population). The mean observed (H_o) and expected heterozygosity (H_e) values for the breeds were in the range of 0.61-0.74 and 0.67-0.75, respectively. In case of Kolhapuri and Sangola, the observed heterozygosity values were higher than the expected heterozygosity values. In the case of Garole, the diversity values were more than those reported by Sodhi et al. (2003; H_o = 0.44, H_e = 0.59). Although varying among populations, the observed mean heterozygosity was lower than the expected mean heterozygosity across all the populations.

All the 31 loci were analyzed for Hardy-Weinberg Equilibrium (HWE) across the four breeds and were found to deviate from HWE when analyzed across populations (Table 2.3). These deviations might have resulted due to the small effective population sizes and the difficulties in collecting enough unrelated individuals. Among the breeds, the number of loci showing a significant deviation from HWE ranged from 5 (Madgyal) to 16 (Bannur), while the locus, OarAE129 deviated in maximum number of populations (6).

2.3.2 Genetic differentiation among populations

The breeds were further analyzed for population differentiation by calculating the fixation indices (F_{IT}, F_{IS} and F_{ST}) for each locus across the populations (Table 2.2). The overall estimates for F-statistics were significantly different from zero (p<0.05). The global heterozygote deficit across all populations (F_{IT}) amounted to 14% (p<0.05), whereas the overall significant (p<0.05) deficit of heterozygote because of inbreeding within breeds (F_{IS}) amounted to 3%. Eight loci (KD101, D9M1722, OarHH35, OarAE129, MMP9, POLBF17, OarJMP8 and HUJ616) contributed

significantly to the heterozygote deficit (F_{IS}) within populations, while 24 loci significantly affected the global heterozygote deficit (F_{IT}).

Table 2.1: Summary statistics presenting the sample size (*n*), flocks sampled per breed, mean allelic richness, average gene diversity, mean observed heterozygosity (H_o), mean expected heterozygosity (H_e) and mean within population inbreeding estimate (F_{IS})

Breed	Sample size (*n*)	Flocks sampled per breed	Mean allelic richness	Avg. Mean gene diversity	Mean *Ho*	Mean *He*	Mean F_{IS}[1]	No. of loci not in HWE
Garole	32	18	5.05	0.69	0.61	0.68	0.110*	11
Kolhapuri	62	30	5.18	0.72	0.74	0.71	-0.026	13
Lonand	40	20	5.42	0.75	0.74	0.74	0.005	14
Madgyal	27	12	5.10	0.72	0.70	0.71	0.032	05
Sangamneri	56	30	5.67	0.76	0.74	0.75	0.019	14
Sangola	38	16	4.96	0.68	0.68	0.67	0.007	12
Madras Red	32	16	5.39	0.71	0.66	0.72	0.096*	10
Bannur	32	16	5.70	0.69	0.68	0.68	0.019	16
Total/ mean	319	158	5.31	0.72	0.69	0.71	0.033	95

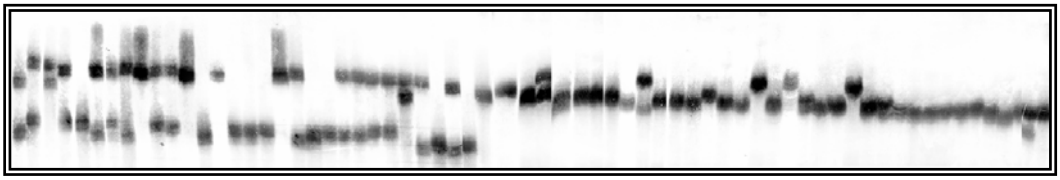
[1] **Mean estimates from Jackknife over loci. * $p< 0.05$ from permutation tests performed using FSTAT program.**

Table 2.2: Chromosome location, annealing temperature and F-statistic analysis of 31 microsatellite loci in Indian sheep populations

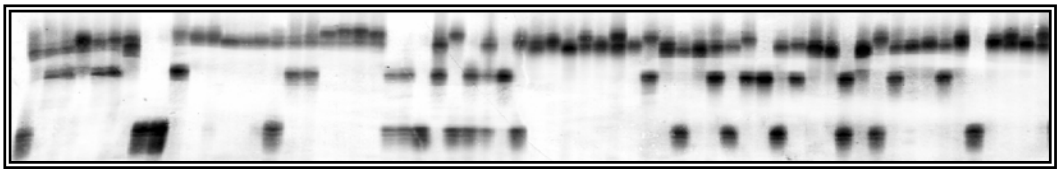
Locus	Chromosomal location	Annealing temperature (°C)	*F* (F_{IT})	θ (F_{ST})	*f* (F_{IS})	Populations not in HWE
KD101	2	58	0.174*	0.097*	0.085*	5
D9M1722	8	55	0.227*	0.156*	0.081*	5
LPLP2	22	58	0.309*	0.275*	0.049	5
CANP3S4	7	59	0.093*	0.057*	0.038	3
BM6506	1	58	0.167*	0.191*	-0.032	3
OarHH35	4	58	0.220*	0.093*	0.140*	4
MAF33	11	58	0.241*	0.256*	-0.021	4
OarFCB11	1	54	0.125*	0.166*	-0.049	2
OarAE129	5	55	0.229*	0.146*	0.099*	6
MAF48	X	50	0.208*	0.134*	0.086	2
MMP9	9	55	0.247*	0.126*	0.139*	4
POLBF17	5	52	0.249*	0.125*	0.141*	3
BM757	9	63	0.184*	0.113*	0.080	1
SPAG7	9	60	0.134*	0.137*	-0.004	1
OarCP38	10	55	0.270*	0.241*	0.038	3
OMHC1	20	52	0.008	0.064*	-0.060	4
OarJMP29	24	52	0.016	0.036*	-0.020	1
OarHH41	10	63	0.049*	0.095*	-0.051	2
BM827	3	55	0.134*	0.093*	0.046	1
BM6526	26	52	0.141*	0.107*	0.038	2
OarHH64	4	55	0.113*	0.063*	0.053	3
BM8125	17	55	0.111*	0.106*	0.007	5
RM4	15	52	0.006	0.072*	-0.070	2
ILSTS005	5	55	0.090*	0.081*	0.009	4
OarHH47	18	60	0.046	0.070*	-0.026	2
OarCP34	3	58	0.083*	0.119*	-0.040	3
OarFCB48	17	55	0.032	0.065*	-0.037	5
OarJMP8	6	52	0.128*	0.079*	0.053*	2
HUJ616	13	52	0.133*	0.077*	0.060*	2
OarVH72	25	55	0.031	0.079*	-0.052	1
OarFCB128	2	60	0.041	0.059*	-0.019	4

*** $p < 0.05$ from permutation tests performed using FSTAT program.**

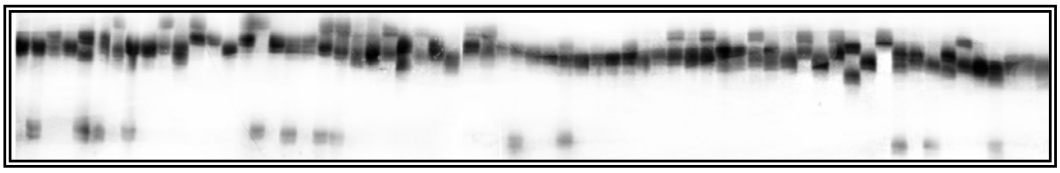
Garole and Bannur (D9M1722)



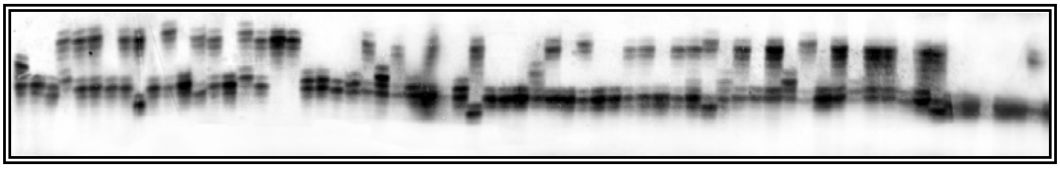
Garole and Bannur (OarHH 64)



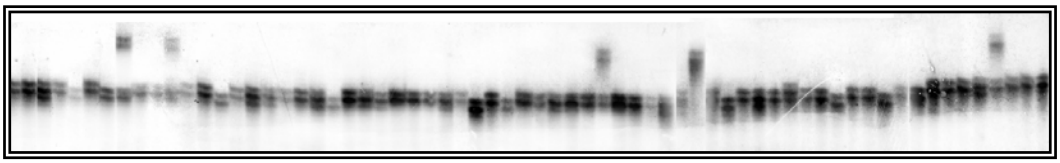
Garole and Bannur (OarHH 35)



Kolhapuri ((POLBF17)



Kolhapuri (OarAE129)



Kolhapuri (OV4)

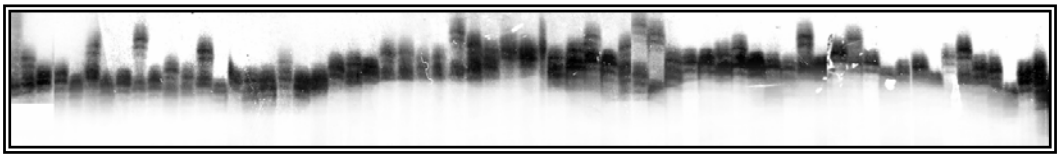


Figure 2.2a: Representative microsatellite profiles for Garole, Bannur and Kolhapuri populations

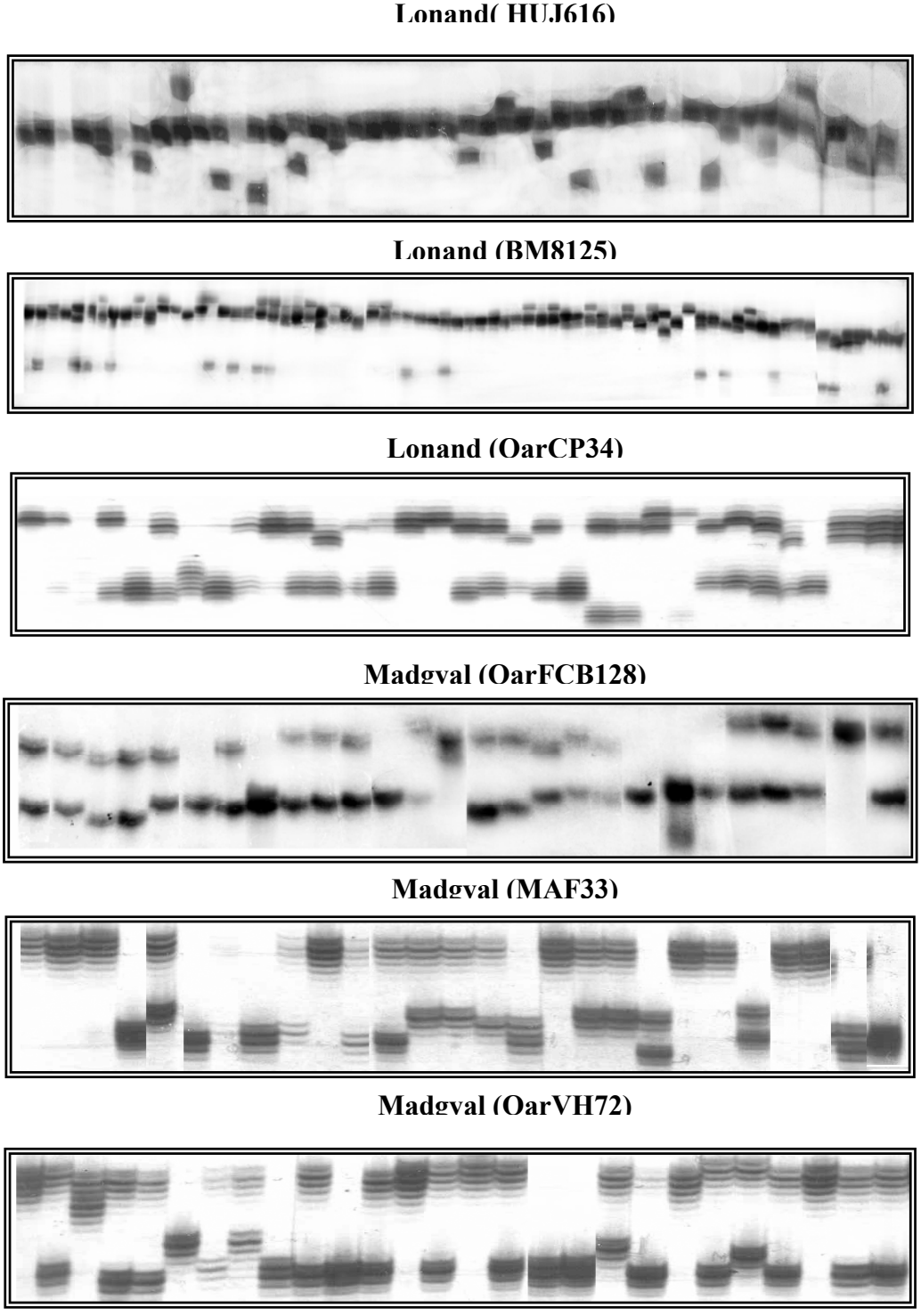
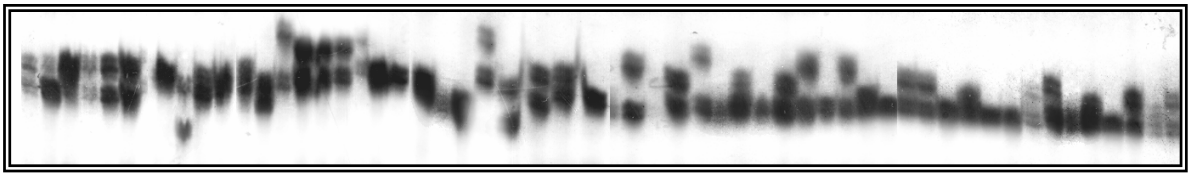
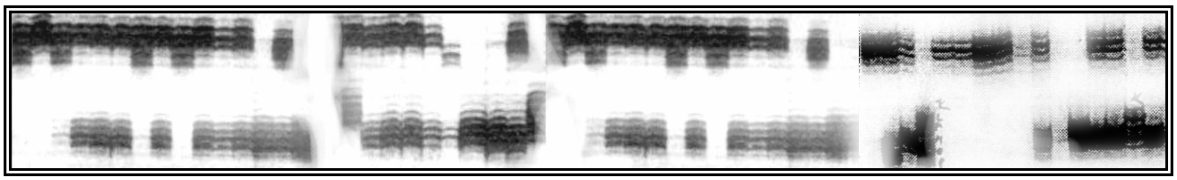


Figure 2.2b: Representative microsatellite profiles for Lonand and Madgyal populations

Sangmaneri (LPLP2)



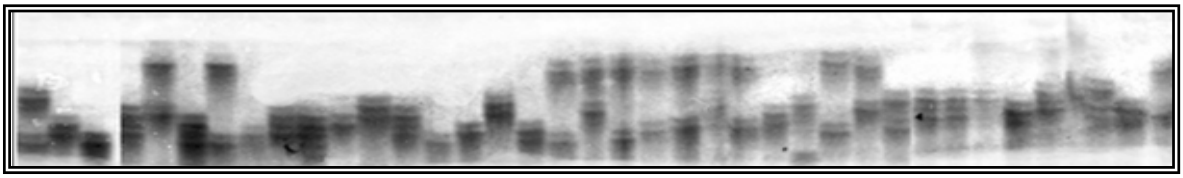
Sangmaneri (BM757)



Sangmaneri (OarJMP8)

Sangola (OarCP38)

Sangola (OMHC1)



Sangola (OarFCB48)

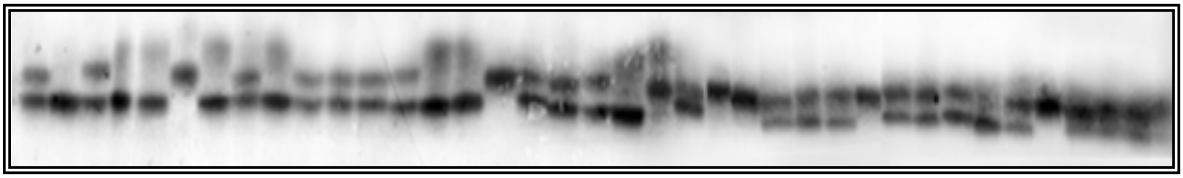


Figure 2.2c: Representative microsatellite profiles for Sangamneri and Sangola populations

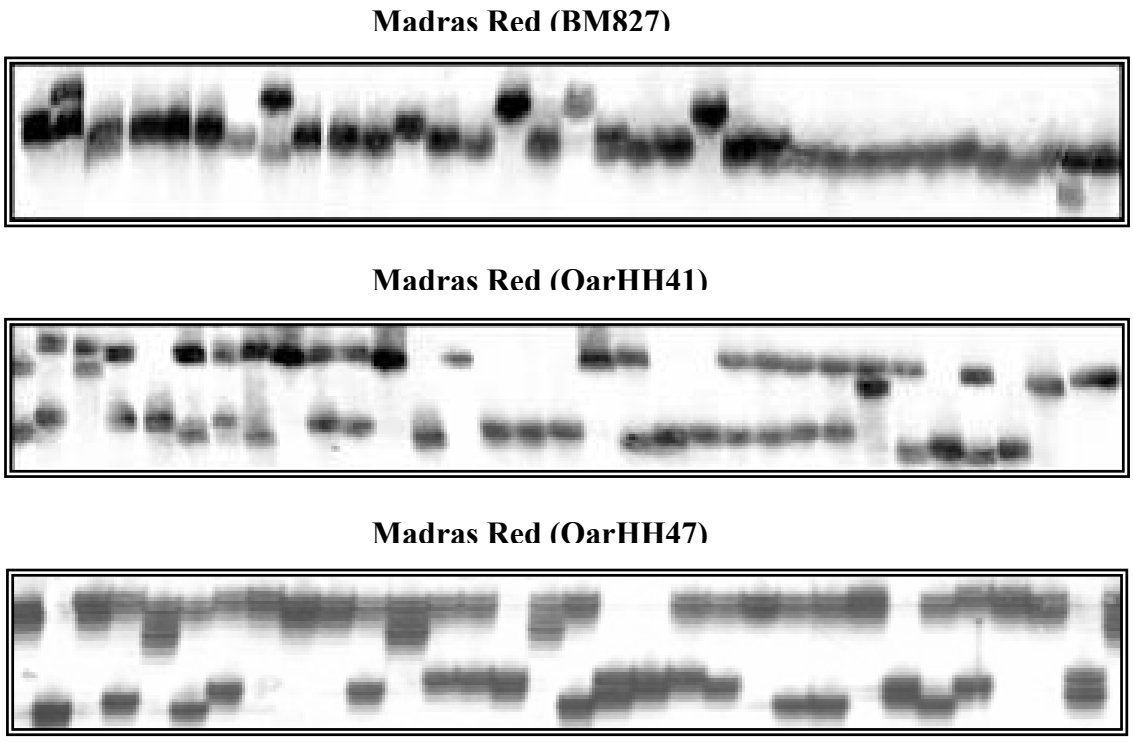


Figure 2.2d: Representative microsatellite profiles for Madras Red population

The F_{ST} values among the breeds revealed a moderate level of genetic differentiation where 12% of the total genetic variation corresponded to the differences among the breeds, while the remaining 88% was the result of differentiation among individuals. The F_{ST} values for each pair of the breeds ranged from 5% (Lonand- Sangamneri) to 15% (Madras Red-Bannur and Sangola-Bannur) [Table 2.4], which indicated that only 5-15 % of the microsatellite variability was due to the subdivision of the population into the breeds, while the remaining variability was within the breeds. The mean F_{IS} values for Garole (0.110) and Madras Red (0.096) indicated a significant heterozygote deficit (p<0.05) (Table 2.1). In contrast, the Kolhapuri strain of Deccani breed exhibited insignificant heterozygosity excess, as indicated by negative F_{IS} value (-0.026). Similarly, the F_{IS} values for other Deccani strains, Lonand (0.005), Madgyal (0.032), Sangamneri (0.019) and Sangola (0.007) (Table 2.1), were positive but not significant. The overall F_{IS} was rather low (3.3%) but significantly higher than zero indicating inbreeding within the populations.

