SYSTEMATICS, PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE GOLDEN JACKAL, *Canis aureus* IN INDIA

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ABBREVIATIONS

%	-	Percent
&	-	And
°C	-	degrees Celsius
µ	-	micro
bp	-	base pair
BSA	-	Bovine serum albumin
CITES	-	The Convention on International Trade in Endangered Species of Wild Fauna and Flora
CR	-	Control Region
Cytb	-	Cytochrome b
DF	-	Degree of freedom
DNA	-	Deoxyribonucleic acid
dNTPS	-	Dexyribo-nucleotide triphosphates
e.g.	-	*exampli gratis* (for example)
EDTA	-	Ethylene diamine tetra acetic acid
et al.	-	*et alia* (others)
etc.	-	*et cetera* (others)
g	-	Gram
HCL	-	Hydrochloric acid
HWE		Hardy-Weinberg equilibrium
IUCN	-	International Union for Conservation of Nature
i.e.	-	*id est* (that is)
km	-	kilometer
kg	-	kilogram
l	-	litre
m	-	milli
mg	-	milligram
mm	-	millimeter
min	-	minute
Na	-	number of alleles

Ne	-	effective number of alleles
PCR	-	Polymerase chain reaction
PTC	-	PCR Thermal Cyclers
pH	-	Potassium hydrogen phthalate
RNA	-	Ribonucleic acid
mtDNA	-	mitochondrial DNA
RPM	-	Revolutions per minute
SE	-	Standard Error
sq	-	square
$	-	Dollar
SNP	-	Single nucleotide polymorphism
STR	-	Short tandem repeat
TE	-	10 mM Tris, 1 mM EDTA liter
viz.	-	*videlicet* (namely)
VNTRS	-	Variable number tandem repeat
WCMC	-	World Conservation Monitoring Centre

EXECUTIVE SUMMARY

The golden jackal (*Canis aureus* Linnaeus 1758), is one of the most widely spread canid species of the family Canidae, occurring in Northern and Eastern Africa, Southeastern Europe and in large parts of Asia Eastward to Thailand. Extensive work has already been conducted to understand morphological, behavioural, and ecological aspects of this species. Despite the wide range of the species no major research has been done till date to understand the molecular genetics of golden jackal. The goal of this study was to elucidate the systematics, phylogeny, population genetic structure, genetic variability and the relationship of golden jackal in relation to other canid species.

In Chapter I, I outline the general characteristics of family Canidae and golden jackal as a typical member of the family. Being the most ancient living family of the order Carnivora and with global distribution throughout the World from Arctic to Tropical forests, Canidae consists of approximately 36 extant species. The members of the family Canidae are called canids. Golden jackal (*Canis aureus*) is a medium-sized canid having a wide range of distribution. Thirteen sub-species of golden jackal are reported among which the European golden jackal (*Canis a. moreotica*) is the largest one. Golden jackals are opportunistic foragers. Due to their tolerance of dry habitats and omnivorous diet, they can live in a variety of habitats. They occupy semi-desert, short to medium grasslands and Savannas in Africa, and forested, mangrove, agricultural, rural and semi-urban habitats in India and Bangladesh. The major threats to golden jackals come from hunting, poisoning and greatly from infectious disease, which have attracted increase attention in recent years. Currently, jackals are slated to be Scheduled III species in India and are placed under Appendix II of CITES. In India they are declared as “species with least concern” and could be considered as “species requiring no immediate protection”.

Chapter II is dedicated to explaining the theory of conservation genetics, molecular genetics, and the molecular markers used in a genetic study. Advancements in DNA technology *i.e.* development of molecular markers has led to the blossoming of genetic analysis of populations in the last decade. Although a large variety of molecular markers are available, here I used the mitochondrial and nuclear markers for achieving the objectives of my study. Mitochondrial DNA is composed of 13 protein coding genes (including Cytb), 22 tRNA genes, 2 rRNA genes, and a noncoding segment called CR. Being the most variable part of the mtDNA, CR provides better resolution when studying closely related species or conspecific population while the conserved nature of Cytb makes it a better marker for studying deeper phylogenetic relationships. Here, I used the universal primers ThrL15926 (5′-GAATTCCCCGGTCTTGTAAACC-3′) and DLH-16340 (5′-CCTGAAGTAGGAACCAGATG-3′) for CR while a canid specific light primer Canid L1 (5′-AATGACCAACATTCGAAA-3′) and a universal heavy primer H15149 (5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′) was used for the Cytb gene. These primers are also used for phylogenetic study in wolves, Serbian red fox, golden jackal, coyote, kit, red and grey fox and dogs.

Other markers used in the present study were nuclear markers *i.e.* microsatellites. Microsatellites are stretches of short DNA sequence in which a motif of one to six bases is tandemly repeated. This property makes microsatellite a highly versatile marker. Microsatellites are multi-allelic in a population and bi-allelic in an individual. They are inherited in a co-dominant mendelian manner and can reveal heterozygote (with two different alleles at a locus) and homozygote (with two copies of the same allele at a gene locus) in each individual. Microsatellites are useful genetic marker because they tend to be highly polymorphic. This variability is mainly due to mutation which occurs through slippage strand mispairing. This variability makes them reliable for population genetic studies. In the present study a panel of 10 microsatellite markers was used which were derived from domestic dog. Among them eight were di-nucleotide while two were tetra-nucleotide and all

markers were labeled with fluorescent dye FAM. These markers are also used for population genetic study in coyote, dog, paternity study in African wild dog, cooperative breeding study in African wild dog and to study the genetics of epilepsy in dog.

Chapter III elucidates the taxonomic affiliation of golden jackal to other jackal species and to resolve their global higher level phylogenetic status in the genus *Canis*. Sixty two samples of golden jackal (55 from India, five from Bulgaria and two from Israel) were collected, preserved in 95% ethanol and stored in -20°C until DNA extraction. For DNA extraction traditional Phenol-Chloroform (PC) method and QIA quick DNeasy blood/tissue kit were used. Compared to the PC, a time consuming procedure QIA quick DNeasy kit method was found quick and more efficient with less comfort. Almost 75% extraction was done with kit while for rest PC method was used. Two regions of mtDNA: an approximately 440 base pair (bp) fragment of the CR (with universal primers ThrL15926 and DLH-16340) and a 412 bp fragment of the Cytb gene (using primers Canid L1 and H15149) were amplified. The PCR conditions used for amplification were optimized by increasing the time for annealing and decreasing for extension as used by Wayne *et al.*, 1997. Amplified products were checked on 2% agarose gel which was followed by cleaning with Qiagen PCR purification kit or ExoSAP. Products were cycle sequenced and cleaned again with ethanol before putting them for sequencing in ABI 3300 automated sequencer (Applied Biosystems). Sequence data obtained for both strands of mitochondrial CR and Cytb for each individual and consistent sequences for all individuals were edited and aligned using Sequencher 4.6 and rechecked visually.

To construct the phylogenetic tree, the sequences of eight canid species for CR and 10 for Cytb retrieved from GenBank. To infer the evolutionary history of golden jackal, three standard methods named Minimum Evolution (ME), Maximum Likelihood (ML), and Neighbor-Joining (NJ) methods were used to construct phylogenetic tree with mitochondrial CR while only Minimum

Evolution (ME) method was used for mitochondrial Cytb. The analysis involved 12 nucleotide sequences for CR and 20 for Cytb and all positions containing gaps and missing data were eliminated. There were a total of 239 positions in final data set for CR and 280 for Cytb. All the analyses were conducted in MEGA5 (Tamura *et al.*, 2011). Maned wolf was used as out group to the rest of the species in Cytb tree. With little variation, both CR and Cytb phylogenetic trees showed the similar results. The grouping of wolflike canids including wolves (Indian Peninsular wolf and Himalayan wolf), Indian feral dog, golden jackal, Ethiopian wolf, side-striped jackal, black-backed jackal, coyote, dhole and African wild dog was well supported in both CR as well as Cytb phylogenetic tree. With good bootstrap supported Cytb phylogenetic tree, within wolflike canids golden jackal, coyote, Ethiopian wolf, African wild dog, grey wolf, Indian Peninsular wolf, Himalayan wolf and Indian feral dogs form a monophyletic clade while side-striped jackal, black-backed jackal, dhole and African wild dog are separate clades. The paraphyletic nature of jackals (golden jackal, side-striped jackal, black-backed jackal and Ethiopian wolf or simien jackal) is clear from the phylogenetic tree. Golden jackal is clearly associated with the larger wolflike canids, the wolves, coyote and simien jackal. It is also evident that golden jackal does not form monophyletic group with other two jackal species, the side-striped jackal and black-backed jackal. The findings are in concordance with all previous phylogenetic studies on wolflike canids done by Wayne *et al.*, (1997); Vila *et al.*, (1999); Zrzavy & Riconcova (2004); Rueness *et al.*, (2011); and Gaubert *et al.*, (2012).