Table 2.3: Loci deviating from the Hardy-Weinberg Equilibrium

Garole	Kolhapuri	Lonand	Madgyal	Sangamneri	Sangola	Madras Red	Bannur
D9M1722	KD101	KD101	KD101	KD101	KD101	D9M1722	LPLP2
LPLP2	CANP3S4	D9M1722	OarAE129	D9M1722	D9M1722	MAF33	BM6506
OarAE129	OarHH35	LPLP2	POLBF17	LPLP2	LPLP2	OarAE129	OarHH35
OarCP38	MAF33	BM6506	OarHH41	BM6506	OarHH35	MAF48	CANP3S4
OMHC1	OarAE129	MMP9	BM8125	OarHH35	CANP3S4	BM6526	MAF33
OarHH64	MAF48	POLBF17		OarFCB11	POLBF17	BM8125	OarFCB11
ILSTS005	BM757	OarHH41		OarAE129	OarCP38	RM4	OarAE129
HUJ616	OarCP38	BM8125		MAF48	OMHC1	OarFCB48	MMP9
HUJ616	OMHC1	ILSTS005		MMP9	ILSTS005	OarJMP8	OMHC1
MMP9	BM6526	OarCP34		SPAG7	OarHH47	OarFCB128	OarJMP29
BM8125	OarHH64	OarFCB48		RM4	OarFCB48		BM827
	OarCP34	OarJMP8		ILSTS005	OarFCB128		OarHH64
	OarFCB48	HUJ616		OarHH47			BM8125
		OarFCB128		OarVH72			OarCP34
							OarFCB48
							OarFCB128

2.3.3 Breed relationships

Nei's genetic distances among each breed pair were estimated, which ranged from 0.20 (between Lonand and Sangamneri) to 0.60 (between Sangamneri and Bannur) (Table 2.4). Principal coordinate analysis was also performed using the Nei's genetic distance matrix, which showed that the four breeds were well separated including the Deccani strains. The Deccani strains, Lonand, Sangamneri and Madgyal, clustered together (Fig. 2.3), while Kolhapuri clustered with Garole and Sangola with Madras Red. The scatter of all the breeds based on the first and second principal coordinates represented 24.81% and 23.33% of the total variation, respectively. The relationship between genetic and geographic distances, examined using the program IBD (Jensen *et al*., 2005), indicated significant isolation-by-distance (IBD) relationship across the sampling range ($r = 0.390$, $p < 0.027$) (Fig. 2.4).

2.3.4 Population subdivision

In order to understand the breed differentiation, an additional population subdivision analysis was performed with the program STRUCTURE version 2.0 (Pritchard *et al*., 2000; Falush *et al*., 2003). The program implements a model-based clustering method to infer population structure using multi-locus genotypes to identify genetic clusters existing within the breeds and to verify the assignment of individuals to their correct breeds. For each individual, STRUCTURE estimates the proportion of ancestry from each of the *K* clusters (inferred populations). Admixture model was used for the analysis and the program was initially run with an arbitrary number of populations, ranging from *K* = 1 to 12, so as to choose the appropriate value of *K* to better model the whole data set.

The best value of *ln* '*Pr(X/K)'* (i.e. the natural logarithm of the probability that a given genotype *X* is part of a given population *K*) was obtained for *K* = 9 (-29188.3). Additionally, the highest posterior probability value (0.8175) was also obtained for *K* = 9. Hence, further analysis was performed using this *K* value for the number of populations. These nine inferred populations would correspond to the ancestral populations from which the recent breeds may have been drawn.

Table 2.4: F_{ST} estimates (above diagonal) and Nei's genetic distance (below diagonal) between each pair of sheep breeds

Population	Garole	Kolhapuri	Lonand	Madgyal	Sangamneri	Sangola	Madras Red	Bannur
Garole		0.11	0.12	0.13	0.12	0.14	0.13	0.14
Kolhapuri	0.36		0.11	0.10	0.10	0.12	0.11	0.14
Lonand	0.47	0.37		0.12	0.05	0.13	0.11	0.13
Madgyal	0.49	0.38	0.50		0.11	0.12	0.07	0.14
Sangamneri	0.46	0.39	0.20	0.45		0.13	0.09	0.14
Sangola	0.45	0.41	0.42	0.41	0.39		0.13	0.15
Madras Red	0.48	0.40	0.46	0.25	0.40	0.45		0.15
Bannur	0.47	0.54	0.50	0.52	0.60	0.50	0.58	

The program computed allelic frequency expected at each locus for the inferred populations and the proportion of each breed in these nine inferred populations. The contribution of each sheep population genomes to the nine genetically inferred

clusters has been illustrated in Fig. 2.5. As clear from the figure, the clusters 5, 8 and 9 were mainly formed by the Garole, Bannur and Madras Red individuals, respectively, demonstrating that these breeds had particular genetic background that could be differentiated. Whereas, the inferred clusters, 1, 2, 4, 6 and 7, were formed by individuals from the Deccani strains (Lonand, Sangola, Sangamneri, Kolhapuri and Madgyal, respectively). Surprisingly, cluster three had nearly equal contribution from all the breeds.

2.4 Discussion

2.4.1 Genetic variability in four Indian sheep breeds

Genetic diversity is reflected by the number of alleles per locus, average number of alleles for all the loci and heterozygosity. A total of 229 alleles were amplified at the 31 loci across the four breeds with an average of 7.39 alleles per locus. According to Takezaki & Nei (1996) and Barker (1994), the markers to be useful for measuring genetic variation should have an average heterozygosity of 0.3 to 0.8 and no fewer than four alleles in the population, in order to reduce the standard errors of distance estimates, respectively. The range of heterozygosity of the markers in this study was between 0.61 and 0.74 with 7.39 average numbers of alleles per locus, and therefore, the markers can be considered as appropriate for measuring genetic variation in the four sheep populations. For the Deccani strains (except Kolhapuri) and Bannur the mean observed and expected heterozygosity values were similar, supported by F_{IS} estimates that were not significantly different from zero (Table 2.1). The average genetic variation observed in each breed (0.68 - 0.75) was slightly higher than that reported for other Indian breeds; Muzzafarnagri: 0.65 (Arora & Bhatia, 2004), Nali: 0.65 and Chokla: 0.66 (Sodhi *et al*., 2006) Hassan: 0.53 (Sharma *et al*., 2006) and Bellary: 0.68 (Kumar *et al*., 2007b) as well as other foreign breeds e.g. Swiss sheep breed Mouflon: 0.45 (Stahlberger-Saitbekova *et al*., 2001), Northern Spanish sheep-Laxta: 0.66, Black-faced Laxta: 0.06, Rubin del Molar: 0.60, Churra: 0.66 and Xalda: 0.57 (Alvarez *et al*., 2004). However, the values were similar to the observed genetic variation in Spanish sheep: 0.77, Awassi sheep from Turkey: 0.75 (Arranz *et al*., 1998) and Castellana: 0.71 (Alvarez *et al*., 2004).

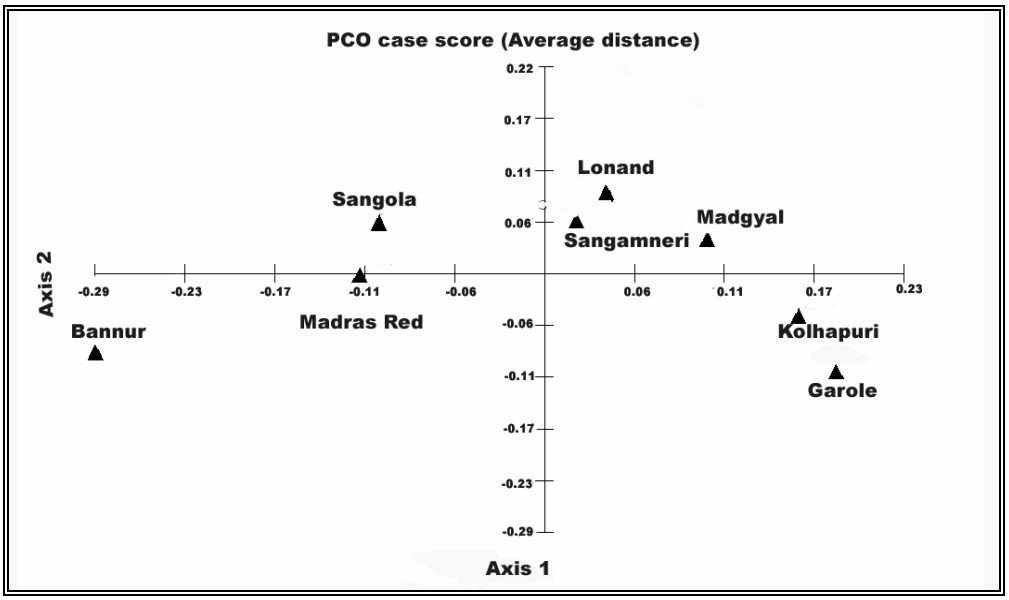


Figure 2.3: Principal Coordinate Analysis of the Nei's genetic distance from the four Indian sheep breeds

The first two coordinates represent 24.81% and 23.33% of the total genetic variation, respectively

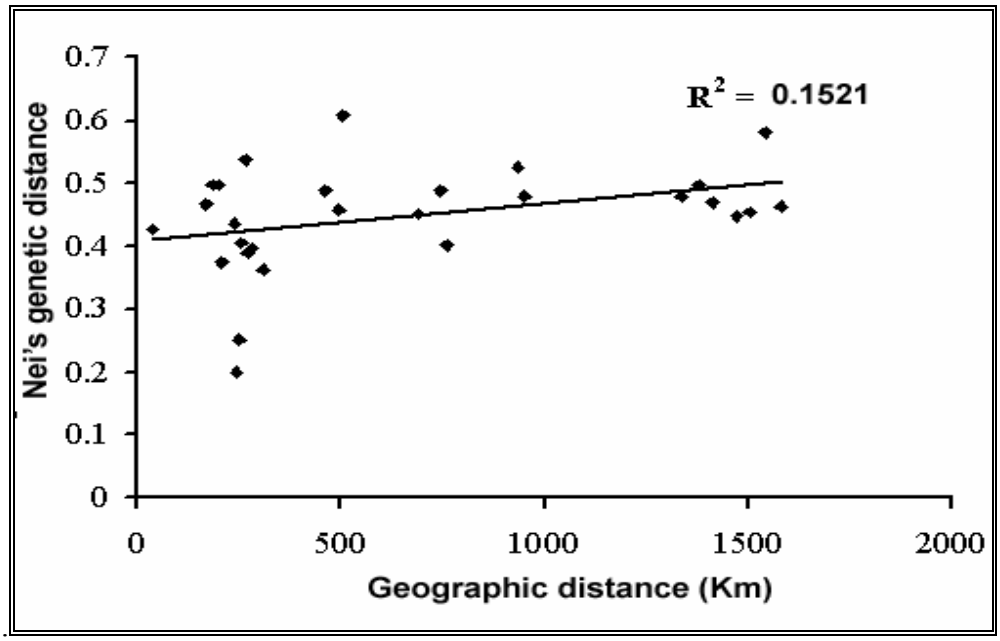


Figure 2.4: Scatter plot of pair-wise Nei's standard genetic distance vs. geographical distance

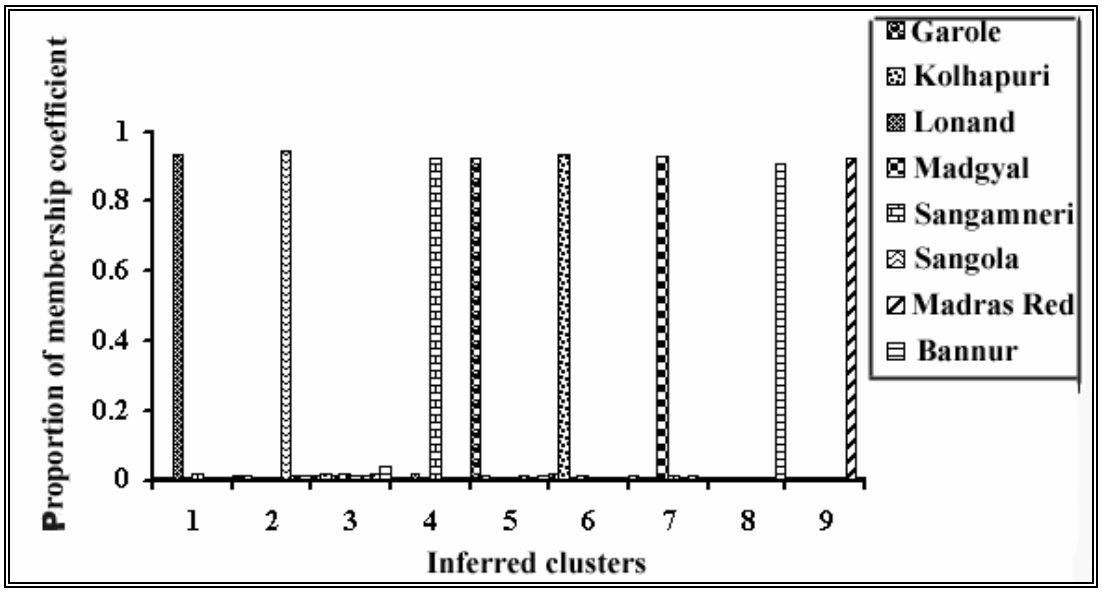


Figure 2.5: Proportion of membership coefficient of the Indian sheep breeds into nine inferred clusters obtained using the STRUCTURE program

The average gene diversity of the Garole breed observed in this study (0.69) was slightly higher than that reported (0.60) by Sodhi *et al*. (2003). However, the present and previous studies cannot be directly compared owing to the different marker sets used; the present study includes 11 markers in addition to those suggested by FAO for genetic diversity analysis. However, broadly the results were comparable to the data published for Indian and European sheep breeds (Arranz *et al*., 1998, 2001; Stahlberger-Saitbekova *et al*., 2001; Sodhi *et al*., 2003; Arora & Bhatia, 2004; Mukesh *et al*., 2006).

Among the Deccani strains, Kolhapuri and Sangola showed higher observed heterozygosity than expected. Additionally, negative F_{IS} value was observed in Kolhapuri, which supports the presence of high heterozygotes in Kolhapuri. Two factors might be responsible for this: the low selection pressure because of the lack of efforts for genetic improvement of these sheep breeds and the probable presence of different genetic lineages within these breeds. Alternatively, this effect might be a result of isolated subpopulations existing within the breed and causing the concomitant excess of homozygotes within the subpopulations. However, when these subpopulations are joined together in one breed/strain, a high heterozygosity is observed at the breed level.

A total of 248 HWE tests (31 markers and 8 populations) were carried out and the heterozygote deficiency analysis revealed that all the four breeds exhibited

significant deviations from HWE at several loci ($p< 0.05$). Although, it is difficult to deduce the exact basis of this departure from HWE, the presence of low frequency of null alleles segregating at these loci might be one of the possible reasons, especially for the locus OarAE129, which showed deviation in six populations. The null alleles could also result from mutations in the primer binding sites failing to amplify in PCR (Callen *et al*., 1993; Pemberton *et al*., 1995). Secondly, the positive F_{IS} values observed could also contribute to the observed deviation. Several factors such as sample relatedness, genetic hitchhiking and population heterogeneity might be responsible for the positive F_{IS} (Nei, 1987). However, it was presumed that the heterozygote deficiency observed in the present study could possibly result from sampling errors (relatedness of few samples under field conditions) than the presence of null alleles in the investigated populations. Considering the demographic/flock structure, wherein the rams may breed with all the ewes in the flock and are housed and grazed together, no controlled mating within the flock is practiced at the shepherd's level. In addition to this, the use of only few breeding rams per flock (3 per 100 ewes) might also be responsible for the relatedness of some samples due to presumed inbreeding. These practices lead to small effective population size or mating between relatives and the difficulties in collecting enough unrelated individuals with consequent genetic drift. Therefore, inbreeding might be the most reasonable cause of heterozygote deficit observed in the present study.

2.4.2 Genetic differentiation among populations

The significant positive F_{IS} values observed in the Garole and Madras Red populations indicated high levels of inbreeding in these populations, which presumably might have resulted from the unplanned and indiscriminate mating prevalent in the breeding region. All the Deccani strains as well as Bannur showed slight heterozygote deficiency, which was however, not significant. The Kolhapuri strain showed marginal heterozygote excess (F_{IS} = -0.026), which could be attributed to the high gene diversity (0.72) as well as the higher observed heterozygosity (0.74) indicating a high gene flow among individuals of this population. Since only 25% of the loci contributed to the average F_{IS} value (0.033), the effect of null alleles in addition to sample relatedness could also be the likely cause of positive F_{IS}.

All the loci significantly contributed to the overall breed differentiation. The level of apparent breed differentiation was moderate but significantly different from

zero (F_{ST} = 12%), indicating a relatively low gene flow among the breeds studied. This moderate differentiation probably results from the bottleneck through reproductive (Takezaki & Nei, 1996) and geographical isolation. Gene flow among the four breeds (Garole, Deccani, Bannur and Madras Red) has probably been restricted by the geographical distance and cultural separation of the communities rearing the animals, in addition to adherence of the shepherds to the traditional sheep husbandry practices to maintain purity of the breeds, resulting in reproductive isolation of the breeds. This was evident even in case of the Deccani strains, which also showed moderate differentiation even though these strains are located in geographic proximity. Further, the large population size of the Deccani breed, and thus presumably large effective population size, might have reduced the effect of drift resulting in moderate genetic differentiation between the morphologically well-differentiated and isolated strains. The remaining 88% of the genetic differentiation was due to the variation within breeds, indicating that the large proportion of variation resides among individuals of breeds rather than among the breeds. The observed genetic differentiation (F_{ST} = 12%) was lower than that observed in the desert bighorn sheep (F_{ST} = 26.0%; Gutierrez-Espeleta *et al*., 2000), and Korean and Chinese domestic goats (F_{ST} = 20.2%, Kim *et al*. 2002). However, it was slightly less than that reported for other Indian breeds (Nali, Chokla and Garole: 18.3%; Mukesh *et al*., 2006) and for other domestic species such as Swiss (F_{ST} = 17.0%; Saitbekova *et al*., 1999) and Asian goats (F_{ST} = 14.3%; Barker *et al*., 2001). Conversely, the F_{ST} values obtained in this study were higher than those reported for other species such as Spanish dog breeds (F_{ST} = 9.9%; Jordana *et al*., 1992), Spanish horse breeds (F_{ST} = 7.8%; Ca˜n´on *et al*., 2000) and South European beef cattle breeds (F_{ST} = 6.8%; Jordana *et al*., 2003).

2.5.3 Assessment of interbreed relationships

The microsatellite marker based phylogenies supported the morphology and geography based classification of the breeds. The PCoA revealed separation of the breeds more clearly among the populations of different geographical locations and the first three principal coordinates explained 65% of the total genetic variation. The Lonand, Sangamneri and Madgyal strains clustered together, which is well explained by their geographic proximity and low genetic distance (Table 2.4). Similar population clustering according to geographic location was previously observed in

microsatellite analyses of humans (Bowcock *et al.*, 1994), cattle (McHugh *et al.*, 1997), chicken (Wimmers *et al.*, 2000) and Indian goat (Rout *et al.*, 2008). Studies using the mitochondrial DNA in Garole, Bannur and Deccani (Lonand and Sangamneri) as discussed in the next chapter (Chapter 3) also revealed geographical clustering of these breeds. Surprisingly, Kolhapuri clustered with Garole and Sangola with Madras Red. As these breeds are located in different geographic regions (Fig. 2.1), one of the possible reasons for this could be common founder populations from which these breeds might have originated. However, it is difficult to prove this as no pedigree records were maintained for the Indian sheep breeds, although cytochrome *b* gene analysis as detailed in the next chapter (Fig. 3.2; Chapter 3) showed that Garole and Kolhapuri shared some haplotypes. The genetic distances among the Deccani strains were similar to the distances among the Garole, Bannur and Madras Red breeds. The extent of genetic distance among populations is generally assumed to reflect the time since their divergence from a common ancestral population. In addition, if the populations show reasonable divergence through genetic distance, then it is implied that there has been sufficient time for the populations to accumulate adaptive differences by differential selection. The Deccani breed has been divided into five strains, which are traditionally bred in different districts of Maharashtra state, which imposes effective reproductive isolation than the geographical isolation. This breeding strategy yields divergence among strains producing an overall heterogeneous population of the Deccani breed, made up of several isolated and homogeneous groups in which even morphological differences could be recognized. This result is partly supported by the mtDNA analysis as discussed in the next chapter (Chapter 3) of the Lonand and Sangamneri strains, which are in geographic proximity and cluster together in PCoA, but do not share a single haplotype, as well as the structure analysis wherein each strain forms a separate cluster.

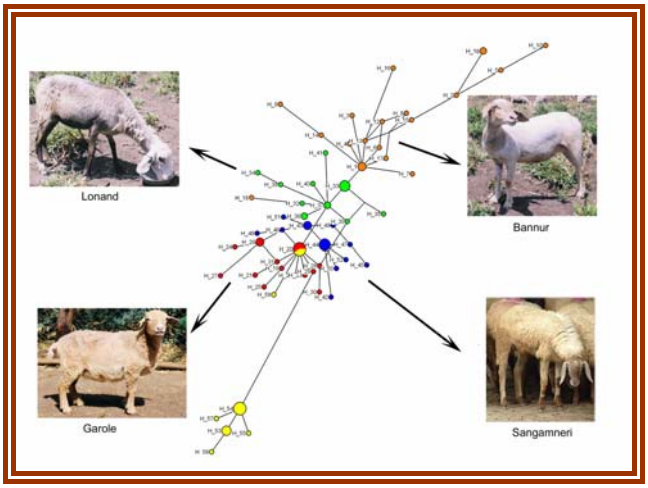
2.5.4 Population structure

The inbreeding values obtained for all the breeds indicated either significant inbreeding and/or a Wahlund effect (genetic structure within breed samples). As the samples were collected from 12-30 flocks per breed, the presence of a hidden genetic structure (not strong enough to be evident using PCoA) cannot be ruled out. Hence, a more robust and recently developed Bayesian MCMC clustering method based on multiple loci (Pritchard *et al.*, 2000) was used to confirm the results of the PCoA. The

result (Fig. 2.5) reveals that each cluster (except cluster 3) represents one breed/strain and supports the traditional morphology/geography based classification. Moreover, the morphological differences within the Deccani strains were also supported by the present molecular data, which further provide the evidence of genetic differentiation of the Deccani strains. The genetic structure observed in the Deccani strains indicates that the strains might be evolving into separate breeds. The structure of the sheep populations obtained using the STRUCTURE program was well supported by IBD ($r = 0.390$, $p<0.02$). The analysis showed that all the breeds were genetically and geographically distant from each other, indicating that isolation by distance played an important role in development of the indigenous breeds in India.

Chapter 3

Phylogenetic relationship and population expansion study using mtDNA



Part of the results from this chapter has been published in Animal Genetics and Journal of Heredity (Received the status of cover page of the journal) while part has been communicated to BMC Genetics

3.1 Introduction

Ever since the domestication, sheep (*Ovis aries* L.) have played an important role in diverse human societies, forming a major component of agro-pastoral systems. The domestic sheep is an important livestock species in India and represent an integral part of the farming system and rural economy. Traditionally, Indian sheep are reared by various communities such as Dhangar in Maharashtra, Guddi in Himachal Pradesh and Raika in Rajasthan, whose traditional occupation is sheep breeding (Karve, 1961). These communities follow strict rules for the migration, distribution and conservation of resources. Sheep are reared under the transhumance system of management, wherein migration is determined by the availability of fodder, the farming system and the climatic conditions. Movement usually does not occur between states, and the migratory route of each shepherd community is often fixed. Such rigid management and the reluctance of the sheep herding communities to give up their traditional practices and customs have likely been responsible for the maintenance of sheep breed diversity in India (Acharya, 1982).

To date, there are very few reports of extensive evaluations of the genetic diversity existing within Indian sheep and all of them revealed high levels of allelic diversity (Arora & Bhatia, 2004; Mukesh *et al.*, 2006; Sharma *et al*., 2006; Sodhi *et al*., 2006; Kumar *et al*., 2007b). Such studies are important to document the population structure, priority for conservation and likely origin of Indian sheep. The sequence variation existing within the mitochondrial DNA (mtDNA) has proven particularly useful for addressing such questions across a number of livestock species. The observation that mtDNA haplotypes group together in distinct clades has revealed insights into the history of domestication in cattle (Loftus *et al.*, 1994; Bradley *et al.*, 1996), pig (Guiffra *et al.*, 2000), goat (Luikart *et al.*, 2001) and horse (Vila *et al.*, 2001). Domestic sheep have a global distribution and a growing number of genetic surveys have been conducted, which report on the haplotypes and mix of mtDNA lineages observed within the indigenous breeds. A general finding has been the presence of multiple mtDNA lineages within breeds, independent of their geographic location or phenotype. A survey of 19 Chinese breeds found that 15 contained a mixture of haplotypes from lineages ‘A’, ‘B’ and ‘C’ (Chen *et al.,* 2006). The clade ‘C’ has also been reported in low frequency in Turkish sheep breeds (Pedrosa *et al.*, 2005) and Portugal (Pereira *et al.,* 2006). However, an investigation of the Near East

breeds reported the presence of five lineages; 'A', 'B', 'C', 'D', and 'E'; Meadows *et al.,* 2007), while an examination of a range of European breeds found the presence of four lineages ('A', 'B', 'C' and 'D'; Tapio *et al.*, 2006a) and the 'A' and 'B' types occur at differing frequencies. The existence of multiple mtDNA lineages and their mixing within breeds has been interpreted as evidence for multiple domestication events and subsequent human-mediated introgression between domestic sheep populations. To date, no mtDNA sequence has been reported from any Indian breed. This is the first study of mtDNA sequence variation within Indian sheep breeds. The objective of this study was to investigate the phylogenetic and phylogeographic structure of three Indian sheep breeds using mtDNA sequence variation. The genetic relationship among the breeds was examined and compared with other European and Asian populations to begin a detailed documentation of India's ovine biodiversity.

3.2 Materials and methods

3.2.1 Sample collection and DNA isolation

This has already been described in chapter 2 (Fig. 2.1 and Table 3.2 & 3.6). Note: For control region analysis two Deccani strains (Lonand and Sangamneri) and for cytochrome *b* gene analysis four Deccani strains (Lonand, Sangamneri, Kolhapuri and Madgyal) were used to represent the Deccani sheep population. The samples of the Madras Red breed were not included in these studies due to paucity of mtDNA isolation from FTA papers.

3.2.2 PCR amplification and mtDNA sequencing

Two regions of the mitochondrial DNA (mtDNA) genome were amplified and sequenced using primers designed from the complete ovine mtDNA (AF010406; Hiendleder *et al.* 1998a).

The primers CytB-F (5′-GTCATCATCATTCTCACATGGAATC-3′) and CytB-R5′-(CTCCTTCTCTGGTTTACAAGACCAG-3′) were used to amplify a 1272 bp region of the mitochondrial cytochrome *b* gene (*cytB*; AF010406 positions 14078 to 15349) and

The primers mtCR-F2 (5′-AACTGCTTGACCGTACATAGTA-3′) and mtCR-R1 (5′-AGAAGGGTATAAAGCACCGCC-3′) were used to amplify a 1246 bp fragment spanning part of the mtDNA control region, the tRNA-Phe and 12S rRNA coding

RNA genes (AF010406 positions 15983 to 592). This region has been referred as the "control region" throughout the thesis. PCR was carried out using 20 µL reaction volumes containing 50 ng of DNA, 0.5 µM of each primer, 100 µM of each dNTP, 1X PCR buffer containing 2.0 mM $MgCl_2$ and 0.2 µL of Titanium *Taq* polymerase (Clontech, USA) in a PTC 200 thermal cycler (MJ Research, USA). The cycling conditions were as follows: initial denaturation at 94˚C for 10 min followed by 30 cycles of 94˚C for 45 s, 60˚C for 45 s, and 72˚C for 45 s with final extension at 72˚C for 10 min. The resulting PCR products were bidirectionally sequenced using the DYEnamic ET sequencing kit and the MegaBACE 1000 DNA Analysis System (GE Healthcare, USA).

3.2.3 Data analysis

The DNA sequences were edited manually by inspecting the electrophorograms using CHROMAS LITE 2.01 (http://www.technelysium.com.au). Total 73 (EF056393–EF056465) and 132 (FJ218019-FJ218150) sequences were obtained from the control region and cytochrome *b* gene, respectively. In addition, the reported *cytB* sequences of domestic and wild sheep were downloaded from GenBank (Representative sequences of type B, C, D and E haplotypes (n = 9, AF010406, DQ852077-DQ852082 and DQ097429-DQ097430) and *O. vignei* (AF034729), *O. orientalis* (AJ867261) and *O. ammon* (AJ867272) and used for a combined analysis with sequences of this study. For comparative analysis of the sheep breeds from different geographic regions, mtDNA control region (1061 bp) and cytochrome *b* (967 bp) sequences available from GenBank (Table 3.1) were included and aligned using CLUSTALX 1.83 (Thompson *et al.,* 1997). All the sequences were trimmed to 967 bp (*cytB*) and 1061 bp (control region), respectively. The alignments were imported in MEGA 3.1 (Kumar *et al.* 2004) and inspected for the presence of singletons (polymorphic sites appearing in only one animal) and parsimony-informative sites (which appear in more than one animal). For the phylogenetic analysis, the singletons were replaced with the consensus base, as they were not considered phylogenetically informative. Diversity parameters such as nucleotide diversity (π) and haplotype diversity (*Hd*) were calculated using DnaSP 4.10 (Rozas *et al.*, 2003). Similarly, sequence divergence between the two mitochondrial lineages ('A' and 'B') and between two geographic regions (Asia and Europe) were calculated in terms of the average number of nucleotide differences between the haplotypes (*D*) and the average

number of nucleotide substitutions per site between the populations (*K*) using DnaSP 4.10.

Table 3.1: GenBank sequences used for the comparative analysis of sheep breeds from different geographical regions

Country	Number	GenBank accession number	
		Cytochrome *b* (967 bp)	**Control region (1061 bp)**
Austria	61	AY87464, AY879468–Y879494, AY879530–Y879553, AY879563–Y879564, AY879572–AY879578	AY879343, AY879347–AY879373, AY879409–AY879432, AY879442–AY879443, AY879451–AY879457
Imported to Austria with European origins	7	AY879518, AY879521, AY879528– AY879529, AY879562, AY879570–AY879571	
Aland Islands	5	AY879465–AY879467, AY879517, AY879561	
Finland	5	AY879520, AY879526	
Russia	11	AY879522–AY879524, AY879509–AY879516, AY879519, AY879524, AY879569	
Spain	6	AY879555–AY879560	(n = 4) AY879436–AY879439
Indonesia	18	AY879495–AY879508; AY879565–AY879568	AY879374 - AY879387 AY879444 – AY879447
China	26	DQ309016 - DQ309021, DQ903208 - DQ903227	
Near East	220	DQ851886 - DQ852082, DQ097407 - DQ097430	
Central Asia	8	AY879527, AY879554, AY879579–AY879584	
Total	**367**		**83**

The population structure and signatures of population expansion were explored using Arlequin 3.11 (Excoffier *et al.*, 2005). First, Fu's *F*s test of selective neutrality (Fu, 1997), which compares the observed haplotype number to the observed number of pairwise differences, was used to establish the presence of population expansion. Secondly, based on the observed distribution of pairwise differences between sequences (i.e. mismatch distribution), the model parameters for the sudden population expansion (Rogers, 1995) were estimated and the fit of the data to the inferred model was tested (Schneider & Excoffier, 1999). The relationships between haplotypes were visualized as a conservative ($\epsilon = 0$) median-joining diagram (Bandelt *et al.*, 1999) constructed using Network 4.1.1.2 (http://www.fluxus-engineering.com).

Nucleotide weighting (ω) was adjusted to reflect the difference in mutational frequency as ω = 20 (transversions) and ω = 10 (transitions). Three networks were generated 1) using the control region mtDNA sequence from 91 Asian animals comprising Indian (73) & Indonesian (18), 2) using cytochrome *b* gene sequences from 132 Indian and reference type B,C, D, and E sequences and 3) using the Indian (132), Asian [272; China (26); Central Asia (8); Indonesia (18) and Near East (220)] and European (95) sequences.

3.2.4 Phylogenetic analysis

Phylogenetic reconstruction was performed using cytochrome *b* gene sequences with multiple methods. Inclusion of reference samples allowed for the identification of the clades clusters 'A' and 'B' as defined by Hiendleder *et al.* (1998a). MEGA 3.1 (Kumar *et al.*, 2004) was used for the construction of bootstrap (1,000 replications) supported neighbor-joining (nj) tree using cytochrome *b* sequences, with Tamura–Nei (Tamura & Nei, 1993) model for the estimation of genetic distance and tree construction. A Bayesian phylogeny tree was constructed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The phylogeny analysis was performed using the software's default priors and (HKY+I+G) model (gamma distribution G = 0.9719, invariable sites = 0.9027) and run for 6 million iterations. Modeltest 3.06 (Posada & Crandall, 1998) was used in order to find the optimal model of DNA substitution using hierarchical likelihood-ratio tests as implemented in PAUP* 4.0b10 for Windows (Swofford, 2003). The Hasegawa–Kishino–Yano (HKY; Hasegawa *et al.*, 1985) evolutionary model (HKY+I+G) with gamma distribution (G = 0.9719) and invariable sites (I = 0.9027) was identified as the best model. For the control region sequences, genetic distance was estimated using Kimura's 2-parameter method, and a neighbour-joining haplotype tree was constructed using MEGA 3.1 (Kumar *et al.*, 2004).

3.2.5 Divergence time using cytochrome b gene sequence

Time-since-expansion was obtained from the equation $t= \tau/2u$ using a nonlinear-stepwise least squares approach as implemented in Arlequin 3.11 (Excoffier *et al.*, 2005) and the 95% confidence interval was provided by 10,000 parametric bootstrap replicates. Tau (τ) is the empirical peak of the mismatch distribution and the $u = m_t \mu$, where m_t is the length of sequence (967 bp) and μ is the substitution rate. The

substitution rate was estimated as 2.27% per million years for the analyzed 967 bp region, with the mean genetic distance calculated using the mean genetic distance between the domestic breeds analyzed here (132 domestic sequences) and Argaliform II *cytb* sequences (*O. ammon* AJ867257-60, AJ867266-76; Bunch *et al.*, 2006) and the assumption of divergence time 4,73,000 years for the most recent common ancestor of the moufloniform (incorporating *O. aries*) and Argaliform II (*O. ammon*) split (Bunch *et al.*, 2006).

3.3 Results

3.3.1 MtDNA sequence variation in Indian sheep

3.3.1a Control region analysis

Breed-specific estimates of genetic diversity (Table 3.2) indicated that Bannur contained the highest and Sangamneri the lowest nucleotide diversity (π) among the analyzed Indian populations. All the breeds contained variable numbers of polymorphic sites (9–17), although the nucleotide diversity (π), average number of differences (*D*) and haplotype diversity (*Hd*) were broadly similar (Table 3.2). Sequences spanning the same mtDNA segment from 83 animals from other Asian and European sheep were used to facilitate comparison against the characterized haplotypes. Analysis of the combined dataset of 156 sequences revealed 71 positions with nucleotide substitutions. Most (41) were phylogenetically informative while the remainders (30) were singletons. In addition to nucleotide substitutions resulting in alleles AF010406.1:m.401A and m.402A in Garole and Sangamneri, as well as allele m.422C in seven animals of Bannur, two indels were observed. Both indels were T insertions; the first one was observed in European and Asian sequences and the second one appeared fixed in the Bannur and Lonand breeds (see positions 16473 and 448, respectively in Table 3.3). As expected, analysis between the Asian and European breeds revealed high sequence diversity (K = 0.231, D = 9.498), which was slightly less than that observed between the two ('A' and 'B') mtDNA lineages (K = 0.268, D = 10.993; Table 3.4).

Table 3.2 Estimates of genetic diversity within the sheep populations based on control region

Breed/ Strain	*n*	Summary statistics[1]					
		Hn	**P sites**	**M sites**	**π (x 10^{-3})**	***Hd***	***D***
Indian populations							
Bannur (Bn)	21	18	17	17	3.55	0.981	3.762
Garole (Ga)	18	13	12	13	2.21	0.941	2.340
Lonand (Lo)	16	10	10	10	2.04	0.900	2.167
Sangamneri (Sn)	18	11	9	9	1.67	0.882	1.771
Population summaries[2]							
All animals	156	73	71	75	6.20	0.962	6.566
Indian animals	73	52	33	36	3.94	0.984	4.168
Asian animals	91	57	37	41	5.12	0.972	5.427
European animals	65	19	21	21	3.27	0.857	3.467
Type 'A' animals	86	55	35	38	3.71	0.977	3.927
Type 'B' animals	70	18	16	16	1.60	0.845	1.696

[1] The number of individuals sampled (*n*), number of observed haplotypes (*Hn*), number of phylogenetically informative sites (P sites), number of mutations (M sites), population nucleotide diversity (π), haplotype diversity (*Hd*) and average number of nucleotide differences (including indels) between haplotypes (*D*) are given for each population.

[2] Summary statistics were calculated following separation of the total dataset into either geographical origin (Indian, Asian or European) or mitochondrial lineage ('A' or 'B').

Table 3.3: Haplotype definitions of Asian and European sheep populations based on control region sequences

Breed	Haplotype	Haplotype definition 11111111111111111111111111111 66666666666666666666666666666 00111111112222333444444445561222224444444455 99023334590146049013455792602017990023488 13 67090257659744643110134359537967121227879 32	Individuals	Haplotype shared with the population	Lineage
Bannur	H_1	TGATACTTCTTTATTCTGGCCTA-TTATCCCCCTTTAATTACA	3		A
Bannur	H_2	.....-.....C..AC..	1		A
Bannur	H_3	.....T.G-.....	1		A
Bannur	H_4	.....C.....-.....	1		A
Bannur	H_5	.....-.....G.AC..	1		A
Bannur	H_6	.....-.....T.....	1		A
Bannur	H_7	.....C....C.....-.....	1		A
Bannur	H_8	...C.....-.....T.....C.....	1		A
Bannur	H_9	.....T.C.....T...-.....T.....	1		A
Bannur	H_10	.....T.C.....-.....C..AC..	2		A
Bannur	H_11	.....A.....-.....G.A...	1		A
Bannur	H_12	.....-.....T.....C.....	1		A
Bannur	H_13	.....-.....C.....	1		A
Bannur	H_14	.....T.C.....-.....	1		A
Bannur	H_15	...C.....-.....C...C..	1		A
Bannur	H_16	.....T.....A....-.....CG.....	1		A
Bannur	H_17	...C.....-.....	1		A
Bannur	H_18	.....-.A...T.....A...	1		A
Garole	H_19	.....G.....-.....AA..-A...	1		A
Garole	H_20	.....-.....AA..-A...	4	JTT(3),FS(2)	A
Garole	H_21	.....G.....-..G.....AA..-A...	1		A
Garole	H_22	C.....-.....AA..-A...	1		A
Garole	H_23	.....-.....G....AA..-A...	1		A
Garole	H_24	...C.....-..G.....AA..-A..T	1		A
Garole	H_25	.....G.....-.A.....AA..-A...	1		A

Breed	Haplotype	Haplotype definition 11111111111111111111111111111 66666666666666666666666666666 00111111112222333444444445561222224444444455 99023334590146049013455792602017990023488 13 67090257659744643110134359537967121227879 32	Individuals	Haplotype shared with the population	Lineage
Garole	H_26	...C.....-..G.....AA..-A...	3		A
Garole	H_27	.T.C.....-..GC.....AA..-A...	1		A
Garole	H_28	..C.....-.....AA..-A...	1		A
Garole	H_29	.....C.....-.....AA..-A...	1		A
Garole	H_30	G.C.....-.....AA..-A...	1		A
Garole	H_31	.....-..G.....AA..-A...	1		A
Lonand	H_32	.....-.A.....A...A...	1		A
Lonand	H_33	.....-.....A.....	5		A
Lonand	H_34	...C.....T...-.....A.G.A...	1		A
Lonand	H_35	..C.....-.....T....A.....	1		A
Lonand	H_36	.....T.....-.....A...A...	2		A
Lonand	H_37	.....-.....A...A...	2		A
Lonand	H_38	...C.....T...-.....A...A...	1		A
Lonand	H_39	..C.....-.....A...A...	1		A
Lonand	H_40	.....-.....T....A...A...	1		A
Lonand	H_41	.....T.C.....-.....A...A...	1		A
Sangamneri	H_42	.....T...-.....T.AA..-A..T	1		A
Sangamneri	H_43	...C.....-.....T.AA..-A...	3		A
Sangamneri	H_44	.....-.....T.AA..-A...	6		A
Sangamneri	H_45	.....T..-.....TT.AA..-A...	1		A
Sangamneri	H_46	...C.....-.....AA..-A...	1		A
Sangamneri	H_47	.....-.....TT.AA..-A...	1		A
Sangamneri	H_48	...C.....C.....-.....AA..-A...	1		A
Sangamneri	H_49	...C.....-.....TT.AA..-A...	1		A
Sangamneri	H_50	....G.....-.....T.AA..-A...	1		A
Sangamneri	H_51	...C.....G.....-.....T.AA..-A...	1		A
Sangamneri	H_52	.....C.....-.....T.AA..-A...	1		A