Chapter IV explains the phylogenetic status of golden jackal and also its relationship with other canid species in India. A total of 55 samples of golden jackals were collected from seven states in India. Same procedure was used for DNA extraction, PCR amplification with CR and Cytb primers, cleaning of products, cycle sequencing, ethanol precipitation and sequencing in ABI 3300 automated sequencer. Forward and reverse sequencing was performed for

each individual and consistent sequences for all individuals were edited and aligned by Sequencher 4.6 and rechecked by eye. To assess the population structure by estimating the genealogy of haplotypes via phylogeographic reconstruction, a phylogenetic tree was constructed to show the relationship of golden jackal with other canids in India. The Neighbor-Joining (N-J) method was used for tree construction. The analysis involved 39 nucleotide sequences and after removing all ambiguous positions, there were a total of 289 positions in the final data set. MEGA5 was used for all the evolutionary analyses. A median-joining network was constructed in the program Network V.4.610 to assess the relationship between the observed haplotypes. Further, to compare molecular diversity of golden jackal across India program DnaSP V5 was used to estimate haplotype diversity (h) and nucleotide diversity (Pi). To investigate expansion or contraction in golden jackal population- Tajima's D statistics was calculated in program DnaSPV5, Fu's Fs statistics was calculated in Arlequin V3.5, and a mismatch distribution was constructed using DnaSPV5. The time since coalescence was also calculated for golden jackal. Large divergence and lack of haplotype overlapping between golden jackal and other Indian *Canis* (Indian feral dog, Indian Peninsular wolf and Himalayan wolf) as depicted in the phylogenetic tree suggests no mtDNA introgression and gene flow between these canids. Thus, there does not seem to be threats of hybridization with the large population of feral dogs and other endangered canids in India. Indian feral dog shows highest nucleotide as well as haplotype diversity (0.0147±0.0016; 0.899±0.037 respectively) among all the canids. For golden jackal these values are 0.0091±0.0007; 0.866±0.034 respectively. Based on the sequence divergence observed, coalescence was estimated at just over 10,465 years ago for golden jackals in India, which coincides with the end of Pleistocene and the onset of Holocene. Relatively high variability in Indian golden jackal CR haplotypes suggests that the habitat and climatic conditions after the estimated coalescence time did not result in the fixation of a single mtDNA haplotypes. Thus, extant population has retained more than one haplotype (15 Control

Region and 7 Cytochrome b haplotypes) upto recently. Higher numbers of haplotypes as well as nucleotide diversity suggests that Indian golden jackal populations have relatively high levels of mtDNA diversity compared to conspecifics in other regions of world. Furthermore, high nucleotide diversity and a star-shaped polytomy of CR haplotypes suggest that they may have undergone dramatic demographic change in the recent past and India may be the centre of radiation of golden jackals if the diversity is confirmed to be higher in India than in other regions of the world. This high CR diversity for Indian golden jackal contrasts with the extremely low genetic diversity at the western most limits of their range in Eastern Europe.

Finally, Chapter V explains the findings of population genetic structure and genetic variability among golden jackal in Western India, Gujarat. Twenty four golden jackals were sampled from Bhal and Kachchh regions (17 from Bhal and VNP and 7 from Kachchh) of Gujarat. The samples were genotyped with 10 polymorphic microsatellite loci derived from domestic dog. Genotyping was carried out using ABI 3130 Genetic Analyzer with GeneScan-500 LIZ as the internal lane size standard. Genemapper V3.7 and Peak Scanner 1.0 softwares were used for allelic identification and sizing. Genetic diversity was quantified in terms of number of alleles, observed (Ho) and expected (He) heterozygosities and polymorphism information content (PIC) by using program Cervus V3.0.3. Genemapper V4.1 was employed to test deviations from HWE, the presence of pairwise linkage disequilibrium, and to calculate F-statistics (*Fst*, *Rst* and *Fis*) to analyse differentiation within and between golden jackal populations. Furthermore, The Bayesian clustering procedure implemented in computer program STRUCTURE V.2.3.3 was used to simultaneously infer the number of distinct genetic clusters suggested by the microsatellite data.

Altogether 78 distinct alleles are found with mean allelic number of 8.8(±2.33). Loci FH2328 yielded the highest number of alleles (Na=13) while loci FH2412 yielded the lowest number of alleles (Na=5) in the golden jackal population.

Out of 10 microsatellites used, 9 loci showed PIC value higher than 0.5 and considered informative for population genetic studies. No evidence for linkage disequilibrium is observed among pairs of loci. The Indian golden jackal yielded observed and expected heterozygosity (Ho 0.81, He 0.82) much higher than those of their Serbian (Ho 0.29, He 0.34), Kenyan conspecifics (Ho 0.41, He 0.52) and also other canids.

A small mean *Fis* value (0.018) concludes very less or no inbreeding between the golden jackal populations. With regards to differentiation, no evidence of significant differentiation between the Bhal and Kachchh population of golden jackal is found. Small values of pairwise *Fst* and *Rst* (0.018 and 0.026 respectively) make them as genetically homogeneous population. Further no evidence for unique genetic clusters within the Bhal and Kachchh populations suggest a weak genetic structure for golden jackal in western India.

Thus, I conclude that even after being separated by a hostile Rann habitat, very low differentiation and interbreeding has resulted in high gene flow between and among the Bhal and Kachchh population of golden jackal. Also the landscape and habitat features were not barriers to their dispersal and therefore, golden jackal forms a continuous and homogeneous population in Western India.

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

GENERAL CONCLUSIONS

LITERATURE CITED

GENERAL INTRODUCTION TO THE SPECIES

I.1 Family Canidae

Among the living families within the Order Carnivora, the Canidae is the most ancient. The family arose in the late Eocene, when no other living families of carnivorans had yet emerged (two arctic families, Miacidae and Viverravidae, have a much older history but none survive to the present time). This is a large group of mostly predatory mammals characterized by their common possession of a pair of carnassail teeth (upper fourth premolar and lower first molar) that are modified to maximize efficiency for shearing skins, tendons and muscles in their prey.

The Canidae consists of approximately 36 extant species categorized into five genera (Van Gelder 1978; Nowak 1991; IUCN/SSC Canid Specialist Group 2001). The members of the family Canidae are called canids, characterized by an inflated entotympanic bulla (bony chamber enclosing the middle ear region) that is divided by a partial septum along entotympanic and ectotympanic suture. Other characteristic features of canids are loss of carotid artery that is situated between the entotympanic and petrosal for most of its course and contained within the rostral entotympanic anteriorly (Wang & Tedford 1994). These basicranial characteristics have remained more or less stable throughout the history of canids, allowing easy identification in the fossil record when structures are preserved.

The contemporary Canidae is the most wide spread family that occurs throughout the world from Arctic to tropical forests (Sheldon 1992) with at least one species present in every continent except Antarctica. A quick perusal of the ranges of all canid species (Macdonald & Sillero-Zubiri 2004)

indicated that over the last century, the geographical range of seven species have increased, eight have decreased and nine have remained stable.

Many canids have distributions that span over a whole continent. Red foxes (*Vulpes vulpes*) and grey wolves (*Canis lupus*) have the most extensive natural range of any land mammal (with the exception of humans and perhaps some commensal rodents). Red foxes are the only canid present in five continents, recorded in a total of 83 countries. Grey wolves occur naturally in North America, Europe and Asia, their range spanning over 62 countries. Two species are present on three continents, namely the golden jackal (*Canis aureus*) and arctic fox (*Alopex lagopus*). And two other, the red fox (*Vulpes vulpes*) and dingo (*Canis lupus dingo*), have reached Australia and Oceania with assistance from mankind (Macdonald & Sillero-Zubiri 2004).

At least 155 of the 192 countries across the globe have (81%), canids including Sudan with the highest number of species (10 species), followed by USA (9 species) and Ethiopia (8 species). The countries that don't host any canid species are island states (e.g., Caribbean island, Madagascar, Malta and most Australian islands). Africa, Asia and South America support the greatest diversity with more than 10 canid species each. Red foxes are sympatric with 14 other canids (from three geographic regions), golden jackal with 13 (from two regions) and Grey wolves with 11 (from three regions) (Macdonald & Sillero-Zubiri 2004).