Breed	Haplotype	Haplotype definition 111111111111111111111111111111 666666666666666666666666666666 001111111122223334444444455612222244444445 5 990233345901460490134557926020179900234881 3 670902576597446431101343595379671212278793 2	Individuals	Haplotype shared with the population	Lineage
CS	H_53	CA........CC..........GT...C.....CAA..-A...	5		B
CS	H_54	CA........CC........T.G-...C.....CAA..-A...	12	JTT(1),FS(5),TMS(4),TSS(2)	B
CS	H_55	CA........CC....C...T.G-...C.....CAA..-A...	1	TMS(1)	B
JTT	H_56	.A........CC........T.GT...C.....CAA..-A...	11	TSS(1)	B
JTT	H_57	.A........CC........T.G-...CT....CAA..-A...	1		B
JTT	H_58	.A........CC........TGG-...C.....CAA..-A...	1		B
JTT	H_59	.....T..........C.......-..........AA..-A...	1		A
FS	H_60	CA.......CCC......A.T.G-...C.....CAA..-A...	1		B
FS	H_61	CA..G.....CC........T..-...C.....CAA..-A...	2		B
FS	H_62	CA........CCG.........GT...C.....CAA..-A...	1		B
FS	H_63	CA........CCG...C...T.G-...C.....CAA..-A...	1	TMS(2)	B
Mu	H_64	CA........CC....C...T.G-...C.....CAA..-A.G.	2		B
Mu	H_65	CA........CC........T.G-...C.....CAA..-A.G.	2		B
TMS	H_66	CA........C.........T.G-..........CAA..-A...	3		B
TMS	H_67	.A........CC........T.G-..........CAA..-A...	1		B
TMS	H_68	..............T.......-..........AA..-A...	5		A
TMS	H_69	............C........-C.........AA..-A...	1	TSS(1)	A
TMS	H_70	CA........CC........TGG-...CT....CAA..-A...	1		B
TSS	H_71	CA........CCGC......T.G-...C.....CAA..-A...	3		B
TSS	H_72	C.........CC........T.G-...C.....CAA..-A...	5		B
TSS	H_73	CA........CC.....A..T.GT...C.....CAA..-A...	1		B

Single Nucleotide Polymorphism (SNP) positions relative to the reference sequence AF010406 are indicated by the vertical numbers. A dash (-) indicates an indel and a dot (.) indicates similar nucleotide regarding the reference sequence. Breed names are abbreviated as Javanese Thin Tail (JTT), Forest sheep (FS), Carynthian sheep (CS), Tyrolean mountain sheep (TMS) and Tyrolean stone sheep (TSS). The numbers in parentheses in the fifth column indicate the number of individuals from each population with the shared haplotype.

Table 3.4 Sequence divergence between the populations based on control region

Populations	K^a	D^b	Shared haplotypes
Asian/European	**0.231**	**9.498**	**17**
Clade A/Clade B	**0.268**	**10.993**	**11**

[a] Average number of nucleotide differences between populations
[b] Average number of nucleotide substitutions per site between population

3.3.1b Cytochrome b gene analysis

The partial cytochrome *b* (967 bp) gene sequences from 132 individuals were analyzed along with the reference lineage specific sequences. Total 31 polymorphic sites were observed out of which 25 were parsimony informative (Table 3.6). The haplotype diversity (*Hd*) within the breeds ranged between 62.0% (Kolhapuri) and 91% (Garole), respectively. The level of genetic diversity observed in India as indicated by the nucleotide diversity (π) value was the lowest (1.87×10^{-3}) in all the countries/regions (other Asian (4.57×10^{-3}) and Europe (2.24×10^{-3}) although the haplotype diversity was comparable (Table 3.6). It is worth to note that the nucleotide diversity for the haplogroup 'A' from Indian populations (1.52×10^{-3}) was more than that from other Asian populations (0.73×10^{-3}). In addition, the sequences belonging to lineage 'A' from Indian and other Asian populations were also examined for genetic variation. A total of 42 and 16 haplotypes (Table 3.6) and 1.45 and 0.707 mean pairwise differences (Table 3.8) were found in Indian and other Asian sequences, respectively.

3.2.2 MtDNA haplotypes within Indian sheep

3.2.2a Control region Analysis

The 41 phylogenetically informative substitutions and two indels defined 73 mtDNA haplotypes, 52 of which were observed in Indian animals. The frequency and breed membership of each haplotype are presented in Table 3.3. Interestingly, there were no haplotypes common across Indian breeds. Even the two strains of Deccani, the Lonand and Sangamneri, which are in geographic proximity (Fig. 2.1, Chapter 2), did not contain a common haplotype among the 34 animals sequenced. The only haplotype that was found in an Indian animal and another breed was H_20, which was observed in four Garole, three Javanese Thin Tail and two European animals. There were five other haplotypes that were shared among the non-Indian breeds while 67 were unique (Table 3.3).

To further investigate the possibility of substructure existing within the clade 'A', the 58 mtDNA haplotypes from Asian individuals were used to construct a median joining network (Fig. 3.1). For each haplotype, clear correspondence was evident between the regions of the network and the breed of origin. This was most obvious for the 18 unique Bannur haplotypes, which were interconnected to each

other by 21 links and to the rest of the network *via* a single connection. Similarly, the Deccani haplotypes were predominantly located in the middle of the network along with the Garole. All but the H_58 Javanese Thin Tail haplotypes were separated from the Indian haplotypes (Fig. 3.1).

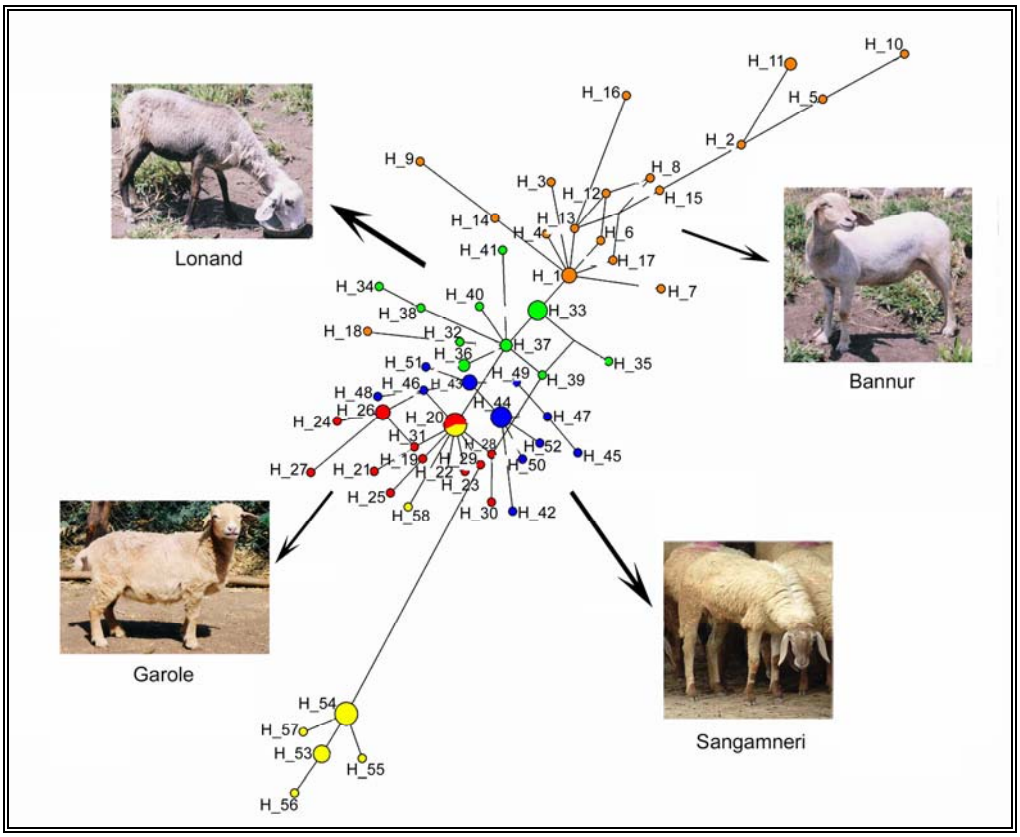


Figure 3.1: Median-joining network (ϵ = 0) of the mitochondrial haplotypes observed in the Asian domestic sheep breeds
Bannur (Orange), Lonand (Green), Sangamneri (Blue), Garole (Red) and Javanese Thin Tail sheep (Yellow). The areas of the circles are proportional to the frequency of the samples.

Table 3.5: Haplotype distribution of Indian sheep based on cytochrome *b* gene sequences

Haplotype	Bannur	Garole	Lonand	Sangamneri	Kolhapuri	Madgyal	Clade	Total
H_1		1	1	1			B	3
H_2		1					A	1
H_3		1					A	1
H_4	10	6	16	15	15	7	A	69
H_5		1					A	1
H_6		1					A	1
H_7		1					A	1
H_8		2					A	2
H_9		1					A	1
H_10		1					A	1
H_11		1					A	1
H_12		1					A	1
H_13		2			1		A	3
H_14		1					B	1
H_15					1		A	1
H_16					1		B	1
H_17					1		A	1
H_18					1		A	1
H_19					1		A	1
H_20				1	1		A	2
H_21					1		A	1
H_22					1		A	1
H_23			1				A	1
H_24	2		1	1			A	4
H_25			1				A	1
H_26			1				A	1
H_27			1				A	1
H_28			1				A	1
H_29	1		1	1			A	3
H_30			1				A	1
H_31			1				A	1
H_32			1				A	1
H_33			1				A	1
H_34			1				A	1
H_35				1			B	1
H_36				1			B	1
H_37				2			B	2
H_38	2			2		1	A	5
H_39	1			1			A	2
H_40				1		1	A	2
H_41						1	B	1
H_42						1	A	1
H_43						1	A	1
H_44	1						A	1
H_45	1						A	1
H_46	1						A	1
H_47							C	1
H_48							E	4
H_49							E	1
H_50							D	2
Total	**19**	**21**	**29**	**27**	**24**	**12**		**141**

Table 3.6: Summary statistic and frequency of sheep mtDNA lineages of the cytochrome *b* gene from different geographic locations

	Summary statistics [1]							Distribution of haplogroups in populations [2] (%)					Among populations	
Breed/ strain	*n*	*Hn*	P sites	M sites	π (x 10^{-3})	*Hd*	*D*	A	B	C	D	E	Shared haplotypes	Unique sequences
Indian Populations														
Bannur	19	8	9	9	1.35	0.725	1.310	100 (8) [a]					5	3
Garole	21	14	9	9	2.90	0.919	2.800	90.5 (12) [a]	9.5 (2) [a]				2	12
Lonand	29	15	16	16	1.77	0.741	1.714	96.6 (13) [a]	3.5 (1) [a]				4.	11
Sangamneri	27	11	10	10	1.74	0.695	1.681	81.5 (7) [a]	18.5 (4) [a]				7	4
Kolhapuri	24	10	9	9	1.09	0.620	1.054	95.8 (9) [a]	14.2 (1) [a]				3	7
Madgyal	9	6	6	6	1.55	0.722	1.500	91.7 (5) [a]	8.4 (1) [a]				2	4
Indian breeds	132	49	31	31	1.87 (1.52) [c]	0.733	1.808	85.71 (42) [a] (51.51%) [b]	7.6 (7) [a]				8	41
Other Asian [d]	272	54	58	60	4.57 (0.73) [c]	0.863	4.421	29.62 (16) [a] (71.26%) [b]	56.6 (27) [a]	14.4 (9) [a]	0.73 (1) [a]	1.83 (2) [a]	5	49
Asia	404	100	77	80	4.00	0.865	3.865	48.4 (56) [a]	40.7 (34) [a]	9.7(9) [a]	0.49 (1) [a]	1.24 (2) [a]	11	89
Europe [e]	95	26	26	26	2.24	0.882	2.167	14.7 (2) [a]	85.3 (24) [a]				6 [f]	20
All populations	500	120	89	94	3.81	0.875	3.686	41.8 (56) [a]	49.0 (52) [a]	7.8 (9) [a]	0.40 (1) [a]	1.00 (2) [a]	14	106

[1] The number of individuals sampled (*n*), number of observed haplotypes (*Hn*), number of phylogenetically informative sites (P sites), number of mutations (M sites), population nucleotide diversity (π), haplotype diversity (*Hd*) and average number of nucleotide differences between haplotypes (*D*) are given for each population.

[2] Summary statistics were calculated following separation of the total dataset into geographical origin (India, Other Asian countries/regions and European countries) as well as distribution of haplogroups in populations (%).

[a] number of haplotypes present in each clade; **[b]** Frequency of the central haplotype in clade 'A'

[c] Nucleotide diversity of the sequences belonging to the haplogroup 'A'; **[d]** Diversity measures were calculated based on the 272 sequences. (26 from china (Chen *et al.*, 2006); 18 from Indonesia (present study; Meadows *et al.*, 2005); eight from Central Asia (present study Meadows *et al.*, 2005); 220 from Near East (Pedrosa *et al.,* 2005; Meadows *et al.*, 2007); **[e]** European sequences include 95 sequences (61 from Austria; 5 from Aland, 5 from Finland, 11 from Russia, 6 from Spain, and 7 from imported animals with European origin (present study; Meadows *et al.*, 2005) **[f]** European haplotypes shared with Asian haplotypes.

Table 3.7: Haplotype distribution table of the domestic sheep breeds from different geographic locations based on the cytochrome *b* gene sequences

	India						Other Asian Countries					Clade	Total
Haplotype	**Bannur**	**Garole**	**Lonand**	**Sangamneri**	**Kolhapuri**	**Madgyal**	**China**	**Central Asia**	**Indonesia**	**Near East**	**Europe**		
H_1		1	1	1			10	1	1	72	27	B	115
H_2		1										A	1
H_3		1										A	1
H_4	10	6	15	15	15	7	4	5	4	36	13	A	113
H_5		1										A	1
H_6		1										A	1
H_7		1										A	1
H_8		2										A	2
H_9		1										A	1
H_10		1										A	1
H_11		1										A	1
H_12		1										A	1
H_13		1										A	2
H_14		1										B	1
H_15					1							A	1
H_16					1							A	1
H_17					1							B	1
H_18					1			1				A	2
H_19					1							A	1
H_20					1							A	1
H_21				1	1							A	2
H_22					1							A	1
H_23					1							A	1
H_24			1									A	1
H_25			1									A	1
H_26	2		1	1								A	4
H_27			1									A	1
H_28			1									A	1

	India						Other Asian Countries					Clade	Total
Haplotype	**Bannur**	**Garole**	**Lonand**	**Sangamneri**	**Kolhapuri**	**Madgyal**	**China**	**Central Asia**	**Indonesia**	**Near East**	**Europe**		
H_29			1									A	1
H_30			1									A	1
H_31	1		1	1								A	3
H_32			1									A	1
H_33			1									A	1
H_34			1									A	1
H_35			1									A	1
H_36			1									A	1
H_37				1								B	1
H_38				1								B	1
H_39				2								B	2
H_40	2			2		1						A	5
H_41	1			1								A	2
H_42				1								A	1
H_43						1						B	1
H_44						1						A	1
H_45						1						A	1
H_46						1						**A**	1
H_47	1											A	1
H_48	1											A	1
H_49	1											A	1
H_50									13			B	13
H_51							3			4		C	7
H_52							1					A	1
H_53							1					A	1
H_54							1					A	1
H_55							1					A	1
H_56							1					B	1
H_57							1					B	1
H_58							1			1	1	B	3
H_59							1					C	1

	India						Other Asian Countries					Clade	Total
Haplotype	**Bannur**	**Garole**	**Lonand**	**Sangamneri**	**Kolhapuri**	**Madgyal**	**China**	**Central Asia**	**Indonesia**	**Near East**	**Europe**		
H_60							1			2		C	3
H_61										1		A	1
H_62										1		A	1
H_63										1		A	1
H_64										4		A	4
H_65										4		A	4
H_66										4		A	4
H_67										2		A	2
H_68										1		A	1
H_69										1		B	1
H_70										1		B	1
H_71										3		B	3
H_72										8	7	B	15
H_73										1		B	1
H_74										4		B	4
H_75										2		B	2
H_76										3		B	3
H_77										2		B	2
H_78										4		B	4
H_79										2		B	2
H_80										2		B	2
H_81										1		B	1
H_82										1		B	1
H_83										3		B	3
H_84										2		B	2
H_85										1		B	1
H_86										5	1	B	6
H_87										1		B	1
H_88										1		B	1
H_89										2		B	2
H_90										3	1	B	7

	India						Other Asian Countries					Clade	Total
Haplotype	**Bannur**	**Garole**	**Lonand**	**Sangamneri**	**Kolhapuri**	**Madgyal**	**China**	**Central Asia**	**Indonesia**	**Near East**	**Europe**		
H_91										17		C	17
H_92										3		C	3
H_93										1		C	1
H_94										4		E	4
H_95										1		C	1
H_96										2		C	2
H_97										2		C	2
H_98										2		D	2
H_99										1		E	1
H_100											5	B	5
H_101											1	B	1
H_102											3	B	3
H_103											4	B	4
H_104											1	B	1
H_105											1	B	1
H_106											1	A	1
H_107											1	B	1
H_108											1	B	1
H_109											2	B	2
H_110											2	B	2
H_111											3	B	3
H_112											1	B	1
H_113											1	B	1
H_114											1	B	1
H_115											1	B	1
H_116											1	B	1
H_117								1				A	1
H_118											1	B	1
H_119											10	B	10
H_120											1	B	1

3.3.2b Cytochrome b gene analysis

Based on the 967-bp sequence fragment between nucleotide positions 14078 and 15349 of *O. aries* (AF0104060), 50 haplotypes were detected defined by 36 parsimony informative sites were detected (Table 3.5). Among the 50 haplotypes in the populations, 37 were observed once, while the most common haplotype (H4) occurred 69 times (Table 3.5). The number of haplotypes identified in each population ranged from six (Madgyal) to fourteen (Lonand and Garole). To understand the relationship among the haplotypes, a median-joining network was constructed, which showed a star shaped pattern (Figure 3.2) for the haplogroup 'A' and 'B'. Majority of the haplotypes from Lonand, Kolhapuri and Garole grouped as per the geographic origin. Very few haplotypes (9 out of 46; Table 3.5) were shared by individuals from different populations across wide geographic regions indicating possible intermixing of the populations from different geographic regions in India (Fig. 3.2 & Table 3.5). However, the Garole breed showed the least intermixing with only one haplotype being shared by the Kolhapuri strain of Deccani, whereas almost all the haplotypes from the Sangamneri strain, except the three 'B' types, were shared. There were high proportions of private haplotypes in Indian populations (37 out of 46; Table 3.5). However, a high number of reticulations remained, suggesting the presence of parallel mutations. Further, in order to compare geographic distribution and diversity of the cytochrome *b* gene haplotypes, 367 mtDNA cytochrome sequences from other geographic regions were analyzed along with the Indian sheep sequences (Table 3.7). Total 56 and 52 haplotypes were identified in 209 and 246 sequences belonging to the 'A' and 'B' lineages, respectively. Nine haplotypes were found in 39 sheep belonging to lineage 'C' from China and Near East. Except the most frequent central haplotypes (H1 and H4), only one of the Indian sheep haplotypes (from the Lonand strain of Deccani) was shared by other regions (shared with a central Asian breed), however, 11 and 6 haplotypes were shared among the sheep breeds from other Asian and European populations (Fig. 3.3 & Table 3.7). Interestingly, one haplotype from the Madgyal strain of Deccani showed close similarity with type 'C' haplotype and was only two mutation steps away from the most common clade 'A' haplotype. Few haplotypes (5) from Sangamneri, Madgyal and Bannur showed clustering with the reported divergent haplotype (DQ309021, sub-clade a5 from Chinese breeds; Chen *et*

al., 2006) from China. Two haplotypes from the Kolhapuri strain showed the linkage between clade 'A' and 'B' haplotypes.

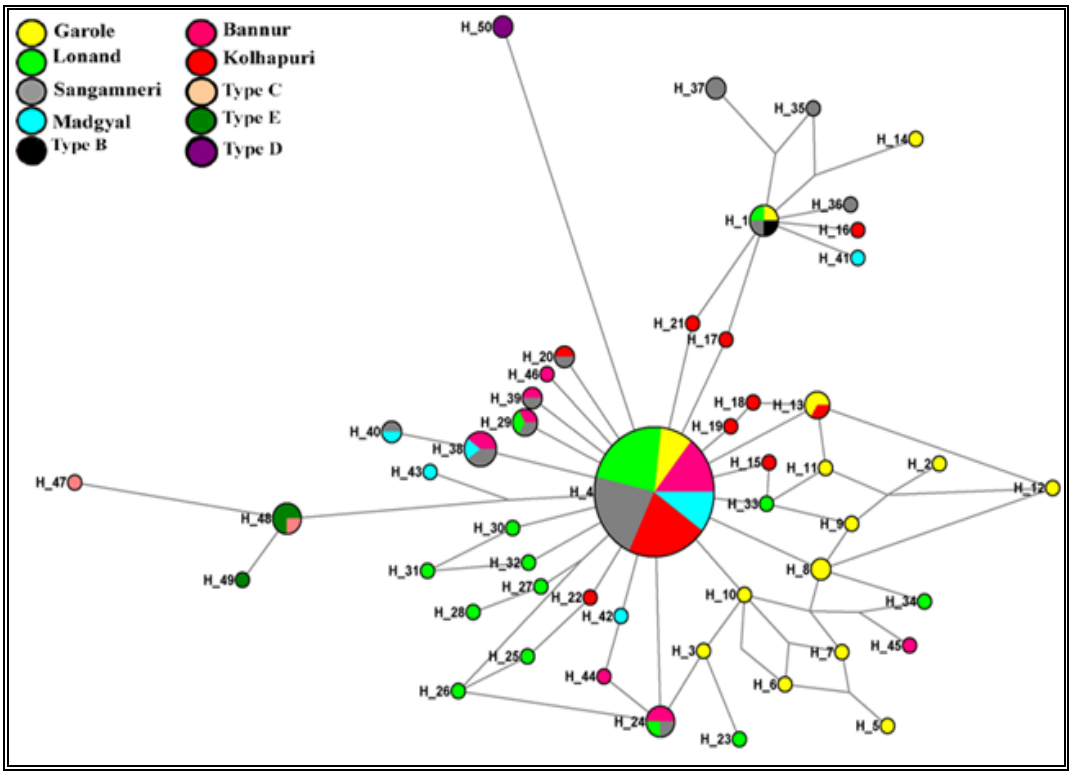


Figure 3.2: Weighted reduced median joining network of cytochrome *b* gene (967 bp) haplotypes from Indian sheep breeds
The nodes size is proportional to the haplotype frequency. The reference ('B' 'C', 'D' & 'E') haplotypes were taken from previous reports (Hiendleder *et al.*, 1998b; Pedrosa *et al.*, 2005; Chen *et al.*, 2006; Meadows *et al.*, 2007).

3.3.4 Population expansion study using cytochrome b gene

The Indian haplogroup 'A' showed star-like phylogeny in MJ network indicating population expansion (Figure 3.2). Two statistical approaches supported this inference. First, the *F*s (Fu, 1997) statistic, which is particularly sensitive to population growth, showed a significant ($p< 0.02$) departure from neutrality in only clade 'A' haplogroup (Fu's *F*s = -27.54; Table 3.8). Second, the observed mismatch distributions were fitted to the sudden expansion model (Rogers, 1995) and the analysis supported population expansion in this haplogroup. The observed mismatch distributions did not deviate from the expectations of the fitted models ($p = 0.99$) according to the sum of squared deviation statistic (Schneider & Excoffier, 1999). The above findings indicate that the clade 'A' has undergone population expansion and this also allowed to us calculate the time since commencement of expansion

which was found to be 26,986 years (95% confidence interval; 4,944 – 1,00,116; Table 3.8).

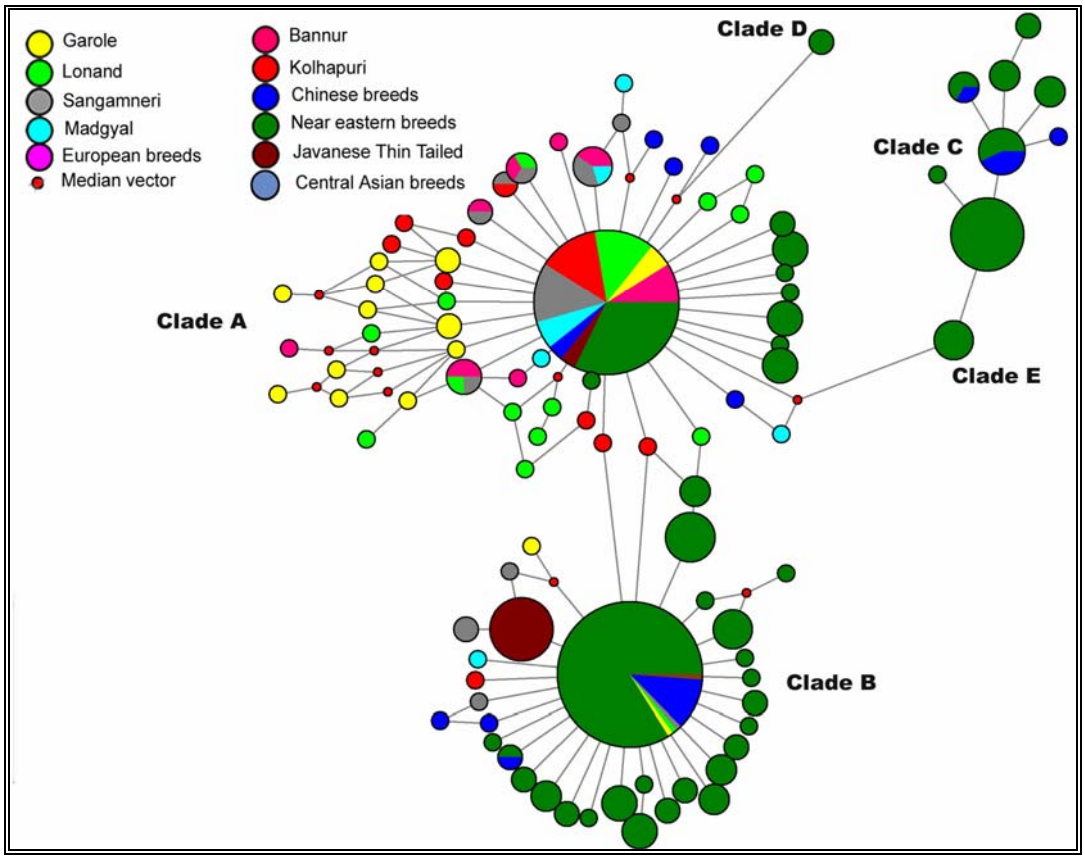


Figure 3.3: Weighted reduced median joining network of cytochrome *b* gene (967 bp) haplotypes from domestic sheep breeds of different geographic locations
The nodes size is proportional to the haplotype frequency. Haplotypes obtained using the published sequences from other Asian and European countries/regions (Table 3.1) were included for comparison with Indian haplotypes.

Table 3.8: Summary statistic of haplogroup 'A' from Indian and other Asian sheep populations

Population	Tau (τ)	SSD	*Fs* value	Pairwise mismatches	Time since commencement of expansion (95% confidence interval)
Indian haplogroup A	1.31	0.00008	-27.54	1.450	26,986 (4,944 –1,00,116)
Other Asian haplogroup A	1.01	0.00364	-21.25	0.707	20,806 (14,523- 30,488)

Tau : empirical peak of mismatch distribution, SSD: sum of the squared differences, *F*s value: Fu's *F*s value

3.3.5 Phylogenetic inference

The phylogenetic analysis was performed using MEGA with Tamura and Nei model for cytochrome *b* gene sequences (50 haplotypes) and Kimura 2-p model for control region sequences (73 haplotypes). In the neighbor joining (NJ) trees the Indian sheep breeds were divided into two clearly separated clusters (Fig. 3.4A & 3.4B). Inclusion of reference sequences allowed the identification of clusters 'A' and 'B' as defined by Hiendleder *et al.* (1998b).

3.3.5a Control region analysis

All the 73 Indian sheep carried a haplotype, which clustered with previously characterized clade 'A' sequences, while most of the European breeds carried clade 'B' sequences. Clade 'C', 'D' and 'E' sequences, reported in low frequency in the Asian, Middle eastern and European breeds (Guo *et al.*, 2005; Pedrosa *et al.*, 2005; Pereira *et al.*, 2006; Meadows *et al.*, 2007) were not observed in the Indian sheep analyzed. Within the clade 'A' branch, four clusters were observed, which loosely discriminated the breeds (Figure 3.4A). All haplotypes of the Bannur breed formed a distinct cluster, while those of the Lonand and Sangamneri strains were predominant in two other clusters. Two Garole haplotypes and one Javanese Thin Tail haplotype shared the Lonand cluster, whereas a few of the Garole (five), Lonand (two) and European (one) haplotypes shared the Sangamneri cluster. The fourth cluster included haplotypes mostly from the Garole breed, along with the shared H_20 haplotype and one haplotype from the Tyrolean mountain sheep (an European breed).

3.3.5b Cytochrome b gene analysis

Based on the reference sequences, the 50 haplotypes were divided into five distinct haplogroups: 'A', 'B', 'C', 'D' and 'E' as revealed by the neighbor joining tree (Fig. 3.4B & Table 3.5). However, among the Indian breeds only two lineages were observed ('A' and 'B'). The tree topology was evaluated using Bayesian clustering method, which resulted in similar tree topology with high support. For the Bayesian analysis the Hasegawa, Kishino and Yano (1985) model with invariable sites and gamma distributed mutation rate variation between sites was found to fit into the data. The tree revealed that haplogroup 'A' was predominant (85.71% animals) and was divided in two sub-clusters (A_1 and A_2). The sub-cluster 'A_1' consists of mainly the shared and the divergent haplotypes, whereas the 'A_2'consist of unique type of the

haplotypes. The haplogroup 'B' was recorded as a rare type (7.58%) in the analyzed Indian sheep breeds and was most frequent in Sangamneri strain of the Deccani breed (18.52%) whereas in the other breeds (except Bannur) it was present in the range of 4-10% (Table 3.6).

3.3.6 Population structure analysis using cytochrome b gene sequences

To further investigate geographical structuring, we performed several AMOVA analyses at different hierarchical levels (Table 3.9). When Indian breeds were grouped as per the geographical regions, only 2.68% of the genetic variation was partitioned among the geographic regions. However, when the breeds were grouped as per the country of origin, (India verses other Asian countries/regions and India verses European countries/regions), 11.0% and 12.61% of the genetic variation was estimated between the countries, respectively, whereas about 89% and 87% variation was within breeds, indicating low but significant geographic structure. However, in the global AMOVA (as per the continents; Asia and Europe) no genetic variance between the two continents was detected.

Table 3.9: Hierarchical distribution of sheep mtDNA variation within and among populations under AMOVA analyses (% of total)

Grouping	Within populations	Among populations within groups	Among groups
No grouping In India	99.49	0.51	-
Three regions in India [a]	99.04	-01.72	02.68
India versus other Asian countries/regions [b]	89.05	-00.05	11.00
India versus Europe [c]	87.30	00.08	12.61
Asia versus Europe	94.81	11.51	-06.33

[a] Three regions in India indicate South (Karnataka state; Bannur breed), East (West Bengal state; Garole Breed) and West (Maharshtra state; Deccani) India.
[b] Other Asian countries/ regions include China, Indonesia, Near East, and Central Asia,
[c] Europe, includes sheep from European countries or regions and sheep with European origins

Figure 3.4A **Figure 3.4B**

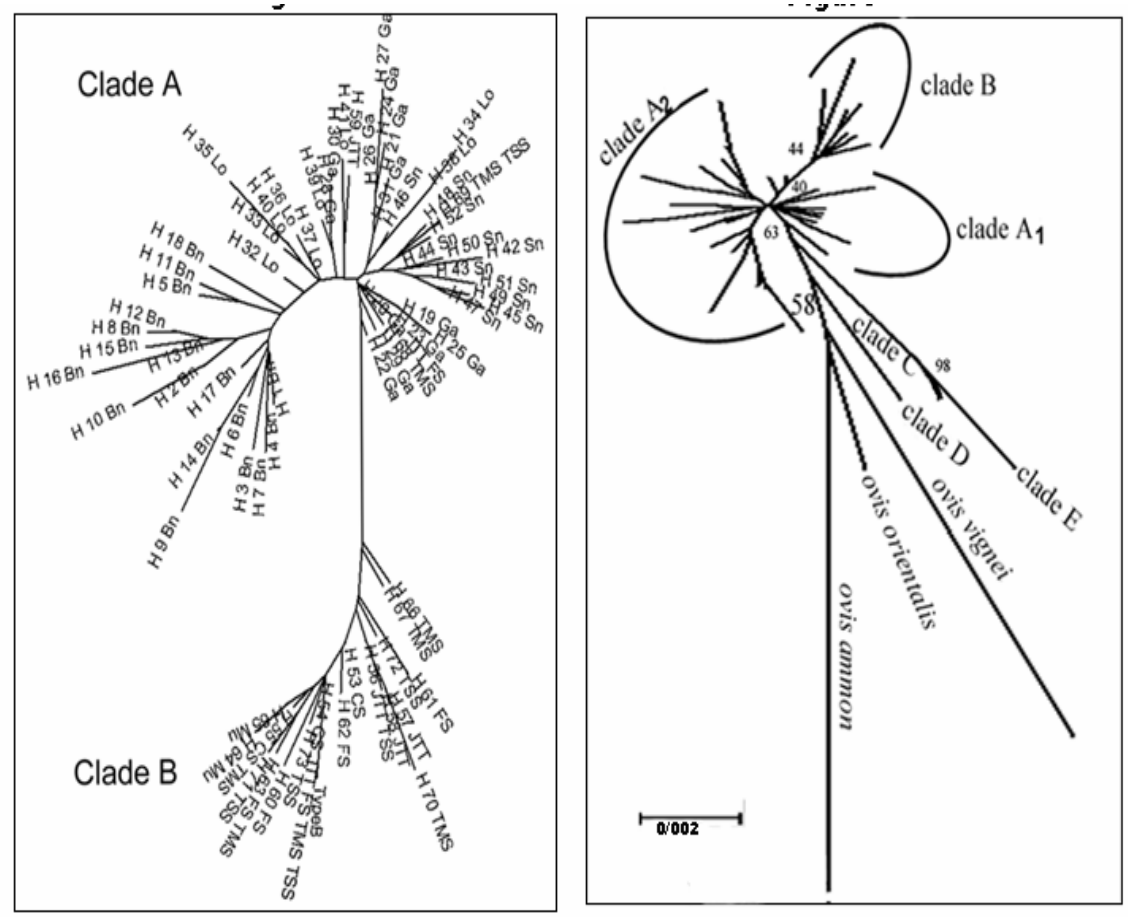


Figure 3.4A: Neighbour-joining tree of 73 mitochondrial sheep haplotypes found within 156 Asian and European sheep including Mouflon, a wild sheep
Breed names are abbreviated as Bannur (Bn), Garole (Ga), Lonand (Lo), Sangamneri (Sn), Javanese Thin Tail (JTT), Forest sheep (FS), Carynthian sheep (CS), Tyrolean mountain sheep (TMS), Tyrolean stone sheep (TSS) and Mouflon (Mu). The Type _B_ sequence refers to the reference mitochondrial sequence (AF010406; Hiendleder *et al.* 1998b).

Figure 3.4B: Neighbor joining tree of Indian sheep haplotypes in relation with wild sheep using cytochrome *b* (967 bp) gene sequence
Analysis of animals from three Indian domestic breeds (n = 132) with wild *Ovis* species [*O. vignei* (AF034729), *O. orientalis* (AJ867261) and *O. ammon* (AJ867272)] and the Clade C (DQ097429-30), D (DQ852081-82) and E (DQ852077-80) haplotypes (Chen *et al.*, 2006; Meadows *et al.*, 2007). Bootstrap values are indicated on the nodes.

3.4 Discussion

3.4.1 Phylogeny of Indian sheep

The present structure of the genetic diversity retains the signature of past demographic events and helps to reconstitute the evolutionary history (Luikart *et al.*, 2003). Recent mtDNA studies carried out in sheep indicate the presence of five lineages, with the haplogroup 'B' being predominant in overall sheep population as well as in European sheep population (Hiendleder *et al.*, 1998a, b, 2002; Tapio *et al.*, 2006a). Most of the Asian breeds analyzed till date (from China and Near East) showed the presence of all the five haplogroups, although the haplotypes 'C', 'D' and 'E' were present at relatively small frequencies (Guo *et al.*, 2005; Pedrosa *et al.*, 2005; Tapio *et al.*, 2006a; Meadows *et al.*, 2007). However, in the present study on the haplogroup'A' (85.71%), was observed in the Indian sheep breeds, characterizing them separate from the other populations sampled to date.

3.4.1a Analysis of the mtDNA control region

The network analysis of mitochondrial control region (Fig. 3.1) revealed striking differences when compared to a similar analysis of Chinese breed clade 'A' sequences (Chen *et al.*, 2006). The Indian network had no clearly defined central haplotype, revealed a lack of shared haplotype and displayed very strong breed structure. This is in direct contrast to the Chinese sheep haplotype network, which had a single high-frequency central haplotype, extensive haplotype sharing and weak breed structure (Chen *et al.*, 2006). It suggests a history for these breeds defined by very low levels of interbreeding between populations and total reproductive isolation. Further, the genetic structure appears to extend even below the breed level. Lonand and Sangamneri, the two strains of Deccani, did not share a single haplotype and occupied distinct but neighboring sections of the haplotype network (Fig. 3.1), indicating that they are genetically separate. This suggests that these strains might actually be different breeds rather than strains of the Deccani breed. This observation was supported by the nuclear markers wherein these strains showed separate clusters in structure analysis (Fig. 2.5, Chapter 2).

Among the Indian haplotypes, only one haplotype (H_20) was shared by four Garole, two Javanese Thin Tail and two European animals (Table 3.3). The Garole

and Javanese Thin Tail have previously been shown to share the same mutation, which underpins the *FecB* prolificacy phenotype (Davis *et al.*, 2002). This suggests that the haplotype H_20 could be the maternal signature of this genetic link. While it cannot be directly inferred from the present molecular data, the transport of Garole south into the Indonesian archipelago is the most likely direction of this migration (Davis *et al.*, 2002).

Interestingly, the control region analysis showed exclusive presence of lineage 'A'. The presence of single lineage in the control region could be reflecting the recent evolution due to associated high mutation rate of this region.

Together, the results indicate a strong genetic structure with mtDNA haplotypes diagnostic of particular breeds. This has relevance to considerations of bio-conservation, as each of the three breeds tested appears to be genetically, as well as phenotypically, distinct.

3.4.1b Analysis of cytochrome b gene

The mitochondrial cytochrome *b* gene has a moderate evolutionary rate and a clear evolutionary pattern that makes it suitable for the studies on the gene flow and phylogenetic evolution at the intra- and inter-specific levels (Kocher *et al.*, 1989; Irwin *et al.*, 1999; Guryev *et al.*, 2001; Cao *et al.*, 2003; Alves *et al.*, 2003). Therefore, in order to understand the history of Indian sheep domestication, this relatively slower evolving cytochrome *b* gene was also analyzed. The nucleotide diversity in cytochrome *b* gene (1.87×10^{-3}) was found to be less than that in the control region (3.94×10^{-3}) confirming that the cytochrome *b* gene is useful for phylogenetic analysis. In contrast to the control region, the network of cytochrome *b* showed presence of two lineages ('A' & 'B') with star shaped phylogeny; however, the 'B' lineage was rare in the analyzed breeds (except Sangamneri strain of Deccani breed). Although, the network showed star shaped phylogeny for both the haplogroups, the Fu's *F*s value was significant only for the haplogroup 'A' (*F*s= -27.54) suggesting population expansion of this lineage in Indian sheep breeds. The majority of the haplotypes from Garole, Lonand and Kolhapuri clustered together reflecting partial structure of breed. In addition, few haplotypes from different breeds from different geographic regions were intermingled, indicating no correspondence between the geographic regions of origin and relationships among breeds. These observations are, however, quite different than those for the control region. Such

weak population structure observed in the present study could be the result of ancient gene flow between the breeds during the process of sheep domestication in India. As the Indian sheep populations have not undergone stringent modern selection strategies, it is reasonable to think that the weak population structure could be the result of historical genetic exchange. The phylogeographic analysis using AMOVA at different hierarchical levels supports this weak geographic structuring among the Indian sheep populations (Table 3.9).