There are five canid species endemic to a single country. Not surprisingly, most are also threatened (Red wolf, *Canis lupus rufus*; Ethiopian wolf, *Canis simensis*; Darwin's fox, *Lycalopex fulvipes*; Island fox, *Urocyon littoralis*; and Hoary fox, *Pseudolopex vetulus*), with the Sechuran fox (*Pseudolopex sechurae*) a near-endemic to Peru. Of the two continents with the highest species diversity, South America harbors nine species (out of 11 species

present confined entirely to south of Panama, while Africa has eight endemic (of 13 species present). Out of 12 canid species found in Asia, only two are restricted in the continent (Macdonald & Sillero-Zubiri 2004)

I.2 Golden jackal, *Canis aureus*: General introduction

Jackal, as a group amongst canids is a widespread species of the family Canidae. There are four species of jackals, golden jackal (*Canis aureus*), black (Silver)-backed jackal (*Canis mesomelas*), side-striped jackal (*Canis adustus*) and the fourth which is very rare called simien jackal or Ethiopian wolf (*Canis simiensis*). The golden jackal means 'golden dog' is largest of the jackals and presumed a true member of the dog family. There are 13 species of golden jackal being recognized (Wozencraft 2005) (Table1.1).

Table1.1- Subspecies of golden jackal

Subspecies	Binomial authority	Description	Range
Canis a. algirensis (Algerian jackal)	Wagner,1841	Sports large, fox-like ears and a lupine face, golden fur with a slight reddish tint, white stain on the throat	Algeria, Morocco and Tunisia
Canis a. anthus (Senegalese jackal)	Cuvier, 1820	Known as the small Black jackal, it is darker than other subspecies	Senegal
Canis a. aureus (Common jackal)	Linnaeus, 1758	Generic subspecies	Live among central range of golden jackal
Canis a. bea (Serengeti jackal)	Heller, 1914	This golden jackal lives in Serengeti National Park, included to be a subspecies	Kenya, Northern Tanzania
Canis a. cruesemanni (Siamese jackal)	Matschie, 1900		Thailand, Myanmar to east India
Canis a. ecsedensis	Kretzoi, 1947		
Canis a. indicus (Indian jackal)	Hodgson, 1833		India, Nepal
Canis a. lupaster (Egyptian jackal)	Hemprich & Ehrenberg, 1833	Sometimes mistaken for the Grey wolf subspecies, with long legs and ears, dirty-yellow fur	Egypt locally
Canis a. moreotica (European jackal)	Geoffroy Saint-Hilaire, 1835	It is among the largest of the golden jackal subspecies, in Hungary and Austria it is known as the Hungarian reed wolf	Southern and Southern-central Europe, especially Greece
Canis a. naria (Sri Lankan jackal)	Wroughton, 1916		Southern India, Sri Lanka
Canis a. riparias	Hemprich & Ehrenberg, 1832		Coast of Ethiopia and Eritrea
Canis a. soudanicus (Variegated jackal)	Thomas, 1903		Sudan and Morocco
Canis a. syriacus (Syrian jackal)	Hemprich & Ehrenberg, 1833	Closely related to *Canis a. lupaster*, but is smaller and more richly colored	Israel, Western Jordan

Recently, a new subspecies of golden jackal was described from the Gaza strip, Palaestine and named *Canis aureus palaestina Khalaf* (Khalaf 2008). Usually there are three jackal subspecies living in the area around Palaestine: the Syrian golden jackal, *Canis aureus syriacus*, the Egyptian golden jackal, *Canis aureus lupaster*, and the Arabian golden jackal, *Canis aureus hardranauticus*. The Palaestinian golden jackal subspecies is morphologically and geographically distinct from these three species by its distinctive coloration on the fur and the moderate size. The size of the Palaestinain jackal is moderate if compared with the larger Egyptian jackal and the smaller Arabian jackal. It is a small race of the Golden or Asiatic jackal, *Canis aureus*. It is smaller than a wolf, larger than a fox and can be distinguished by its relatively smaller, rufous ears and shorter black-tipped tail. The dorsal colour is usually variable black, yellowish-gray or brown-yellowish tinged with rufous, greyer on the back, which is grizzled with varying amounts of black. A dark band runs along the back from the nose to the top of the tail. This mane becomes wider on the back, extending into the lateral surfaces. There are two dark bands across the lower throat and upper breast. There is also a reddish phase. The under parts are almost white or yellowish-brown. The winter coat is longer and greyer. The tail is relatively short, usually with a black tip. Now the Egyptian jackal (*Canis aureus lupaster*) present in the North Africa is also considered a subspecies of grey wolf (*Canis lupus*) in comparison to golden jackal. The evidences were explained by Nassef 2003 and Rueness *et al.*, 2011.

Distribution of three jackals, namely side-striped, black-backed and simien jackal is limited to Africa, as side-striped jackal is native to Central and southern Africa (Wozencraft 2005) and black-backed jackal inhabits two areas of the African continent. One region includes the southernmost tip of the continent including South Africa, Namibia, Botswana and Zimbabwe; other is along the Eastern coastline including Kenya, Somalia and Ethiopia. Simien jackal is endemic to Ethiopia, where it is one of several species of

mammals restricted to Afro-alpine grassland and health lands (Yalden & Largen 1992), whereas, the golden jackal is widely spread in North Africa and North-East Africa, occurring from Senegal on the west coast of Africa to Egypt in the East. In the northern region it is found in Morocco, Algeria and Libya, while in South it expands to Nigeria, Chad and Tanzania. They have expanded their range from the Arabian Peninsula into Western Europe to Austria and Bulgaria (Genov & Wassiley 1989; Shledon 1992) and eastwards into Turkey, Syria, Iraq, Iran, Central Asia, the entire Indian subcontinent, then East and South to Sri Lanka, Myanmar, Thailand and parts of Indo-China region (Jhala & Moehlman 2008). The worldwide distribution of the golden jackal is shown in Figure 1.1.

Figure.1.1- Red shaded areas showing the worldwide distribution of golden jackal (www.iucnredlist.org)

Golden jackal, a typical representative of the genus *Canis* (Clutton-Brock 1976), is generally 70 to 105centimeters (28-42inches) in length, with a tail length of about 25centimeters (10inches). Its standing height is approximately 38 to 50centimeters (16-20inches) at the shoulder. Average weight is 7 to 15kilograms (15-33pounds), with males tending to be 15% heavier than the females (Ivory 1999; Moehlman & Hofer 1997).

Basic coat color of golden jackal is golden but varies from pale creamy yellow to a dark tawny hue on a seasonal basis. The pelage on the back is often a mixture of black, brown and white hair. Jackals inhabiting rocky, mountainous terrain may have a greyer coat shade (Sheldon 1992). The belly and under parts are a lighter pale ginger to cream. Unique lighter marking on the throat and chest make it possible to differentiate individuals in a population (Macdonald 1979a; Moehlman 1983). Melanistic and piebald forms are sometimes reported (Jerdon 1874; Muller-Using 1975). The tail is bushy with a tan to black tip. Legs are relatively long and feet slender with small pads. Scent glands are present on the face, anus and genital regions. Females have 4-8 mammae (Macdonald 1992). The skull of the golden jackal is more similar to that of the coyote (*Canis latrans*) and the grey wolf (*Canis lupas*) than that of the black-backed, side-striped and simien jackal (Clutton-Brock *et al.*, 1976). The dental formula reported for golden jackal is 3/3-1/1-4/4-2/3 = 42.

The black-backed jackal (*Canis mesomelas*) is distinguished from other members of the genus *Canis* by a dark saddle extending from neck to tail in bold contrast to the rufous head, flanks and legs (Estes 1991; Stains 1974; Van de Merwe 1953). Its head resembles that of a dog with a pointed muzzle and erect, large and pointed ears (Smithers 1983). General colour is reddish brown to tan, redder on flanks and legs. The Black-backed jackal is longer and taller than the golden jackal. Its skull size is similar to other jackals with usual length of 141-147mm (Van Valkenburgh 1994) and mean adult cranial

volume of 56ml (Sheppy & Bernard 1984). It was also reported that skull of the black-backed jackal from East Africa is shorter in total length and wider and less variable in 16 other measures than from Southern Africa (Valkenburgh & Wayne 1994).