The reason for such confounding results between the two analyzed regions could be attributed to the differential evolution of the two segments. Analysis of closely related mammalian mitochondrial genomes by Pesole *et al.* (1999) revealed that various functional regions are under different evolutionary constrains and hence display different mutation rates. The control region shows rapid and species specific evolutionary pattern revealing recent evolution compared to the cytochrome *b*. Thus, the high mutation rate of control region together with the unique animal husbandry practices and selection under different climatic and geographic conditions (Acharya *et al.*, 1982) might have resulted in the haplotypes diagnostic of the particular breeds revealing the strong population structure of Indian sheep. In contrast, the cytochrome *b* gene analysis showed partial breed structure and weak population structure, reflecting the possible ancient gene flow between the analyzed breeds. Therefore, this study emphasizes the need to analyze multiple regions of mtDNA to draw conclusions about pattern of the evolution.

3.4.2 Origin of Indian sheep

A number of questions remain to be answered concerning the number, timing and location of domestication events that gave rise to modern domestic sheep. Various theories have been put forth in order to explain the domestication of sheep (Zeuner, 1963; Hiendleder *et al.*, 1998a; Hiendleder *et al*., 2002; Guo *et al*., 2005, Pedrosa *et al.*, 2005; Chen *et al*, 2006; Meadows *et al*, 2007). The global existence of at least five mtDNA lineages may have arisen following a single domestication event, which contained a heterogeneous ancestral population. Or, alternatively, each mtDNA lineage might be the result of biologically, geographically and/or temporally distinct events. The finding in this study that multiple breeds from the Indian subcontinent contain a single predominant mtDNA lineage ‘A’ provides evidence for the second hypothesis. The time since commencement of expansion for the Indian haplogroup

'A' (26,986 years) was much ancient than that estimated for the lineage 'B' (Meadows *et al.*, 2007) as well as for sheep domestication (Ryder, 1984). However, this dating needs some caution as the confidence interval associated with it was much wider (4,544 – 1,00,116), hence not permitting firm dating. Furthermore, the predominance of the lineage 'A' in the Indian breeds was different than the multiple lineages reported in other Asian and European countries (Tapio *et al.*, 2006a; Chen *et al.*, 2006; Meadows *et al.*, 2007) could be questionable. The predominance of the lineage 'A' in Indian breeds could arise from high levels of relatedness and a low effective population size (*Ne*) within the animals sequenced. However, sequence analysis of the mtDNA from Indian sheep showed high genetic diversity within the breeds. The haplotype diversity in the analyzed breeds was not only comparable and/or higher than that in other European and Asian breeds (Table 3.2 & 3.6), but also the haplotypes in the Indian breeds were due to mutations mostly at positions different from the mutational positions observed within the European breeds and the other Asian breed, Javanese Thin Tail (Table 3.3). Further, clade 'A' sequences from the cytochrome *b* gene were highly divergent. Considering multiple sampling of phenotypically distinct breeds and moderate-to-high levels of both observed nucleotide (π) and haplotype (*Hd*) diversity (Table 3.2 and 3.6), low *Ne* appears unlikely. The only alternative explanation could be a single origin for the three Indian breeds with a rare subsequent introgression from European breeds expected to carry the clade B mtDNA lineage (Pereira *et al.*, 2006; Tapio *et al.*, 2006a). Of course, analysis of additional Indian breeds is required to determine if this is a general phenomenon of Indian sheep breeds.

3.4.3 India as the centre of domestication of lineage 'A'

It is generally expected that the genetic loci from ancestral population would show higher nucleotide and haplotypic diversity compared to a derived population (derived from a subset of the genetic types of ancestor) (Troy et al., 2001; Savolainen et al., 2002). In order to understand the origin of the lineage 'A', we compared the evolutionarily important cytochrome b sequences belonging to lineage 'A' from Indian and other Asian populations. Indian haplogroup 'A' showed 1.45 substitutions per site whereas it was 0.707 in other Asian populations suggesting higher genetic variation in Indian haplogroup 'A' compared to the other Asian countries/regions (Table 3.8). Also, the nucleotide diversity of the sequences belonging to haplogroup

A from India (1.52 x 10^{-3}) was higher than the haplogroup 'A' sequences from the other Asian populations (0.73 x 10^{-3}). In addition India population had considerably higher proportions of unique haplotypes (41 within lineage 'A') than the other Asian countries/regions (15 within lineage 'A') [Table 3.7]. The frequency of the central haplotype (H4) was lower in Indian population (51.51%) than in the other Asian populations (71.26%). Further, in India, 13 and 9 haplotypes were at a distance of two and three or more mutation steps away from the central haplotype, respectively, whereas in other Asian population there were only 2 (from China) haplotypes with maximum of two mutation steps away from the central haplotype. Moreover, the distribution of the haplogroup 'A' from Indian and other Asian and European population in the median joining network (Fig. 3.3) indicated that the Indian haplotypes were distributed throughout, whereas European and other Asian countries/regions haplotypes restricted to part of the network. Such pattern of distribution could be because of independent domestication of lineage 'A' in India and subsequent gene flow to the other Asian and European populations. This indicated ancient nature of lineage 'A' in Indian sheep population supported by the larger (26, 986 years) time since commencement of expansion for Indian lineage 'A'.Furthermore, out of 49 haplotypes from Indian breeds, 5 were closely related to the divergent haplotype belonging to the sub-clade a5 (DQ309021) from the other Asian population (China; Chen et al., 2006) while one to haplotype 'C'. These findings indicated higher diversity in the lineage 'A' sequences leading to the possibility of a diverse pool of pre-domesticated animals of lineage 'A' in India. Further, it also suggests that two independent domestication events might have occurred in sheep lineage 'A' and India might be one of those domestication centers. However, due to the lack of samples of domestic sheep and *O. orientalis* from Indus Valley and Central Asia and the quite confused taxonomy of *Ovis* genus with the lack of data on the distribution of *O. orientalis* from India, the results presented here do not permit to confirm and hence, infer the exact geographic locations of lineage 'A' domestication.

Chapter 4

Identification and analysis of prolificacy gene in few Indian sheep breeds and its introgression into Deccani breed

The contents of this chapter have been published in Current Science

4.1 Introduction

The Indian sheep industry is mainly based on the production of lamb meat and contributes about 37% to the total meat production (FAO, Production Book'98). Profitable and sustainable sheep production is a high priority for India and genetic improvement of sheep for meat production is one of the important developmental priorities. Enhancing the reproductive rate of sheep is a logical approach to improving economic efficiency of meat production. However, the litter size of almost all Indian sheep breeds, except the Garole, is very low and is thus a major constraint to sheep meat production. It has been shown that the Garole breed possesses the same prolificacy gene mutation as in the Booroola (Davis *et al.,* 2002), supporting the theory put forth by Turner (1882) that the Bengal sheep that arrived in Australia in 1792 might be the probable source of *FecB* mutation in Booroola**.** However, Garole has low growth rate and body weight and poor survival in harsh semi-arid environment (Present study, Nimbkar *et al.,* 2003b) and hence it is not suitable for meat production in semi-arid environment of India. Therefore, other Indian sheep breeds having more desirable characteristics for meat production and the ability to survive in semi arid environments need to be screened for the *FecB* mutation.

The Deccani is the main sheep breed of Maharashtra state. It is a coarse wool breed and one of the largest contributors to the meat production of India (Khan *et al.,* 2002). There are about 3 million Deccani sheep in Maharashtra, reared in flocks of 30-100 breeding ewes by small holder farmers and landless flock owners. However, the reproductive performance of the Deccani is very low with an average litter size of 1.04 and hence, genetic improvement of reproductive performance of this breed is necessary. There are three main methods of bringing about genetic change in litter size: selection within breeds, the use of between breed variability and the use of major genes such as the Booroola (Piper & Bindon, 1982a, b). Increasing prolificacy in sheep *via* selection within the available breeds is a very slow process and only about 20% improvement in lambing could be achieved. In addition, the heritability of litter size in sheep is about only 0.1; however, the coefficient of variation is relatively high in many breeds, indicating a potential for one to two percent annual rate of genetic gain for single trait selection (Bradford, 1985; Elsen *et al.,* 1994). For example, the average rates of genetic improvement of 1.3% of the mean per year have been realized in Merino, Romney and Galway breeds, mainly because of the high

coefficient of variation in these breeds (Land *et al.*, 1983). However, the fecundity of Deccani is very low and there is not much genetic variation (with respect to fecundity) to exploit by selection. In such a case, the use of other breed resources or major genes is likely to be a faster and more efficient option. Introduction of a prolificacy gene into non-prolific sheep breeds having other desired traits may effectively increase the reproductive performance of local breeds. Therefore, a diallel cross-breeding programme using the Deccani, Bannur and prolific Garole breeds was conducted at the Nimbkar Agricultural Research Institute (NARI), Phaltan, Maharashtra state, India, during 1996-99. The Bannur breed was included because it has the best body conformation for meat production. The resulting progeny (¼ Garole ewes) were screened for *FecB* mutation using the PCR-RFLP test (Wilson *et al.*, 2001). Ovulation rate (OR) and litter sizes (LS) were analyzed to study the effect of *FecB* gene introgression in these ewes.

4.2 Materials and Methods

4.2.1 Sample collection and DNA isolation

This has already been described in Chapter 2 (Fig. 2.1).

4.2.2 Breeding program

Crossbreeding between Deccani (D)/Bannur (B) and Garole (G) was carried out at NARI. Ten F1 rams (D/B x G) were produced by crossing Deccani (D)/Bannur (B) ewes with nine Garole (G) rams. Each of the ten F1 rams was then single-sire mated to Deccani (D), Bannur (B) and Deccani x Bannur (D x B) or Bannur x Deccani (B x D) ewes to produce ¼ Garole {G x D/B x D or G x D /B x (D x B) or G x D/B x (B x D) progeny (12 to 27 daughters per sire representing ten progeny groups). Two hundred and twenty seven females resulting from this backcrossing were included in the analysis of the *FecB* mutation. Ninety of these ¼ Garole ewes were further inseminated with Deccani ram semen and the resulting progeny were analyzed for the same mutation. Furthermore, the test was extended to 340 samples collected from shepherds' flocks where homozygous ($FecB^{B}/FecB^{B}$) crossbred rams had been introduced by NARI. These experiments were performed to study the extent of management of the increased prolificacy of the crossbred animals under local environment of sheep rearing in Maharashtra.

4.2.3 Determination of ovulation rate

The ovulation rate of the ewes was determined by laparoscopy at 4 to 7 days after natural oestrus, twice before and after first lambing at NARI. The ovulation rates (OR) of pure Garole, Deccani and heterozygous and non-carrier Deccani - Garole crosses were determined.

4.2.4 PCR amplification

Sixteen nanogram DNA per sample per reaction was amplified in 4 µL volume containing 1X PCR buffer (10 mM Tris-HCl; pH-8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% w/v gelatin), 100 µM of dNTPs, 2.5 ng (7 µM) of forward and reverse primers, 0.22 µg of TaqStart antibody (Clontech, USA) and 0.1 U of AmpliTaq DNA polymerase (Perkin Elmer, USA). The reaction consisted initial cycle of denaturation (95ºC, 2.5 min) followed by 30 cycles of denaturation (95ºC, 10 s), annealing (62ºC, 30 s) and extension (72ºC, 30 s) and one final cycle of extension (72ºC, 2.5 min) in a PTC 200, thermal cycle (MJ Research, USA). The amplified PCR product was subjected to *Ava*II digestion by adding a restriction enzyme cocktail (8 µL) containing 1X RE buffer M (GE Healthcare, USA), 44 mM $MgCl_2$, acetylated BSA (1.2 µg), water and 0.4 U *Ava*II enzyme (GE Healthcare, USA) and incubated for 3 hours at 37ºC. Following digestion, the fragments were electrophoresed on 3% MetaPhor agarose gel and scored for the mutation depending upon the size and number of bands as homozygous mutation ($FecB^B/FecB^B$, 110 bp); heterozygous ($FecB^B/FecB^+$; 110 bp and 140 bp) and homozygous wild type ($FecB^+/FecB^+$; 140 bp).

4.3 Results

The representative amplification profiles of $FecB^B/FecB^B$, $FecB^B/FecB^+$ and $FecB^+/FecB^+$ genotypes for each population are shown in Figure 4.1a and 4.1b and the results are summarized in Table 4.1 The *BMPR-IB* mutation ($FecB^B/FecB^B$) was found only in the Garole sheep population. Of the ten backcross progeny groups with ¼ Garole genes generated at NARI, 37-63% carried one copy of the Booroola allele ($FecB^B/FecB^+$) (Table 4.2). Considering all the backcrossed ewes together irrespective of their individual group, nearly half (49.53%) of the ¼ Garole ewes were heterozygous ($FecB^B/FecB^+$) as is the expectation for the Mendelian inheritance of a single copy of the mutation present in one parent. Further analysis of 194 progeny

with 12.5% Garole genome from *FecB* heterozygous sires revealed the presence of one copy of *FecB* in 90 animals (46.39%) and the wild type genotype in 104 animals (53.60%). Among the 340 progeny of $FecB^{B}/FecB^{B}$ rams in local shepherds' flocks, 55% were found to be $FecB^{+}/FecB^{+}$ and 45% $FecB^{B}/FecB^{+}$. One copy of *FecB* increased OR from 1.03 to 2.02 whereas the litter size (LS) in 25% Garole ewes increased by 0.52, 0.61 and 1.03 for the first, second and third parities, respectively. Single and twin lambs in this study were 28% and 44%, respectively, with the low incidence of triplet (2.5%).

Table 4.1: *FecB* genotypes of pure breeds

Breed/Strain	$FecB^{B}$ / $FecB^{B}$	$FecB^{B}$ /$FecB^{+}$	$FecB^{+}$/ $FecB^{+}$	Total
Garole	124	12	-	136
Deccani subtypes				330
Kolhapuri			20	20
Lonand			150	150
Madgyal			20	20
Sangamneri			20	20
Sangola			20	20
Bannur			26	26
Madras Red			20	20

Table 4.2: Segregation of the *FecB* mutant allele in the Garole crossbred animals

Family No.	Garole grand sire (Tag number)	Crossbred sire (Tag number)	% of heterozygous mutant allele in the crossbred progeny ($FecB^{B}/FecB^{+}$)
1	16258	417	57.14
2	16260	78803	35.71
3	16258	112	36.36
4	16252	404	41.17
5	16259	409	57.89
6	16253	R180	38.46
7	16260	27811	47.82
8	193	D98047	50.00
9	106	28709	57.24
10	R140	D98105	39.28

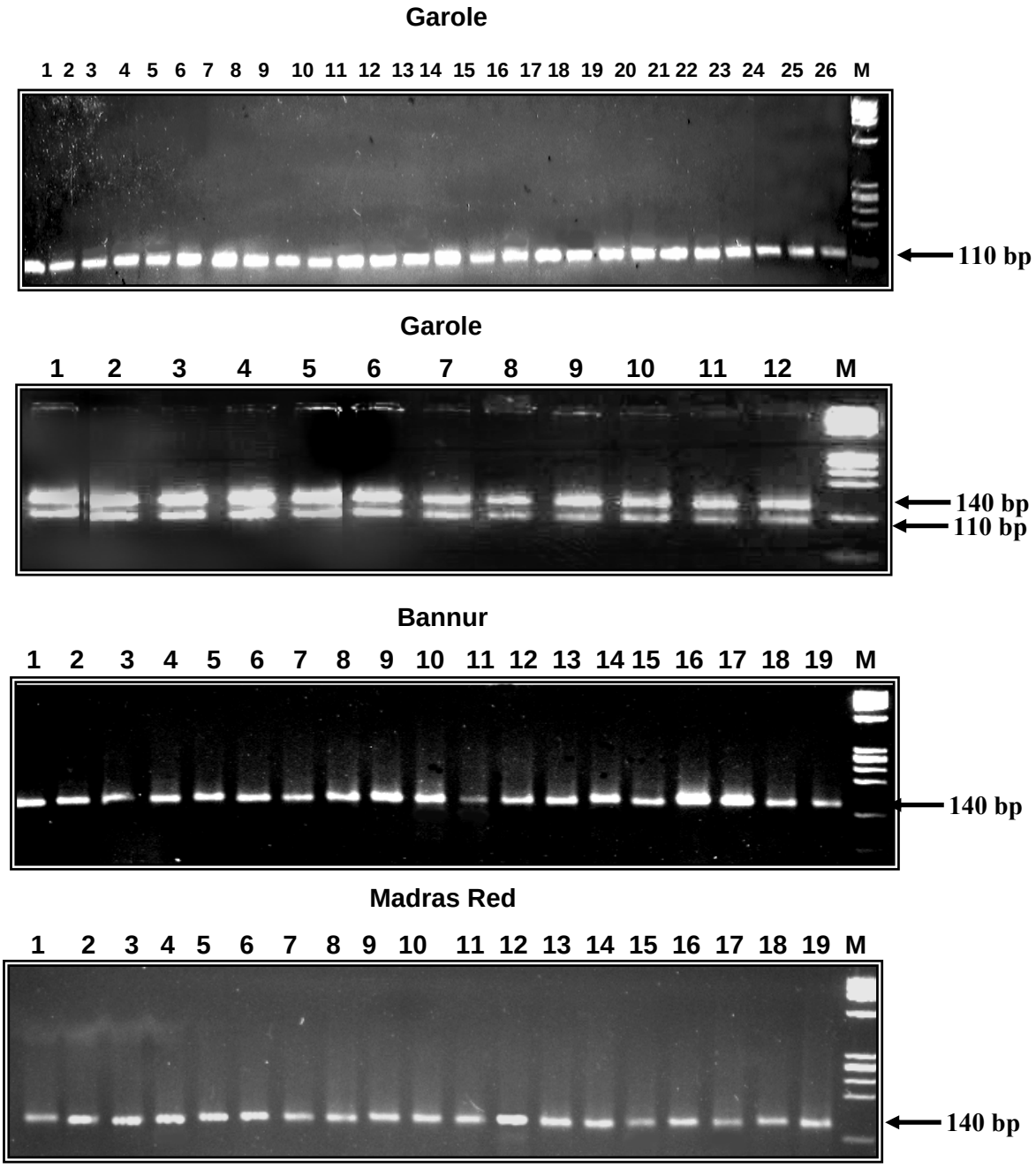


Figure 4.1a: *BMPR-IB* mutation detection in Garole, Bannur and Madras Red sheep population using PCR-RFLP method
(140 bp band represents *FecB*$^{+}$ allele while the 110 bp band indicates *FecB*^{B} allele)

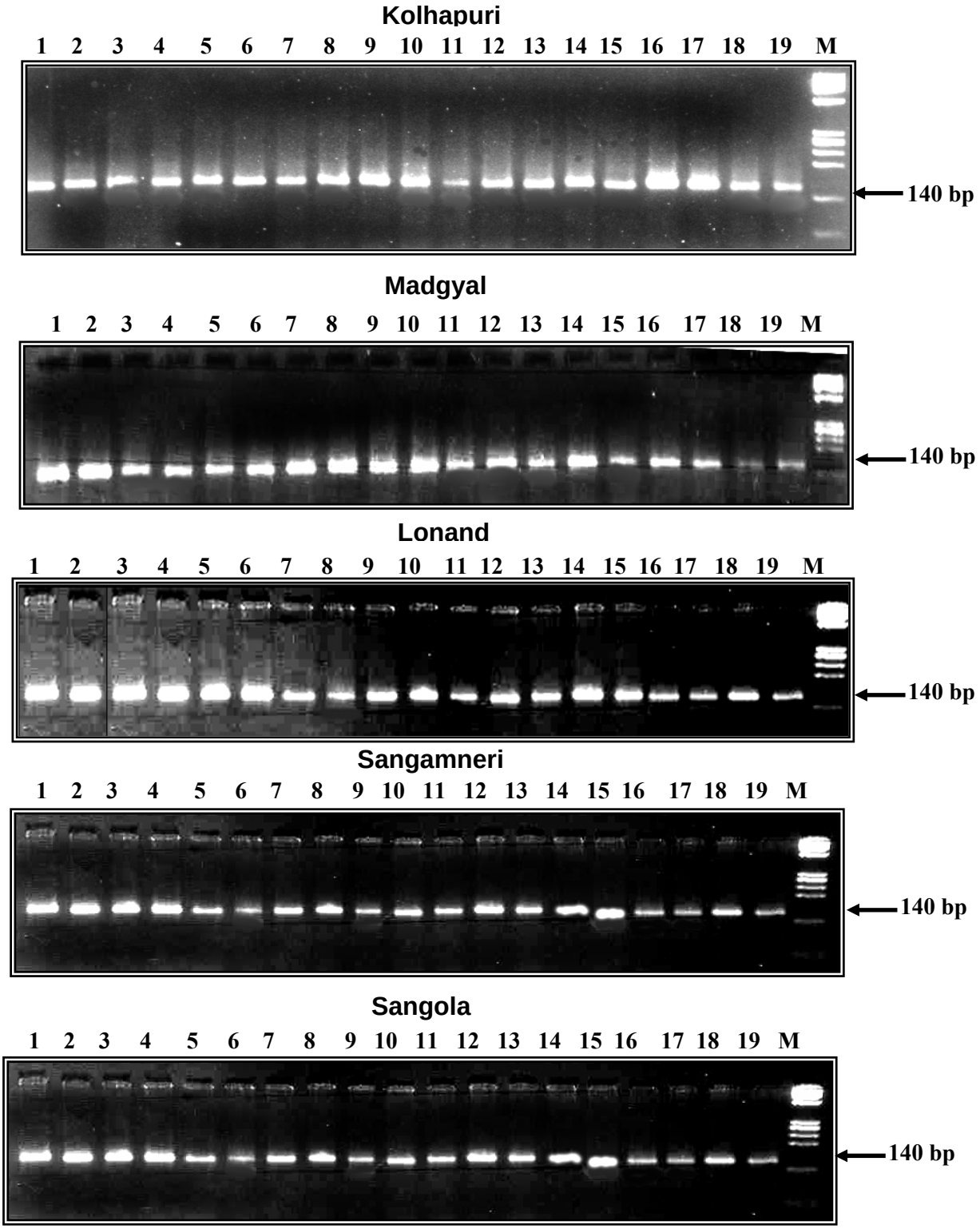


Figure 4.1b: *BMPR-IB* mutation detection in Deccani strains using PCR-RFLP method

(140 bp band represents *FecB*$^{+}$ allele while the 110 bp band indicates *FecB*^{B} allele)

4.4 Discussion

4.4.1 FecB gene mutation screening

The research for increasing the prolificacy of sheep *via* utilization of the major genes was started in 1980 and till date many prolificacy genes have been identified. The discovery of the *FecB* mutation led to the development of a commercial DNA test, and prompted researchers to screen other prolific sheep breeds to determine whether the same mutation is also responsible for their high prolificacy. A number of prolific breeds all over the world were screened and besides Booroola, the mutated gene was found to be present in Garole (India; Davis *et al.*, 2002), Javanese Thin Tailed (Indonesia; Davis *et al.*, 2002), Small Tailed Han (China; Liu *et al.*, 2003; Wang *et al.*, 2003; Jia *et al.*, 2005; Yan *et al.*, 2005; Davis *et al.*, 2006), and Hu sheep (China; Wang *et al.*, 2003, 2005; Yan *et al.*, 2005; Davis *et al.*, 2006; Guan *et al.*, 2006). However, other Indian breeds were not screened for the mutation. Hence, the screening was carried out in some other Indian breeds *viz*., Deccani (represented by five strains), Madras Red and Bannur breeds along with the Garole breed. However, as expected, the mutation was observed only in the prolific Garole sheep breed and was absent in other breeds analyzed (Table 4.1 and Figure 4.1a, b). The result suggests that probably there were no natural avenues for transfer of the mutation from Garole into the other breeds analyzed here. This is not surprising considering the vast distance (1500 to 2000 km) between the Garole's native place in northeastern India and that of the other breeds in central and southern India. The twelve samples of Garole showing $FecB^{B}/FecB^{+}$ genotype indicated that these animals are unintended crossbred animals.

To date, the prolificacy gene has been introgressed from the Booroola Merino into several breeds in different countries to improve the reproduction rate while maintaining desirable levels of performance for other traits (Davis *et al.*, 1991a; Meyer *et al.*, 1994a; Southey *et al.*, 2002). However, earlier attempts of breeding for developing and establishing new breeds of sheep incorporating the *FecB* gene and having consistent and predictable product prior to the development of DNA tests required a lot of effort and time and could not meet the growing demands of the market. The speed and efficiency of selection was increased by the use of molecular markers associated with *FecB* gene in selecting the desired genotypes in the breeding

programs (Montgomery *et al.*, 1993; Gootwine *et al.*, 1998; Gootwine *et al.*, 2001). However, the indirect DNA tests required prior information about the *FecB* status of at least one parent to develop the pedigree, which needed progeny testing and the analysis of the ovulation rate data. It was also essential that the animal carrying the *FecB* gene should inherit the whole chromosomal region spanned by the markers since recombination between the markers hampered detection of the *FecB* status of the animal. Three microsatellite markers Oar-AE101, Oar-HH55 and BM1329 commonly used for the early identification of *FecB* carriers resulted in 10% error due to recombination between the markers (Montgomery *et al.*, 1996). The discovery of the mutation in the *BMPR-IB* (*ALK6*) receptor and subsequent development of the PCR-RFLP test was the major breakthrough for the animal production industry. This test overcomes the disadvantages of the marker test and is easy to perform. Early detection of allelic status of the *FecB* gene and no need to compare the phenotype data with the marker data are some additional advantages. Finally, a large number of samples can be tested in a short period, which would help the animal husbandry researchers to design breeding strategies and to advise shepherds appropriately in time.

4.4.2 FecB gene introgression analysis

This is the first study of the use of a breed other than the Booroola Merino to introduce the *FecB* gene into a non-prolific breed and the comparison of heterozygote ($FecB^{B}/FecB^{+}$) and non-carrier ($FecB^{+}/FecB^{+}$) animals based on genotypes obtained using the forced RFLP test for the Booroola mutation. The OR of the *FecB* homozygote (mostly pure Garole), *FecB* heterozygote (Garole-Deccani crosses) and non-carrier ewes (Deccani and its crosses with Garole) as determined at NARI, were 3.37, 2.09 and 1.08, respectively (Table 4.3). These studies showed that the introgression of one copy of the Garole gene ($FecB^{B}$) increased the prolificacy of Deccani sheep by about 0.7 lambs born/ewe lambing and the OR by nearly one corpus luteum per pair of ovaries, which was lower than that estimated by Piper *et al.* (1985). However, similar results have been observed in Garole x Malpura crosses (Sharma *et al.*, 2004; Kumar *et al.*, 2006, 2007a) and Javanese Thin Tailed crosses (Inounu *et al.*, 1993) [Table 4.3] as well as other crosses involving the Booroola strain, *viz.,* Booroola Merino x Rambouillet and Booroola Merino x Awassi, where prolificacy was increased by 0.65 and 0.6 lambs born/ewe lambing (Gootwine *et al.*, 1998;

Southey *et al.*, 2002). Further the effect of one copy of the *FecB* gene on LS in ¼ Garole ewes was 0.52, 0.61 and 1.03 for the first, the second and the third parities, respectively, suggesting that the effect of the gene appears to increase with parity.

4.4.2.1 FecB gene expression

A notable difference between this and other studies of sheep flocks into which the *FecB* gene has been introgressed, is the low incidence (2.5%) of triplet or higher order litters. Southey *et al.* (2002) reported the most common litter size class for $FecB^{B}$ / $FecB^{+}$ ewes among Booroola Merino-Rambouillet crosses as 3, with the exception of 87.5% Rambouillet ewes. A majority of lambings of ¾ Romney or Perendale and ¼ Booroola Merino $FecB^{B}/FecB^{+}$ ewes were also three or more lambs (Meyer *et al.*, 1994a). This could be due to the higher average litter size of the background breeds and suggested that the effect of the gene varies according to the breed into which the gene is introgressed. However, in the studies reporting the higher estimates of LS (>/= 0.9), the ewe genotyping was based on the ovulation rate criterion of Davis *et al.* (1982) (i.e. the ewes with at least one OR observation of >3 were classified as carrying one copy of the gene and the ewes with at least one OR observation >5 were classified as homozygous for the gene). Therefore, the difference in LS estimates is likely to be either due to misclassification of ewes or to differences in the method of estimating the mean OR and LS for the different genotypes. Studies based on genotype determination using microsatellite markers or the direct DNA test (Gootwine *et al.,* 2001; Gootwine *et al.,* 2003; Schulze *et al.,* 2003) reported the increase in LS with one copy of the gene to be in the range of 0.5-0.7. Piper & Bindon (1982b) reported the litter size among Booroola x medium non-Peppin Merino (MNP) ewes to be higher by only 0.57 than that of pure MNP ewes. It is, however, not clear from the article, if the Booroola sires of the crossbred ewes were homozygous $FecB^{B}/FecB^{B}$ rams since it was published before the presence of the single prolificacy gene in the Booroola Merino was confirmed.

A few other undesirable characteristics such as a reduction in body size, decrease in growth rate and an increase in embryo mortality were observed in the Garole crossbred animals (Present study, Nimbkar *et al.*, 2003b). In many cases, Booroola crossbred lambs have also been reported to have inferior growth rate and small mature body size (Smith *et al.*, 1993; Smith *et al.*, 1996; Walling *et al.*, 2000) and a reduction in milk yield (Gootwine *et al.*, 1995). While it is likely that some of

these negative traits are due to multiple births/multiple lambs reared per ewe rather than the effect of the *FecB* mutation itself. Therefore, using molecular markers, it is desirable to transfer only the minimum possible Garole genome by selecting for productivity traits and using the PCR-RFLP test to select for increased prolificacy and with minimum linkage drag.

The present study suggest that there might be some synergism between the effects of *FecB* and the genome of the Deccani sheep since these sheep have been observed to have higher milk production and consequently a better ability to rear lambs than the Garole sheep (Nimbkar observed; unpublished data).

4.4.3 Dampened expression of the FecB gene in Garole

The mean ovulation rate (5.7) and litter size (2.6) of the Booroola are quite high (Piper & Bindon, 1985) as compared to that of Garole, though the same mutation is present in both the breeds indicating that the expression of the gene is dampened in Garole. The damped effect of *FecB* in Garole and ¼ Garole ewes could be due environmental differences (e.g. plane of nutrition mainly lower level and poorer quality of nutrition available to the sheep in the tropics.), a breed effect or the presence of other genetic factors such as modifier genes or novel mutations within the *BMPR-IB* gene that dampen the expression of the *FecB* trait in the Garole sheep. In West Bengal, which is the natural habitat of the Garole sheep, the mean litter size is 2.23 (Ghalsasi & Nimbkar, 1993; based on information collected from farmers); whereas, in the semi-arid environment of Maharashtra, the mean litter size is 1.74 (Nimbkar *et al.*, 1998). Alternatively, the genetic effects enhanced by the environmental factors might be contributing to such suppressed expression of the gene in Garole sheep. The effect of the *FecB* gene on prolificacy has been considered to be undesirably large and higher lamb mortality due to the higher incidence of multiple births in Booroola crossbreds has been widely reported in the literature (Davis *et al.*, 1991a). Although the effect of the *FecB* gene in this study is moderate, it also means that it is more manageable in a shepherd's flock. Therefore, such dampened expression of *FecB* in Garole and Garole crossbred animals is desirable in Indian sheep rearing practices as well as economy.

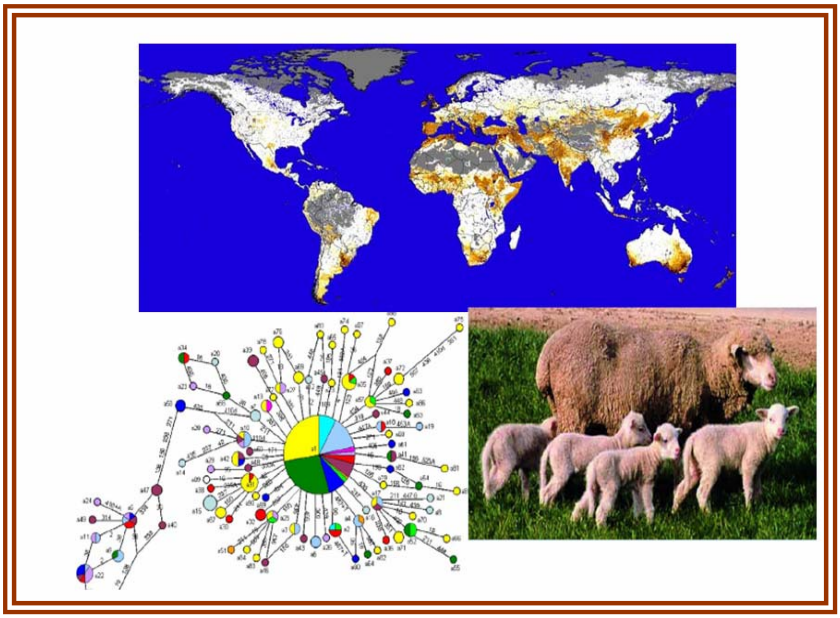
Table 4.3 Mean ovulation rate (OR) and litter size (LS) of *FecB* carrier (heterozygous and homozygous) and non-carrier ewes of various breeds produced by crossing with *FecB* carrier breeds other than the Booroola Merino (number of ewes in brackets)

Breed /cross	$FecB^{+}/FecB^{+}$		$FecB^{B}/FecB^{+}$		$FecB^{B}/FecB^{B}$		Method of genotype detection	Reference
	OR	**LS**	**OR**	**LS**	**OR**	**LS**		
Garole	-	-	-	-	-	3.37	DNA test	Nimbkar *et al.*, 1998
Deccani	-	-	-	-	-	1.08	DNA test	Present study
¼ Garole, ¾ Deccani or Bannur	1.03±0.04[1] (57)	1.01 (69)	2.02±0.07[1] (69)	1.83 (71)	3.37±0.26[1] (53)	2.01±0.07[1] (53)	DNA test	Present study, Nimbkar *et al.*, 2003a
Garole X Malpura carrier	-	1.02	-	1.87	-	-	not detected	Sharma *et al.*, 2004
Javanese Thin Tailed	1.39 (22)	1.38 (21)	2.73 (19)	2.31 (18)	-	-	OR	Bradford *et al.*, 1986
Javanese Thin Tailed	-	1.24±0.42[1] (552 records)	-	1.95±0.64[1] (494 records)	-	2.59±0.94[1] (217 records)	OR and LS	Inounu *et al.*, 1993

[1]standard deviation
(Source: Nimbkar, 2006)

Chapter 5

Summary and future directions



5.1 Indian sheep genetic diversity

Genetic diversity of four Indian domestic sheep breeds *viz.,* Garole, Deccani (represented by the Lonand, Sangamneri, Kolhapuri, Sangola and Madgyal strains), Bannur and Madras Red was assessed using nuclear (microsatellites) and cytoplasmic markers (mtDNA; control region and cytochrome *b* gene). Both the systems revealed high genetic variability in the Indian breeds.

5.1.1 Nuclear marker (microsatellite) analysis

A significant genetic structure among the breeds was observed when nuclear markers such as microsatellites were used for diversity analysis. The pattern of genetic differentiation was broadly consistent with the isolation by distance ($r = 0.3900$, $p<0.027$) suggesting limited genetic exchange among the breeds. However, significant inbreeding was observed in Garole and Madras Red sheep population. The Principal Coordinate Analysis (PCoA) distinguished the four breeds as well as the Deccani strains. Further, the Bayesian clustering approach identified each breed as one lineage, with nine clusters all together. Diversity patterns were concordant with geographical origin and supported the traditional breed classification system

The results obtained from the molecular data generated in this study revealed contribution of Indian sheep husbandry practices towards maintenance of breed identity and diversity. The results also supported the morphology or geography based classification of the Indian breeds and provided new relationship among the Indian breeds. Such population structure needs to be maintained in breed genetic management in order to conserve the diversity of breeds in relation to environmental adaptation and specific performance. However, care must be taken to avoid inbreeding within the breeds by frequent change of breeding rams.

5.1.2 Cytoplamic marker (mtDNA) analysis

This study represents the first characterization of mitochondrial DNA diversity within three breeds of Indian sheep (four strains of the Deccani breed, as well as the Bannur and Garole breeds) from different geographic regions and with divergent phenotypic characteristics. A total of 2028 bp of the mitochondrial genome sequence containing two regions; 1) a 1061 bp fragment spanning the control region, a portion of the 12S rRNA gene and the complete phenyl tRNA gene and 2) a 967 bp fragment of the cytochrome *b* gene were sequenced from 73 and 132 animals, respectively and

compared with the corresponding published sequence from the European and the Asian breeds. Analysis of 156 sequences (Indian, other Asian and European) from control region (1061 bp) fragment revealed 73 haplotypes, 52 of which belonged to the Indian breeds whereas 50 haplotypes were found in 132 Indian animals from the partial cytochrome *b* gene fragment. All the breeds showed considerable nucleotide and haplotype diversity; however, the lineage 'A' sequences of Indian breeds showed higher nucleotide and haplotype diversity as compared to the lineage 'A' sequences from the other Asian populations. Phylogenetic analysis showed presence of two lineages ('A' and 'B') in the Indian sheep breeds, with predominance of lineage 'A' (85.71%). The Indian network of the control region had no clearly defined central haplotype and revealed a lack of shared haplotype. These results indicate a strong genetic structure with mtDNA haplotypes (from control region) diagnostic of particular breeds. Whereas, the network profile of cytochrome *b* showed star shaped phylogeny for both the lineages; however, only 'A' lineage had undergone population expansion as indicated by the mismatch distribution analysis and the Fu's *F*s value (-27.54). Weak population structure was observed using the cytochrome *b* gene in the Indian sheep breeds and could be due to the possible historical gene flow among the breeds. The time since commencement of expansion for the Indian lineage 'A' sequences was 26,986, which was far predated than the sheep domestication time.

These evidences suggest India as the domestication centre for lineage 'A'. They also indicate that the Indian sheep are distinct from the other Asian and European breeds studied so far. These results have implications for the conservation of India's ovine biodiversity and suggest a common origin for the breeds investigated. However, the sampling effort may still be insufficient to see the whole distribution of haplogroups, other than 'A', because of their low frequency. Analysis of more number of Indian sheep breeds from more geographical regions needs to be performed in order to supplement and confirm the proposed hypothesis of India being the centre for lineage 'A' of sheep.

Overall, the networks for Indian sheep (Fig. 3.1 and Fig. 3.2) suggest a history for these breeds defined by very low levels of interbreeding between populations. This is entirely consistent with Indian sheep husbandry, which contains long-standing and strong cultural and traditional barriers and discourages genetic exchange between the breeds. In addition, the breeds under investigation appear to have undergone selection in different climates and in different geographic regions of the country.

5.1.3 Prolificacy gene analysis in the Indian sheep breeds

DNA samples from four Indian sheep breeds *viz.,* Garole, Deccani, Bannur and Madras Red were screened by PCR-RFLP to determine the presence of the *FecB* mutation in these breeds. The Garole was the only breed, which carried the *FecB* mutation. The *FecB* PCR-RFLP test was found to be fast, accurate and useful as a tool for making breeding decisions.

The *FecB* gene was introgressed from Garole sheep into Deccani breed and the performance of the crossbred animals was monitored in subsequent generations. Approximately half of the first backcross ewes (progeny of *FecB* heterozygote F1 rams) was found to carry one copy of *FecB* mutation, as expected. The introgression of one copy of the *FecB* gene increased the ovulation rate by 1.0 and litter size by 0.7. The expression of the gene was thus found to be dampened in Garole and its crosses. Such moderate increase in prolificacy is manageable under Indian sheep rearing conditions.

5.2 Need and challenges in sheep genetic resources management

Maintaining breed diversity enables people to exploit diverse ecological or economic niches. This is particularly the case in marginal and environmentally fragile areas, such as drylands, where most livestock is located and kept mostly by poor farmers/shepherds.