Other species of jackal is side-striped jackal (*Canis adustus*). It is a medium sized canid with overall grey to buff-grey in colour. Its head is grey-buffy white while the ears are dark buffy. The back is grey, darker than the underside and the flanks are marked by the indistinct white stripes running from elbow to hip with black lower margins. The boldness of marking varies greatly between individuals; those of juveniles are less and well defined than those of adults. The legs are often tinged rufous, and the predominantly black tail nearly always bears the distinctive white tip, which Kingdon (1977) suggests may be a “badge” of the species’ nocturnal status. Its skull is similar to that of black-backed jackal, but flatter, with a longer and narrower rostrum and having a distinct sagittal crest and zygomatic arches of lighter build (Atkinson & Loveridge 2004). As a result of elongation of the rostrum, the third upper premolar lies almost in line with the others and not at an angle unlike black-backed jackal (Skinner & Smithers 1990). This jackal is reported to have three to seven subspecies from the continent (Allen 1939; Kingdon 1997).

Fourth and very rare, the simien jackal or Ethiopian wolf (*Canis simiensis*) is the largest member of the genus in Africa and is distinguishable from other jackals by its larger size, relatively long legs, distinctive reddish coat and white under parts, throat, chest and tail marking (Gottelli & Sillero-Zubiri 1990, 1992). It has elongated skull with a slender elongated nose (Gray 1868). The facial length is 58% of the total skull length. The skull is very flat in profile, with only a shallow angle between frontals and nasals. The teeth, especially the premolar, are small and widely spread. The sharply pointed canines are average 19mm long (14 to 22mm), and the carnassials (P4 and M1) are relatively small (Clutton-Brock *et al.*, 1976). With probably fewer than 500 individuals surviving (Gottelli & Silleo-Zubiri 1992), this distinctive carnivore is

considered the rarest canid in the World and is classified by IUCN as "endangered" (Ginsberg & Macdonald 1900). More than half of the species' population lives in the Bale Mountains National Park. There is no fossil record of this canid yet.

The jackals in Europe are distributed in small and scattered populations, mainly along the mediterranean and Black Sea Coast of the Balkan Peninsula (Demeter & Spassov 1993; Krystufek *et al.*, 1997). The golden jackals are found in the Caucasus, Turkish Thrace, Greece, Bulgaria, Albania, along the eastern Adriatic Coast and in Romania. Serbia has recently been recolonized (Mitchell-Jones *et al.*, 1999). Over the first half or the 20th century, the population of the golden jackal declined dramatically due to habitat fragmentation and intensive hunting pressure. Population density decreased in core areas (Bulgaria, Serbia and Greece) as well as at the edges of its distribution range from where the golden jackal will be completely disappeared within the next 50 years (Krystufek *et al.*, 1997).

Under intensive conservation, apart from Greece, where the species has been listed as vulnerable in the national red list (Giannatos *et al.*, 2005), the golden jackal has expanded its European distribution range, most notably in Bulgaria, where there was a 33-fold increase in the area inhabitated by jackals between the 1960s and the 1980s and which now supports the largest jackal population in Europe (Genov & Wassilev 1989; Krystufek & Tvrtkovic 1990; Krystufek *et al.*, 1997; Giannatos 2004; Humer *et al.*, 2007). During this period, stable population was established in Romania (200 individuals) and in Hungary (1000 individuals) (Demeter 1984; Humer *et al.*, 2007). The stabilization and growth of the Balkan population resulted in the expansion of the species to central and Western Europe.

The presence of golden jackal was first recorded in Italy in 1984 (Lapini & Perco 1988), in Slovenia in 1985 (Krystufek & Tvrtkovic 1990), in Austria in

1987 (Bauer & Suchentrunk 1995; Spitzenberger 2001), in Slovakia in 1989 (Hell & Bleho 1995; Hell & Rajsky 2000; Rajsky *et al.*, 2005) and in Germany in 1996 (Möckel 2000). Along the Dalmatian coast, a rapid expansion of the golden jackal took place in the 20th century and after 1980, Jackals have also established permanent territories in Istria (Krystufek & Tvrtkovic 1990). From the Pelješac Peninsula (Southern Dalmatia, Croatia), the presence of jackals was mentioned in the literature for the first time in the 19th century and they are known to be present here ever since (Krystufek & Tvrtkovic 1990). In a recent survey, jackals were present throughout the Peninsula (Krofel 2008), though the number of jackals on the Peninsula was somewhat decreased after temporary higher hunting pressure (D. Denac, pers. comm.).

Rarely, records of vagrants are also reported from central Europe, e.g., Slovakia (T. Pataky pers. comm.), Austria (Bauer & Suchentrunk1995), Italy (Lapini *et al.*, 1993) and Slovenia (Krystufek *et al.*, 1997). Recently, a female jackal was shot in Upper Savinja Valley, Northern Slovenia (Krofel *et al.*, 2008). According to the available information, it is not possible to reliably ascertain whether this was a territorial animal or a vagrant. However, since there were neither reports of other jackals observed in this region nor any vocalization heard before or after the killing, it seems more reasonable to conclude that it was a vagrant. The unusual aspect of this record is the location in Northern Slovenia, which is further away from areas of permanent jackal presence in Croatia then other known records of jackals during last decade in Slovenia (Krofel *et al.*, 2008). The Asiatic or golden jackal, *Canis aureus* in Thailand has been found in some of the country's protected forests such as Khao Nang Wildlife Research Centre (Conforti 1996; Simchareon 1998), Thung Yai and Huai Kha Khaeng Wildlife Sanctuary in western Thailand (Robinson *et al.*, 1995).

Jackal has also been reported as an imported mythic symbol in literature. The Egyptian god of embalming, Anubis, was portrayed as a jackal-headed man

or as a jackal wearing ribbons and holding a flagellum, a symbol of protection, in the crook of its arm. Anubis' head is always shown as a black jackal or dog with long ears and pointed muzzle, even though real jackals are typically tan or light brown. To the Egyptians, black was the color of regeneration, death and the night. The reason for Anubis' animal model being canine is based on what the ancient Egyptians themselves observed of the creature- dogs and jackals often haunted the edges of the desert, especially near the cemeteries where the dead were buried. Infact, it is thought that the Egyptians began the practice of making elaborate graves and tombs to protect the dead from desecration by jackals. Like-wise, the Greek god Hermes and the Monster Cerberus are thought to derive their origins from the golden jackal. In India, jackals feature in ancient texts like the *Jatakas* and *Panchatatra* that abound with animal stories. The jackal normally is portrayed as an intelligent or wily creature in these stories. Some tribes here believe in the existence of a horn-like growth called *Shiyal-shingi* which appears on the heads of some jackals. The possession of this growth is considered a sign of good fortune. Coffee beans that have passed through the gut of a jackal are believed to have an added flavour, and these are collected and marketed in certain parts of Southern India (Jerdon 1874, A. J.T. Johnsingh pers. Comm.). The jackal is also mentioned frequently in the Bible, where it is portrayed as a sinister creature, most notably in Pslam 63: 9-11 where it is stated that non-believers would become food for the jackals.

The social organization of golden jackals is extremely flexible depending on the availability and distribution of food resources (Macdonald 1979a; Moehlman 1983, 1986, 1989; Moehlman & Hofer 1997). Jackals have a monogamous pair as a social unit in their society, which defends its territory from other pairs. These territories are defended by vigorously chasing intruding rivals and making landmarks around the territory with urine and faeces. The territory may be large enough to hold some young adults who stay with their parents until they establish their own territory. Thus, the

primary social unit of golden jackal is the pair bond between the mated male and female, which persists throughout life. They also howl together to show that there is a bond between them. They also use different howls to locate one another. They both take care of the young, but the male does even more. Of a total of 270 recorded jackal sightings in the Bhal and Kachchh areas of Gujarat, India, 35% consisted of two individuals, 14% of three, 20% of more than three and the rest of single individual (Jhala & Giles 1991). Moehlman & Hofer 1997 gave average group size as 2.5 in Serengeti, Tanzania, while average pack size in Velavadar National Park, India was 3.0 (n=7) (Jhala & Giles 1991).

Affiliative behaviours like greeting ceremonies, grooming, and group vocalizations are common in jackal social interactions (Van Lawick & Van Lawick-Goodall 1971; Golani & Keller 1975). Vocalization consists of a complex howl repertoire beginning with 2-3 simple, low-pitch howls and culminating in a high-pitched staccato of calls. Jackals are easily induced to howl and a single howl evokes responses from several jackals in the vicinity. Golden jackals often emit a warning call that is very different from that of their normal howling repertoire in the presence of large carnivores like tigers, hyaenas and wolves (Jerdon 1874). In India, howling is more frequent between December and April, a time when pair bonds are being established and breeding occurs, perhaps suggesting a role in territory delineation and defense (Jaeger *et al.*, 1996).

Reproductive activity commences from February to March in India & Turkmenistan, and from October to March in Israel (Golani & Killer 1975; Ginsberg & Macdonald 1990). In Tanzania, mating typically occurs from October to December with pups being born from December to March (Moehlman 1983, 1986, 1989). The Golden jackal of the Serengeti court at the end of the dry season and produce pups during the rainy season. As with other canids, mating results in a copulatory tie that lasts for several minutes

(Golani & Mendelssohn 1971; Golani & Keller 1975). Timing of births coincides with abundance of food supply; for example, the beginning of the monsoon season in northern and central India, and the calving of Thomson's gazelle in the Serengeti (Moehlman 1983; Ginsberg & Macdonald 1990). Females are typically monoestrus, but there is evidence in Tanzania of multiple litters. The gestation period (pregnancy) lasts for about nine weeks (63 days) (Sheldon 1992). Moehlman & Hofer (1997) gave mean litter size as 5.7 (range=1-8) in Tanzania, while in Bhal areas in India, average litter size was 3.6 (range=2-5; n=11) (Jhala unpubl.) Just before giving birth, the female digs a nursery den. There may be upto nine pups in a litter, but two to four is the usual number. Weight of a pup is about 200gm at birth. The newly-born pups are covered in soft fur but are blind and helpless for the first few days. Their eyes open after about ten days. For the first three weeks they feed on nothing but their mother's milk and she never leaves them alone. For another five weeks the pups continue to suckle, but also eat regurgitated food the parents swallow prey they have caught. At the age of three months, the den is no longer used and pups begin to follow the parents as they begin to learn the territory and hunting. At six months they are prepared to hunt alone. Even though pups have begun to gain independents, the parents still care for and play with them. Pups attain maturity at eleven months.

Both male and female members of a pair have important roles in maintaining their territory and in raising the young when one parent dies, it is unlikely that the rest of the family will survive. However, in most jackal families, there are one or two adult members called "helpers". Helpers are jackals who stay with the parents for a year after reaching sexual maturity, without breeding, to help take care of the next litter. These helper associations are probably responsible for reports of large joint hunting. Within the family, helpers are subordinate to parents. Helpers strengthen the family in several ways. The presence of a single adult at the den provides considerable protection: adults both "rumble growl" and "predators bark" to warn the pups to take refuge, and

a single adult can successfully drive off large predators. Helpers also bring food to a lactating mother and improve the provisioning of the pups directly by allowing the parents to spend more time foraging alone or hunting as a pair. Families with helpers may be able to defend and exploit a carcass more successfully than an individual would be able to. The female jackal initiates all den changes. Though the males are predominantly monogamous, females reserve their aggression for female intruders, preventing the sharing of the male and his parental investments. However, this “monogamy” might be behavioral but not sexual. In a fascinating study on a related canid species, the Ethiopian wolf, *Canis simiensis*, Claudio-Sillero-Zubiri and others found that while the male and female wolves would stay together and continue to care for the young (with their probably closed related pack member), 70% of the copulation were not between the bonded male and female pairs but with wolves from different packs, which might be preventing inbreeding of this particularly isolated species.

Paternal care has never been reported as absent in any canid species. Male parental care, usually in the context of biparental investment in monogamous pairs, is probably universal in canids. Kleiman & Malcolm (1981) reviewed literature up to 1979 and found reports of male care in 17 of 35 species. Paternal care has subsequently been reported in two more fox species, *Vulpes bengalensis* (Johnsingh 1978) *Vulpes macrotis*, now recognized as distinct from *Vulpes velox* in which male care was reported by Seton (1909). However, quantitative data on parental care in wolf, *Canis lupas* (Harrington & Mech 1982; Fentress & Ryon 1982), golden jackal, *Canis aureus* (Moehlman 1983), black-backed jackal, *Canis mesomelas* (Moehlman 1983), red fox, *Vulpes vulpes* (Macdonald 1979a), arctic fox, *Alopex lagopus* (Garrott & Eberhardt 1982), maned wolf, *Chrysocyon brachyurus* (Rasmussen & Tilson 1984) and African wild dog, *Lycon pictus* (Malcolm & Marten 1982) have been reported. In only cases of black-backed jackal, *Canis mesomelas* and bat-eared fox, *Otocyon megalotis* (Malcolm, personal observation, Nel 1978),

males have been recorded spending more time at the den than females. In arctic foxes, there was no difference in the food brought to the four dens by males and females. However, in both golden and black-backed jackals, females were recorded regurgitating more frequently than males (Moehlman 1983). Moehlman (1986, 1989) synthesized data on canid behavioural ecology and hypothesized that adult sex ratio, dispersal, mating and neonate rearing systems are size related. In small canids (< 6 kg), the adult sex ratio in social groups is biased toward females, young males tend to emigrate, and females stay in their natal ranges as helpers until a breeding opportunity arises. Medium sized canids (6-13 kg) have an equal adult sex ratio and an equal emigration rate, and both sexes may be helpers. Large sized canids (> 13 kg), excluding the maned wolf, *Chrsocyon brachyurus*, exhibit an adult sex ratio skewed toward males, female emigration and male helpers.

The diet of golden jackal is catholic, as they are opportunistic foragers. The diet consists of 54% animal food and 46% plant food (Ivory 1999). Like most predator, they use to scavenge rather than hunt since hunting is both energetically expensive and at times even dangerous. Hunting is usually carried out at night and normally they do not attack large animals but prefer small to medium sized prey such as rabbits, rodents, birds, insects, fish and monkeys (Jhala & Moehlman 2008, Kruuk 1972; Lamprecht 1978; Macdonald 1979b; Poche *et al*., 1987; Demeter & Spassov 1993; Yom-Tov *et al*., 1995; Lanszki & Heltai 2002; Lanszki *et al*., 2006).

Golden jackals have excellent sense of hearing which they put to good use in locating their prey hiding in grassland. It has been observed to hunt ungulates 4-5 times its body weight, though it more commonly targets young specimens. In the Serengeti, the golden jackal is a major predator of gazelle fawns (Ivory 1999), while in India, the golden jackal often kills black buck calves (Jhala & Moehlman 2008). Although it is common for Jackals to hunt alone, they do

occasionally do so in small groups, usually consisting of 2-5 individuals. Working in pack greatly increases the chances of making a successful kill (Jhala & Moehlman 2008). The golden jackal is often seen scavenging. They are ever alert to scavenging opportunities provided by kills of larger predators such as leopard (*Panthera pardus*), lion (*Panthera leo persica*) and tiger (*Panthera tigris*). When they spot a large predator making a kill, the jackals rush in to eat any remaining meat. Groups of 5-18 jackals have been seen frequently large ungulating carcasses. If other scavengers such as hyaenas and vultures have also arrived, the jackals bury as much meat as they can. Jackals also reach for turtle nests along coastal beaches and feed on the eggs. Usually larger mammals such as water buffalo (*Bulbalus bulbalis*), sambar (*Cervus unicolor*), spotted deer (*Axis axis*), wild boar (*Sus scrofa*) are inaccessible to the jackals by their sheer size. But whenever these animals die or are killed, jackals are assured of a rich and plentiful supply of food. So, why bother to go to all that trouble and run unnecessary risks, when nature offers such a windfall in the shape of a dead animal. Bad meat is not poisonous since harmless bacteria bring out decomposition. Nevertheless, jackals are much less dependent on carrion than is commonly supposed. Like dogs, they bury surplus food but return to it within a day to retrieve it using their sense of sight and smell. When animal food is not available, they use to feed on fruits and berries, as during the breeding season in India, the jackal feeds predominantly on fruits (Jhala & Moehlman 2008).

However, living in some parts of India and Bangladesh, the jackals can even subsist on garbage (Jhala & Moehlman 2008). They can also exploit man-introduced food sources such as small live stock and so could cause noticeable damage to poultry farms. There are literatures available for live-stock predation by golden jackal. In Israel, the farmers claim to lose an average of 1.5-1.9% of the calves born each year to golden jackal (*Canis aureus*) predation. The economic value of the total cattle losses in 1993 was estimated to be about US$ 42,000 (Yom-Tom *et al.*, 1995). This high

predation rate is actually caused by the farmers themselves, through the illegal dumping of domestic animal carcasses, a primary source of food for jackals, whose population has in turn thrived and augmented. As a matter of fact, in the decade 1978-1988, the number of jackals increased from a density of $0.2/km^2$ to $2.5/km^2$ and the current amount of meat dumped by farms is calculated to be enough to support a population density of $3.8/km^2$ predators. However, the jackals were also considered a serious problem in Bulgaria, where 1053 attacks on small stock, mainly sheep and lambs were recorded between 1982 and 1987. Along with this, some damage to newborn deer in game farms was also reported (Giannatos *et al.,* 2005). This high predation rate is thought to be the consequence of a jackal population explosion due to the high availability of human produced food and/or habitat manipulation (Yom-Tom *et al.,* 1995). This means that the conflict is expected to escalate if illegal waste dumping is not prohibited and the predator population is not controlled (Yom-Tom *et al.,* 1995) and also the preventive measures to avoid predation are lacking in both the cases. Genov & Wassiley 1991, reported that in Bulgaria most of the attacks on live-stock happened in the flocks of sheep that grazed unattended at night in pastures, while in Israel, the cattle grazed unattended all the year round in paddocks and gave birth in the field, so the opportunistic jackals would learn to exploit newborn calves, taking advantage of their high numbers (Yom-Tom *et al.,* 1995). However, even without preventing measures, the highest damage by jackals from Bulgaria is minimal when compared to the domestic losses by wolves (Giannatos *et al.,* 2005).