During the past decade a large number of genetic diversity studies using molecular markers were carried out in domestic sheep breeds all over the world. Asia is the domestication centre (Near East) for sheep and also has biologically diverse sheep population, making it an important region for sheep diversity studies. However, very little efforts have been made to study the sheep breed diversity from this region. Limited reports are available from few countries such as India, China, Pakistan, and Indonesia as well as the Near East region. On the contrary, an example of large-scale analyses of the sheep genetic diversity, the Econogene project (http://lasig.epfl.ch/projets/econogene/) from the European countries is available. The extensive mtDNA mapping of European sheep has facilitated to identify the progenitor for these sheep breeds, whereas the Asian sheep progenitor still remains unknown. The results from present study indicate that the Indian sheep have unique evolutionary pattern. Similar studies from the nearby countries such as Pakistan, Bangladesh and Afghanistan sheep populations will contribute to diversity and to

explore exchange patterns. Therefore, there is a need for a close coordination between large-scale projects and extensive genetic mapping of the available breeds from this region. Such information will aid to understand a global estimate of existing domestic sheep genetic diversity. It can also assist to uncover the history of Asian sheep breeds as well as to define objective (scientific) criteria for deciding whether breed populations that occur in different countries belong to a common gene pool or suggest a different evolutionary pattern. In addition, such data also represent a source for identifying unique phenotypic qualities and the most pure populations which can then be the focus of conservation and further development.

5.3 Marker assisted selection: A step towards genetic improvement in sheep

The molecular characterization of the breeds plays an important role in the genetic improvement. Molecular techniques (e.g. MAS) serve as an aid to avoid excessive inbreeding while at the same time permit the targeted functional variation. Selection programs for economically important traits can increase the frequency of genes desirable for the productivity and profitability of local breeds. Genetic improvement of the reproductive traits *via* the introgression of the prolificacy gene is one such example. Various prolificacy genes from a number of sheep breeds throughout the world have been identified. However, the Booroola gene (*FecB*) is the most widely used. The recent development of the marker test to identify the *FecB* mutation has opened an excellent opportunity to use this gene. Marker assisted selection to improve the prolificacy of many breeds in various countries is going on. In the present study, the introgression of the Garole *FecB* gene into Deccani was carried out. The introgession process was monitored using the PCR-RFLP method while keeping the percentage of Garole genome to the minimum. The increase in prolificacy of the Deccani breed was found to be moderate and manageable. Thus, the ease of application and the potential of extra gain are the greatest for such direct markers. This has also resulted in great optimism for the use of molecular markers in planning breeding programs. Therefore, research to find many more such direct markers related to desired production related traits would be beneficial for the genetic improvement of the livestock. The International Sheep Genomics Consortium (ISGC) is currently engaged in "HapMap" analysis for global sheep breeds (http://www.sheephapmap.org/http://www.sheephapmap.org/). This approach has been estimated to provide assembled and ordered sequence for the ovine genome. It

would also detect probable SNPs with defined genomic locations. The information generated will help in the analysis of genetic variation within the sheep genome. Identification and cataloguing thousands of single nucleotide polymorphisms (SNPs) will help to develop a research tool to speed up the gene discovery process in sheep.

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Research experience

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- **V.C. Pardeshi**, N.Y. Kadoo, M.N. Sainani, J.F. Maddox and V.S. Gupta. Genetic diversity assessment using microsatellite markers revealed significant regional uniqueness of Indian sheep breeds. Communicated to Animal Genetics.
- **V.C. Pardeshi**, N.Y. Kadoo and V.S. Gupta. Phylogenetic analysis of Indian sheep breeds using cytochrome *b* gene sequence. Communicated to BMC Genetics.

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- **V.C. Pardeshi**, J.M. Maddox, N.Y. Kadoo, Gupta V.S. (2008) Genetic modulation of the *FecB* gene expression submitted to the Helen Newton Turner memorial international workshop on 'Using the *FecB* (Booroola) gene in sheep breeding programs', to be held in NCL, India from 10-12 November, 2008.
- C. Nimbkar, P.M. Ghalsasi, B.V. Nimbkar , P.P. Ghalsasi, V.S .Gupta, **V.C. Pardeshi**, J.F. Maddox, J.H.J van der Werf, G.D. Gray, S.W Walkden-Brown. (2008) Biological and economic consequences of introgression of the *FecB* (Booroola) gene into Deccani sheep submitted to the Helen Newton Turner memorial international workshop on 'Using the *FecB* (Booroola) gene in sheep breeding programs', to be held in NCL, India from 10-12 November, 2008.
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Posters presentations

- **V. Pardeshi**, N. Kadoo, M. Sainani, and V. Gupta. A study of genetic diversity in important sheep breeds of India (2006). In Symposium on National Biodiversity and Ecosystem Information Infrastructure: Challenges and Potentials. Pune, India, January 30 – February 2.

- J. Kijas, J. Meadows, **V. Pardeshi**, V. Gupta, C. Drögemüller, C. Moran, B. O'Rourke and P. Arthur (2006). Genetic basis of the Australian Merino. In International Society of Animal Genetics (ISAG). Porto Seguro, Brazil on August 20-25.
- **V. Pardeshi**, M. Sainani, V. Gupta, P. Ghalsasi, C. Nimbkar, B. Nimbkar, J. Maddox and S. Walkden-brown (2005). A study towards genetic diversity and genetic basis of prolificacy in important sheep breeds of India International Conference on Plant Genomics & Biotechnology: Challenges & Opportunities. Raipur, India October 26-28. (received **gold medal** for the poster)

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SYNOPSIS OF THE THESIS TO BE SUBMITTED TO THE UNIVERSITY OF MUMBAI FOR THE DEGREE OF Ph.D. IN BIOTECHNOLOGY

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Topic of research	A study towards genetic diversity and genetic basis of prolificacy in important sheep breeds of India
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(V. S. Gupta) (Varsha C. Pardeshi)

A study towards genetic diversity and genetic basis of prolificacy in important sheep breeds of India

Synopsis

For the Degree of Doctor of Philosophy
in Biotechnology

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Organization of thesis

Title: A study towards genetic diversity and genetic basis of prolificacy in important sheep breeds of India

Chapter 1: Introduction and review of literature

Chapter 2: Molecular genetic diversity study of geographically and economically important Indian sheep breeds using nuclear markers

Genotyping of four breeds *viz.,* Garole, Deccani (represented by five subtypes, Lonand, Madgyal, Sangamneri, Sangola and Kolhapuri), Bannur and Madras red using 31 markers and genetic diversity analysis using various population genetics softwares'

Chapter 3: Phylogenetic relationships and population expansion study using mtDNA

Sequencing of the cytochrome *b* gene and D-loop region of mtDNA from three breeds Bannur, Garole and Deccani and sequence data analysis using various softwares

Chapter 4: Identification and analysis of prolificacy gene in a few Indian sheep breeds and its introgression into the Deccani breed

d) Prolificacy gene mutation screening in the four sheep breeds *viz.,* Garole, Bannur, Deccani and Madras red using PCR-RFLP method
e) Introgression study of the prolificacy gene from Garole into Deccani
f) Dampening effect of the prolificacy gene

Chapter 5: Summary and future approaches

Bibliography

Introduction

Sheep (*Ovis aries L.*) constitutes an important species of livestock and plays an important role in the rural agricultural economy of arid and semi arid zones in India. India is endowed with large and biologically diverse sheep population. However, this indigenous genetic diversity is under utilized in the most conventional systems. In the marginal environments local livestock breeds are crucial for rural livelihood since they provide a wide range of products while requiring relatively low levels of input. Moreover, these indigenous breeds are well adapted to the harsh environments and have additional characteristics like disease resistance. But in conventional breeding programme such advantageous traits were underutilized due to failure to identify the animals carrying the most advantageous traits. Conservation of these local breeds is gaining the highest priority for future use of mankind. Also, these breeds are not very clearly defined. The classification based on phenotype alone is not precise since the apparent phenotypic differences may be due to adaptation to specific agro-climatic conditions

Breed characterization and their genetic improvement for the conservation and better utilization require the knowledge of genetic variation available both, within and between the sheep breeds. Molecular markers that are neutral in their behavior are ideal markers to reveal diversity within and between breeds and gaining importance in such analysis in various species. Study of molecular variation within and between breeds can help in making objective management decisions regarding genetic resources of sheep breeds and conservation of unique populations for future breeding strategies. Among the various problems, low fertility in sheep is the most important constraint of the meat industry to derive benefits. One of the approaches to increase meat production is to introduce prolificacy gene into non- prolific sheep breeds having desired traits. Therefore, in the present study four economically important sheep breeds, viz, Garole, Deccani (five strains Lonand, Madgyal, Sangamneri, Sangola, and Kolhapuri), Bannur and Madras red were used for diversity analysis. Prolificacy gene screening was carried out and the prolific breed Garole was used for its introgression into non-prolific breed, Deccani.

Molecular genetic variation study of Indian sheep breeds with autosomal nuclear markers

The Indian sheep population, represented by 42 breeds in diverse ecological conditions, is ranked forth in the world (Acharya, 1982). It is important to conserve the domestic breeds in order to maintain or increase meat production to keep pace with global environmental changes, provide opportunities to meet future market demands, possibilities to offer livelihoods for people, both locally and globally, together with cultural-historic and scientific reasons (Oldenbroek, 1999). Breed characterization is the primary step in any conservation program. Conventionally, the Indian sheep breeds are classified based on the phenotypic characters or Agro-ecological regions. However, the accuracy of phenotypic characterization of domestic sheep is often affected by influence of the environment and the underlying genetic complexity. Therefore, assessment and characterization using a neutral system is essential. A number of studies have been initiated all over the world to characterize the domestic breeds using different molecular techniques (Hoelzel, 1998; Brudford *et al.*, 2003; Luikart *et al.*, 2003). Microsatellite markers, by virtue of their co-dominant and multi-allelic nature, prove to be efficient in genetic diversity studies, pedigree evaluation and genetic mapping as compared to other molecular markers like RAPD, RFLP and ISSR (Goldstein and Pollock, 1997). Hence, microsatellites have become the markers of choice in characterization of sheep breeds (Buchaman *et al.*, 1994; Arranz *et al.*, 1998, 2001; MacHugh *et al.*, 1997; Saitbekova *et al.*, 1999; Diez-Tascon *et al.*, 2000; Barker *et al.*, 2001). Such data can also be useful to make objective decisions regarding conservation of breeds and to pyramid breed specific genes to develop economically beneficial composite breeds. In the case of sheep populations in East and South Asia, extensive research has been done and a vast amount of valuable data on morphological and ecological characters, genetic diversity and phylogenetic relationships have been accumulated (Geng et al, 2002, 2003; Sun *et al.*, 2002, 2003, 2004a, b, 2005; Lu *et al.*, 2004, 2005a, b, c; Yang *et al.* 2004a, b, c; Du *et al.*, 2005). However, only a few studies have been performed to characterize the Indian sheep breeds using reliable molecular markers (Sodhi *et al.*, 2003, 2006; Arora and Bhatia, 2004; Mukesh *et al.*, 2006; Sharma *et al.*, 2006; Kumar *et al.*, 2007b).

In the present study genetic diversity of four Indian domestic sheep breeds namely, the Garole, Deccani (represented by the Lonand, Sangamneri, Kolhapuri,

Sangola and Madgyal strains), Bannur and Madras Red, was assessed using 31 microsatellites markers. Three hundred and nineteen (about 27-62 individuals per breed) animals were genotyped to ascertain their historical relationships by applying recent statistical methods. All the breeds displayed high within breeds genetic diversity reflected by high mean allelic diversity and heterozygosity and were genetically distinct (F_{ST}: 0.12 ± 0.01). The Principal Components Analysis (PCoA) distinguished the four breeds as well as the Deccani strains. A Bayesian clustering approach identified the each breed as one lineage, with about nine clusters all together. Overall pattern of isolation by distance was evident (r = 0.390 P<0.027) suggesting limited dispersal and extensive philopatry of the breeds studied. Diversity pattern were concordant with geographical origin and supports traditional classification system.

Phylogenetic relationships and population expansion study using mtDNA

The sequence variation existing within the mitochondrial DNA (mtDNA) has proven particularly useful for addressing questions such as population structure, priority for conservation, and likely origin across a number of livestock species. Domestic sheep have a global distribution and a growing number of genetic surveys have been conducted, which report on the haplotypes and mix of mtDNA lineages observed within the indigenous breeds. A general finding has been the presence of multiple mtDNA lineages within breeds, independent of their geographic location or phenotype. A survey of 19 Chinese breeds found that 15 contained a mixture of haplotypes from lineage A, B and C (Chen *et al.* 2006). Investigation of five Turkish breeds reported the same result (Pedrosa *et al.* 2005), while examination of a range of European breeds found the presence of both type A and B lineages occurring at differing frequencies. The existence of multiple mtDNA lineages and their mixing within breeds has been interpreted as evidence for multiple domestication events and subsequent human mediated introgression between domestic sheep populations To date, no mtDNA sequence has been reported from any Indian breeds.

This study represents the first characterization of mitochondrial DNA diversity within three breeds of Indian sheep (Deccani with two strains, Bannur and Garole) from different geographic regions and with diverged phenotypic characteristics. A total of 2028 bp fragment including cytochrome B gene (967 bp) and control region (1061 bp fragment spanning the control region, a portion of the 12S rRNA gene, and

the complete phenyl tRNA gene) was used to study the diversity. Both the regions evolve with different rates; the control region is known to be a hyper-variable region whereas cytochrome *b* gene region is more conserved. The control region was sequenced from 73 animals and compared with the corresponding published sequence from European and Asian breeds and the European Mouflon (*Ovis musimon*). Analysis of all 156 sequences revealed 73 haplotypes, of which 52 belonged to the Indian breeds. Only one Indian haplotype was shared with European and other Asian breeds, whereas the Indian breeds had no haplotypes in common. All 52 Indian haplotypes belonged to the mitochondrial lineage A with complete absence of other lineages. In contrast, the cytochrome *b* region showed both; Clade 'A' and Clade 'B' along with unique sequences per breed. The results indicate that although the founder sheep population for the Indian breeds had both 'A' and 'B' haplotypes. The network analysis of the cytochrome *b* gene sequences showed star shaped phylogeny indication that the populations are examining also supported by the Fu's *F*s values (-27.54). The time since commencement of expansion of clade A was also much more than reported (26,986). These results points towards a separate domestication of the Clade 'A'. Therefore, these Indian sheep are distinct from other Asian and European breeds studied so far. The relationships among the haplotypes showed strong breed structure and almost no introgression among these Indian breeds consistent with Indian sheep husbandry, which discourages genetic exchange between breeds. These results have implications for conservation of India's ovine biodiversity and suggest a common origin for the breeds investigated.

Prolificacy gene screening and introgression study in sheep breeds of India

Profitable and sustainable sheep production is a high priority for India and genetic improvement of sheep for meat production is one of the important developmental priorities. Enhancing reproductive rate is a logical approach to improve the economic efficiency of meat production. However, the litter size of almost all Indian sheep breeds, except the Garole, is very low and is thus a major constraint to sheep meat production. Introduction of a prolificacy gene into non-prolific sheep breeds having other desired traits may effectively increase the reproductive performance of local breeds. In early 2001, Wilson *et al.* identified the mutation that causes super prolificacy of Booroola, an Australian prolific sheep breed, which was confirmed by groups in France and the U.K (Mulsant *et.al.*, 2001; Souza *et.al.*, 2001, respectively).

They also developed the PCR-RFLP method for screening the breeds for the prolificacy gene (also called as *FecB* gene) mutation. This test was carried out in Garole, Bannur, Deccani (represented by five strains Lonand, Madgyal, Sangamneri, Sangola, and Kolhapuri) to screen for the mutation. The mutation was found to be present only in Garole. Crossbreeding between the Garole (G) and the Deccani (D) /Bannur (B) was carried out at Nimbkar Agricultural Research Institute (NARI) in order to determine whether a composite sheep having medium-high prolificacy which can survive in the semi arid environment of the Deccan plateau in Maharashtra state could be developed. Ten F1 rams (G x D/B) were produced by crossing Deccani (D)/ Bannur (B) ewes with nine Garole (G) rams. Each of the ten F1 rams was then single-sire mated to Deccani (D), Bannur (B) and Deccani x Bannur (D x B) or Bannur x Deccani (B x D) ewes to produce ¼ Garole {G x D/B x D or G x D /B x (D x B) or G x D/B x (B x D) progeny (12 to 27 daughters per sire representing ten progeny groups). Two hundred and twenty seven females resulting from this backcrossing were included in the analysis for the *FecB* mutation. Ninety of these ¼ Garole ewes were further inseminated with Deccani ram semen and the resulting progeny were analyzed for the same mutation. Furthermore, the test was extended to three hundred and forty samples collected from shepherds' flocks where homozygous $FecB^{B}$/ $FecB^{B}$ crossbred rams had been introduced by NARI. These experiments were performed to study the extent of management of the increased prolificacy of the crossbred animals under local environment of sheep rearing in Maharashtra.

Of the ten-backcross progeny groups with ¼ Garole genes generated at NARI, 37-63% carried one copy of the Booroola allele ($FecB^{B}$/$FecB^{+}$). Considering all the backcrossed ewes together irrespective of their individual group, nearly half (49.53%) of the ¼ Garole ewes were heterozygous ($FecB^{B}$/$FecB^{+}$) as is the expectation for the Mendelian inheritance of a single copy of the mutation present in one parent. Further analysis of 194 progeny with 12.5% Garole genome from *FecB* heterozygous sires revealed the presence of one copy of *FecB* in 90 animals (46.39%) and the wild type genotype in 104 animals (53.60%). Among the 340 progeny of $FecB^{B}$/$FecB^{B}$ rams in local shepherds' flocks, 55% were found to be $FecB^{+}$/$FecB^{+}$ and 45% $FecB^{B}$/$FecB^{+}$. The ovulation rate (OR) of the *FecB* homozygote (mostly pure Garole), *FecB* heterozygote (Garole-Deccani crosses) and non-carrier ewes (Deccani, Bannur and their crosses with Garole) as determined at NARI, were 3.37, 2.09 and 1.08,

respectively. The effect of one copy of the *FecB* gene on litter size (LS) in 25% Garole ewes was 0.52, 0.61 and 1.03 for the first, second and third parities, respectively. These studies showed that the introgression of one copy of the Garole gene ($FecB^{BB}$) increased the prolificacy of Deccani sheep by about 0.7 lambs born/ewe lambing and the OR by nearly one corpus luteum per pair of ovaries, which was lower than that estimated by Piper *et.al* (1985) suggesting the dampened expression of the *FecB* gene in Garole and its crosses.

To study the extent of variability in the FecB locus of Garole and its expression

The mean ovulation rate (5.7) and litter size (2.6) of the Booroola are quite high as compared to the Garole, though the same mutation is present in both the breeds. The differences may be due to environmental differences (e.g. plane of nutrition), a breed effect or the presence of other genetic factors such as modifier genes or novel mutations within the *BMPR-IB* gene that dampen the expression of the *FecB* trait in the Garole sheep. In West Bengal, which is the natural habitat of the Garole sheep, the mean litter size is 2.23 (based on information collected from farmers), where as in the semi arid environment of Maharashtra, the mean litter size is 1.74 suggesting that the genetic effects enhanced by the environmental factors might be contributing to further suppressed expression of the gene in the Garole sheep.

In order to study additional genetic factors (other than Q249R mutation) responsible for suppressed expression of prolificacy gene eight primer pairs spanning the whole cDNA sequence of the *BMPR-IB* gene using Primer Premier 3 software were designed and synthesized. After PCR amplification no size variation was observed between low and high effective breeding value (EBV) animals. Therefore, direct PCR product sequencing was carried out to check the sequence variation. The sequence was identical to the published cDNA sequence suggesting no variation/mutation within the *FecB* gene from Garole that could assign suppressed expression of prolificacy.

Alternatively, dampening of *FecB* expression may be due to the variation in the flanking region of the *FecB* locus. Hence, 4 microsatellite markers (JL 26, JL 2, JL 36 and JP27) closely linked to *FecB* locus were synthesized and used to check variation in this region using 16 Garole samples with high and low EBVs. However, except JP 27 all other three primers exhibited monomorphic pattern. Although, JP 27

marker showed polymorphic pattern, no specific alleles related to high and low EBVS animals were observed. This would further suggest analyzing the 5' upstream and 3' downstream expression regulating elements of *FecB* gene in Garole.

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