The feeding ecology of the golden jackal (*Canis aureus* Linnaeus. 1758) and its interspecific trophic relationship with the sympatric red fox (*Vulpes vulpes* Linnaeus. 1758) was investigated in an area of recent range expansion of the golden jackal in Hungary, Central Europe (Lanszki *et al*., 2006). Diet composition was determined by scat analysis over 4 years. Compared with jackals, foxes consumed more small mammals (mean biomass consumed:

jackal 77%, fox 68%) and to a lesser extent plant matter (6% and 18% respectively). Based on prey remains found in scats, small mammal specialization over a 2-year period and seasonal predation upon wild boar piglets (mainly by jackal), seasonal fruit eating (mainly by the fox), and scavenging on wild or domestic ungulates (both predators) were found. The tropic breadth of both species was very narrow and the fox proved to be more of a generalist. The food overlap index between the two canids was high (mean, 70%) and varied with the decreasing availability and consumption of small mammals. Alongwith the golden jackal, the red fox, *Vulpes vulpes* is a commonly occurring predator in Israel. Although the jackal is larger than a fox, their dietary habits are identical and are therefore in direct competition with one another. Foxes generally ignore jackal scent or tracks in their territories, though they will avoid close physical proximity with jackals themselves. Studies have shown that in areas where jackals became very abundant, the population size of foxes decreased significantly, apparently because of competitive exclusion (Scheinin *et al*., 2006). Borkowski *et al*., 2011 studied the diet composition of golden jackal in central Israel. The result showed that the main food category was ungulates (39.4% frequency of occurrence), 80% of which were domestic animals-which were assumed mostly consumed as carrion. Other common food types included fruits (31.3%), birds (30%), small mammals (23.5%) and invertebrates (21.2%), while garbage was found in only 9.1% of the scats.

In India, golden jackals have been known to appropriate the dens of Bengal foxes (Jhala & Moehlman 2008). Conversely, jackals are shown to vacate areas inhabited by the larger grey wolf. Wolves are often actively intolerant of jackals in their established territories and have been known to approach jackal - calling stations at a quick trotting pace, presumably to chase off the competitors (Giannatos *et al*., 2005). However, there are occasions when jackals scavenged on wolf kills without evoking any aggressive responses from the larger canids (Jhala & Moehlman 2008). The golden jackal remains

have been found in spotted hyaena scat, though hyaenas have shown to have a distance to golden jackal flesh, consuming them only starving (Kruuk 1972). Even though the golden jackal (*Canis aureus*) is the most common wild canid in India, little information is available on its ecology (Jhala & Moehlman 2008). Aiyadurai and Jhala (2006) conducted a study on home range, habitat use, food habits and ranging patterns of golden jackals in Velavadar National Park and the surrounding Bhal region of Gujarat, India. The mean home range of jackals in Velavadar National Park was 29.77 sq km. The home ranges were much larger than those reported for jackals in Bangladesh 0.6-1.1 sq km by Poche *et al*., (1987) and 0.5 sq km by Jaegar *et al*., (2001) and in Ngorongoro crater 5.1 sq km by Poche *et al*., (1987), but similar to those reported in Serengeti 10.34-23 sq km by Van Lawick-Goodall & H. Lawick-Goodall (1971). The average home range overlap in the Bhal jackal was 14-16%, whereas the core areas of each jackal were almost exclusive. Feeding ranges of several jackals in the Bhal overlapped as also reported by Van Lawick and Van Lawick-Goodall (1971). Jackals were observed to range over large distances in search of food and suitable habitat. Jackals were reported to use nine different habitats, namely grassland, medium *Prosopis*, dense *Prosopis*, village outskirts, saline wasteland, haplophytic scrub, fallow fields, mud flat and others (road edges, canal etc.). There was a marked difference in the habitat selection of jackals between night and day. Village outskirts were preferred at night, while grassland and *Prosopis* thickets were selected during the daylight hours. Scat analysis showed that blackbuck (33%) and cattle (32%) form the major food items followed by vegetation matter (24%) and hare (12%). Blackbuck and cattle remains combined, comprised more than 60% of the prey remains in jackal scats. Later on, Patil & Jhala 2008 studied movement patterns and habitat use by golden jackal in Bhal region of Gujarat. In an average, the jackal was found to travel about 8.6 km every night (with an average rate of 0.74 km per hour) and 9.55 km per 24 hours. Aiyadurai (2006) has also reported night forays of the jackals in Bhal to be around 6.2 km per hour with an average

rate of 0.7 km per hour. The jackal was observed to travel in excess of 20 km during one night. They also made observation on the activity and habitat use by jackal. They found resting as the major activity during the daytime while during night it was movement. Feeding was performed for a very short duration during the night hours, when it was actually moving in search of food. They also found that most of resting time was spent in the dense and medium *Prosopis* thickets while most of the moving time in open field and sparse *Prosopis* patches.

Majumder *et al*., 2011 studied food habits and temporal activity patterns of the golden jackal and jungle cat (*Felis chaus*) in Pench tiger reserve, Madhya Pradesh, India. They found that rodents contributed the maximum in the diet of the two predators (40% in golden jackal and 63.6% in jungle Cat). The estimated dietary overlap between jackal and jungle cat was 0.9 (90%). With regard to temporal activity pattern, jackal showed variation in activity pattern as they were found more active in the early morning and at night while jungle cat was found active mostly in the night hours. The same study was carried out by Chourasia *et al*., 2012, who observed food habits of golden jackal and striped hyena (*Hyaena hyaena*) in Sariska tiger reserve in Rajasthan. They found vegetative matter contributed maximum (17.57%) in jackal's diet followed by rodents (15.77%), chital (10.81%), sambar (5.41%) and nilgai (4.05%), while nilgai and domestic cattle contributed maximum (24.76% each) in the diet of striped hyena, followed by sambar (17.14%), chital (16.19%) and vegetative matter (10.48%). The estimated dietary overlap between jackal and striped hyena was 67%.

Due to the tolerance against dry habitats and omnivorous diet, the golden jackals occur in wide variety of habitats. These ranges from the Sahel desert to the evergreen forests of Myanmar and Thailand. They occupy semi-desert, short to medium grassland and Savannahs in Africa, and forested, mangroved, agricultural, rural and semi-urban habitats in India and

Bangladesh (Clutton-brock *et al.*, 1976, Poche *et al.*, 1987). A greater number live around human settlements with abundant food resources and shelter (Prater 1980). This adaptability permits jackals to have the ability to forego water (Kingdon 1977) and they have been observed on Pirotan Island in the Gulf of Kutch, India where there is no fresh water. Jackals can commute between this Island and the mainland by traversing through mangroves and small Islands that are exposed during extreme low tides. Jackals have been reported at elevations of 3,800m in the Bale Mountains of Ethiopia (Sillero-Zubiri *et al.*, 2004) and are well-established around hill stations at 2,000m in India (Prater 1980). High densities of jackals are observed in areas with abundant food and shelter. In India, jackal populations achieve high densities in pastoral areas such as Kachchh, Maharashtra, Rajasthan and Haryana. Based on known density estimates for parts of India and considering that about 19% (*i.e.*, about 637,000km^2) of the geographical area of India as forest cover, jackal populations (and that jackals are also found outside forested habitats) has a minimum population estimate of over 80,000. It seems not unreasonable for the Indian subcontinent (Jhala & Moehlman 2008).

Responses of golden jackals (*Canis aureus*) to broadcasted howling were investigated in rural Bangladesh. In the study carried out in 1996, two hypotheses were tested: that the howl response shows the same annual trends reported for other *Canis*, being high during the season of pairing-mating when territories are being established, and low during the denning season when there is a risk to vulnerable young from advertising the location of their den to rival conspecifics, that the frequency of approach responses (confrontation) varies inversely with howl responses and is higher during denning when howling is low. Thus, the results support both hypotheses and are consistent with the primary function of howling being as a passive means of territory maintenance whereby dominant animals advertise their locations to facilitate mutual avoidance between groups and thereby reduce accidental confrontation.

The jackals in Africa have been studied intensively as compared to the Asia (Kruuk 1972). Data on species' presence in Ethiopia, Somalia and Eritrea are found in Funaioli (1971) and Yalden *et al.*, (1980, 1996). Status and distribution of the species are discussed in Ginsberg & Macdonald (1990). They also gave information on the species' ecology. Some data on the species' distribution and ecology in Morocco are analyzed by Aulagnier & Thévenot (1986), Kerrouani *et al.*, (1996) and Ribi (1992). The main reference for the ecology of golden jackal in Africa are Lamprecht (1978) and Moehlman (1978, 1979, 1983). Both the authors studied coexisting jackals in the Serengeti, the former focused on diet and foraging behaviour while the later on their social behaviour. Moehlman (1983) made some very interesting observations on various aspects of social behaviour, such as social dominance, subordination relationships, communal howling, courtship and hunting. Ferguson (1978, 1981), Row-Row (1982), Loveridge & Macdonald (2001) have studied the socioecology, home range and movement patterns of black-backed jackal, *Canis mesomelas* in South Africa.

Krukk (1972) observed social relationships between spotted hyaenas with that of the Golden and black-backed jackals. The only study on the golden jackal's ecology in Central Africa is one by McShane & Grettenberger (1984) that investigated its feeding habits. Variation in habits, diet and behaviour are also found in different environmental conditions as shown by Macdonald (1983) in Israel. The evidences for scale-free patterns in the foraging trajectories of side-striped jackals, *Canis adustus*, widely distributed African canid were presented by Atkinson *et al.*, (2004). They used radio-tracking techniques for their study. Loveridge & Macdonald (2003) investigated the niche separation along habitat use, activity and dietary axes between two sympatric jackal species *Canis adustus* and *Canis mesomelas* in north-west Zimbabwe. It was found that dietary overlap was high, especially in the dry season, but activity periods differed between the species, with *C. adustus*

being more nocturnal than *C. mesomelas* and the two jackal species used different habitats.

I.3 *Canis aureus*: Status in India

I.3.1 Human-jackal conflict

Though the golden jackal is not big enough to make humans as prey, there are still incidences of jackals attack and aggression towards humans. Scientific literature as well as news paper clips appeared where jackals are reported attacking humans, even there are incidences of death of persons attacked by jackal. Generally it is seen that in the places where jackals live in large spaces as Savannah forests or remote deserts areas, these conflicts happened hardly ever, but in areas densely populated by people as also jackals and other mammals, attack on humans are more common than in earlier. Experts reported that the main reason behind human-jackal conflict is the industrialization and urbanization of forest cover area resulting in habitat shrinkage and to cope with this situation jackals are moving towards to human habitat (Jhala & Moehlman 2008). The other reason for this conflict may be human itself. The illegal dumping of garbage and sometimes carcasses of turkeys, hens and cattle near their agricultural farms provide a rich food for the potential predators like jackals, resulting mainly in cattle predation and conflict with humans also (Yom-Tom *et al.,* 1995).

In India, such incidences are also reported. Recently, in September 2009, a jackal has spread panic in Sitadih village under Angada block in Bihar. Four villagers were injured following attacks by the jackal (http://timesofindia.indiatimes.com/four-hurt-in-jackal-attack/). The forest officials have also mentioned a jackal attack on 20 residents of Lali village under Namkom block in Bihar in July 2009. A jackal was also reported killing an old man and injured 24 people in Buxan village in Azamgarh District, Uttar Pradesh in November 2009. The similar case also came in light in Bihar's

Gopalganj District, where nearly 100 people have been bitten and injured by jackal in June 2007. The veterinarian pathologists suggested that an animal (in this case jackal) launching seemingly pointless attack on humans is surely a rabid. It is claimed to injure human being in the mad phase of disease and after this phase it will die soon.

I.4 *Canis aureus:* Threats

The golden jackal is a widespread and fairly common species, found at high densities in suitable areas and able to thrive even close to human settlement (IUCN 2009; Macdonald 2006; Sillero-Zubiri *et al.*, 2004). Over its entire range except in protected areas like National Parks and Sancturies, the jackal population is continuously declining. The main threat to the species comes from the reduction in forest covered area and food non-availability (IUCN 2009; Sillero-Zubiri *et al.*, 2004). The traditional land use practices like livestock rearing and dry farming that were earlier conductive to the survival of jackals and other wildlife are now being steadily replaced by industrialization and intensive agriculture; wilderness areas and rural landscapes are being rapidly urbanized resulting in less food availability and reduced habitats. Jackal populations adapt to some extent to this change and may persist for a while, but eventually disappear from such areas (Giannatos 2004).

Persecution and hunting are important factors for their declination. In India, pastoralists occasionally use poison to kill predators like wolves and leopards that predate on livestock and jackals are killed by scavenging such poisoned kills. Sometimes illegal poisoning of jackals are also reported where they predate on livestock and damage crops (Yom-Tov *et al.*, 1995), but the species is not generally as damaging as the red fox (*Vulpes vulpes*) or grey wolf (*Canis lupas*) (Giannatos 2004) and may even benefit humans by scavenging waste and controlling pest species such as rodents and rabbits (Nowak 1991).

Some tribal communities like the *kolis, vaghris* in Gujarat and Rajasthan and *nari kuravas* in Tamil Nadu to kill and eat jackals. Beside this, there is a threat from organized poaching for skin, fur and tail which are sometimes marketed. Predation by other carnivores also poses a threat to golden jackal. In Kutch, jackals are predated by striped hyaenas (*Hyaena hyaena*). Spotted hyaenas also have been observed to kill and feed on golden jackals (Kruuk 1972; Kingdon 1977). Singh (1987) reported that pythons (*Python morulus*) were a major predator of jackals in Corbett National Park, India. Jackals are often chased and sometimes killed by feral dogs when they approach human habitation. Other than this, besides dogs, jackals are supposed to be the most common road kills on rural roads in India (Sillero-Zubiri *et al*., 2004) and the incidence of road kills increases during the breeding season from February to March (Jhala & Sharma 1997).

Now-a-days, a greater threat is coming from infectious diseases which have attracted increased attention in recent years and can be an important as well as intractable extinction risk for many species (Funk *et al*., 2001). A number of reviews have highlighted how disease issues are particularly relevant to canids. Canids are the source of human disease such as rabies, Leishmaniasis or hydrated cysts (*Echinococcus granulosus*). Moreover, threatened canids have suffered high disease-related mortality (Young 1994; Funk *et al*., 2001; Cleaveland *et al*., 2002, 2003; Woodroffe *et al*., 2004). Since jackals live in close proximity to human habitation, they have been reported to be susceptible to a large spectrum of canine pathogens commonly found in domestic dogs (*Canis familiaris*), including rabies (Foggin 1988), *Babesia canis* (Van Heerden 1980), *Ehrlichia canis* (Van Heerden 1979), *Leishmania donovani* and *Toxoplasma gondii* (Van der Merve 1953), *Ancyclostoma canium* and *Echinococcus granulosus*. Jackals in India are often infected with diseases like rabies, distemper and rabid jackals frequently attack domestic livestock, dogs and humans (Jhala pers. Comm.). Skin diseases like mange and parasites like ticks and fleas are common in jackals

in areas where they occur at high densities. In Tanzania, golden jackals had positive serological test resulted to canine parvovirus, canine herpesvirus, canine coronavirus and canine adenovirus (W.B. Karesh pers. Comm.), while in a serological survey conducted in Kenya (Alexander *et al*., 1994), all the three species of jackals (golden, side-striped and black-backed jackals) had antibodies for canine parvovirus, canine distemper, rabies and ehrlichiosis. Golden jackals are also reported to be infected (80%) with seven species of helminth parasites in Bangladesh (Shaikh *et al*., 1982).

Based on these surveys, it is concluded that as jackals are most abundant wild carnivores, then also being more susceptible to canine pathogens, they could serve as an important indicator of species to monitor the exposure of rare and endangered canids to specific canine diseases.

I.5 *Canis aureus*: Legal protection

Currently, jackals are slated to be Scheduled III species in India and are placed under Appendix II of CITES. In India they are declared as “species with least concern” and could be considered as “species requiring no immediate protection”. However, no hunting of any wild animal is permitted under the current legal system in India, jackals are afforded the least legal protection mainly to control trade of skin, fur and tail with caution and knowledge that populations throughout its range are likely declining.

CONSERVATION GENETICS: AN OVERVIEW

II.1 Introduction

Biodiversity is currently being lost at a rapid pace as a consequence of human activities (Kerr & Currie 1995; Novacek & Cleland 2001; Ceballos & Ehrlich 2002). The past century has seen rapid increase in human population and in the impact of human activities on the environment. This has lead to dramatic habitat loss, degradation and fragmentation resulting in many species of organisms becoming extinct or coming to the verge of extinction (IUCN 1996a; IUCN 1996b; WCMC 1992). According to the IUCN red list of threatened species (http://www.iucnredlist.org) about 25% of extant mammalian species are threatened with extinction. The high rate of extinction of a species has made its conservation an essential issue of the 21st century (Hedrick 2001). The conservation of an endangered species depends mainly on direct protection of the species in question and preservation of its habitat. Nonetheless, since 1980s, conservation genetics or the application of genetics to the preservation of species has received increasing attention (Frankel & Soulé 1981; Frankham 1995a; Frankham *et al.*, 2002; Ralls & Ballon 1986). Thus, Conservation genetics is a research field in which, among other things, molecular techniques are used to increase the knowledge of species and populations threatened with extinction. It comprises research on genetic processes that are typical of small populations, management, examination of taxonomic status, and application of genetic analysis in forensics and monitoring (Frankham *et al.*, 2002).

Human-associated factors such as habitat destruction, hunting and competition from introduced exotic species are the driving force behind the majority of recent extinctions. In addition to these factors, species are also vulnerable to stochastic factors that may cause extinctions even when the species is protected and there appears to be adequate habitat. These

stochastic factors can be extrinsic and include biotic elements such as the influence of predators, competitors and pathogens (Roelk-Parker *et al.*, 1996), or abiotic elements such as floods, fires and droughts (Raup 1991). Other stochastic factors such as demographic and genetic stochasticity (Frankham *et al.*, 2002) are intrinsic to species. Demographic stochasticity arises from chance fluctuations in birth rates, death rates and sex ratios (Avise & Nelson 1989). Genetic stochasticity includes inbreeding depression resulting from unavoidable mating between relatives, loss of genetic variation and the accumulation of deleterious alleles. The effects of genetic stochasticity depend on the effective population size. As stochastic factors are more important in small populations, they can exacerbate a situation once human factors have reduced a species' population size (Gilpin & Soule 1986). Therefore, in small captive populations, where extrinsic factors that may cause extinctions are usually minimized, demographic and genetic stochasticity play important roles.

There has been much discussion and controversy over the contribution of genetic factors to protecting endangered species. Some authors have suggested a limited role of genetic factors (Caro & Laurensen 1994; Lande 1988), while others have supported a greater role (Frankham & Ralls 1998; Hedrick 2001; O'Brien *et al.*, 1983). There is now a growing body of evidence demonstrating that the fates of small populations are linked to genetic changes. Inbreeding has been shown to affect fitness in captive animals of conservation interest (Ralls & Ballou 1983). Crnokrak & Roff (1999) reviewed a number of studies demonstrating inbreeding depression in wild populations. Other studies have found that genetically impoverished endangered populations often do not show signs of recovery until crossed with individuals from other populations (Westemeier *et al.*, 1998). Such studies underline the importance of genetic factors in the protection and recovery of endangered species.

Frankham *et al.*, (2002) have identified 11 major areas in conservation genetics: i) inbreeding depression, ii) loss of genetic diversity, iii) population fragmentation and reduced gene flow, iv) accumulation and loss (purging) of deleterious mutations, v) genetic adaptation to captivity, vi) taxonomic uncertainties, vii) outbreeding depression, viii) defining management unit within species, ix) forensic species identification, x) genetic drift and xi) understanding species biology.

II.2. Deoxyribonucleic Acid (DNA): Structure and Function

DNA is organized in chromosomes that are contained in a cell nucleus (nuclear DNA), and in mitochondria organelles present in the cell cytoplasm (mitochondrial DNA, mtDNA) it takes the form of double helix built by four nucleotides: Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). The linear order in which these four nucleotides follow each other in the double helix is called nucleotide sequence. This simple structure is extremely stable and allows the DNA to act as a template for protein synthesis and replication.

The two new double helix are identical, each one formed by a parental chromatid and by a complementary chromatid. In this way DNA sequences are faithfully copied and the genetic information coded in the sequences is preserved during cell duplication. The process of replication is not perfect and some nucleotide mutations may be inserted by chance. Mutations modify DNA sequences and generate genetic variability. The genome of vertebrates and many other living organisms is largely made of non-coding DNA sequences that apparently have no function. Genes, sequences present in single copy or in families made up of a small number of copies of the same gene, constitute the functional, non-repetitive DNA and codify for proteins. DNA sequences that make up the gene are organized in functional domains, have the role of regulating the transcription: the first part of the gene is made up of a promoter, a sequence of a few dozen nucleotides which is recognized by RNA polymerase. This is followed by coding sequences (exons) that normally alternate with tracts of sequences that are transcribed, but not

translated (introns). The gene ends with termination sequences that interrupt RNA synthesis.

II.3. Molecular markers in conservation genetics

The recent past has witnessed a rapid development in applications of molecular genetic techniques having the potential to help make faster well-informed decisions in conservation (O'Brien 1994). Advancements in DNA technology, *i.e.*, development of molecular markers have allowed a finer precision to investigation. Varieties of molecular markers have become available since 1950s and have been applied to many areas in biology such as population genetics, forensics, paternity testing and gene mapping. However, it is only since the 1980s that these molecular markers have become widely used in the field of conservation genetics.

A molecular genetic marker is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. Now molecular markers are potentially used to identify parents, offspring and close relatives in a single group or populations, to quantify the genetic variability of present and past populations, to reconstruct the phylogenetic relationship of taxa and to match samples of individuals to each other and to species or populations for forensic purposes. Some commonly used molecular markers are RFLP (Restriction fragment length polymorphism), AFLP (Amplified fragment length polymorphism), RAPD (Random amplified polymorphic DNA), VNTR (Variable number tandem repeat), SNP (Single nucleotide polymorphism), STR (Short tandem repeat), Microsatellite polymorphism and Mitochondria DNA (mtDNA) polymorphism.

II.4. Mitochondrial DNA (mtDNA)

The analysis of mitochondrial DNA (mtDNA) polymorphisms represents the most commonly used means for revealing phylogenetic relationships among

closely related species, and among populations of the same species (Avise & Lansman 1983; Wilson *et al*., 1985; Avise 1986). With few exceptions, the animal mitochondrial DNA is a small (15-20kb) circular molecule, composed of 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes and a noncoding segment of about 1000 bp, called Control Region that initiates replication and transcription.

Many features of mtDNA *viz*., large quantity in the cell, small genome size, maternal inheritance and extremely low probability of paternal leakage (Lopez *et al*., 1996; Cummins *et al*., 1997), high mutation rate than nuclear genome, high copy number and change mainly through mutation rather than recombination (Eyre-Walker & Awadalla 2001), makes it the biological material of choice for mirroring the evolutionary past of a species. Moreover, the mtDNA diversity can be efficiently used to infer both, relatively recent as well as ancient evolutionary events by selectively analyzing independent (Control Region) or dependent (essentially functional genes like Cytochrome b) domains. These attributes make mtDNA analysis a powerful tool in the molecular systematics, which in recent years has extensively and successfully been utilized to understand the phylogeography (Vila *et al*., 1999; Zachos *et al*., 2009; Wayne *et al*., 1997), population structuring (Bowen *et al*., 1994) and taxonomic relationships of a large number of animal species (Xu & Árnason 1997). Other uses have included establishing interspecific hybridization (Gottelli *et al*., 1994; Land & Lacy 2000), identification of species from hair and faecal samples (Foran *et al*., 1997; Paxinos *et al*., 1997), and the detection of illegal hunting and collecting.

The use of mtDNA beyond these applications is more limited (Bruford *et al*., 2003), especially because it only provides information about the female lineage (Avise 2004). This limitation is a drawback when studying domestic animals because male-mediated gene flow is usually more pronounced among them. For example, the application of mtDNA markers in domestic dogs (Okumura *et al*., 1996; Tsuda *et al*., 1997; Vila *et al*., 1999; Savolainen

et al., 2002 & Pires *et al*., 2006) has showed little correspondence between mitochondrial lineages, geographic structure or traditional breed classification, and many breeds contain haplotypes shared by other breed scattered over different phylogenetic clades.

II.4.1 Mitochondrial Control Region (CR)

The control region is the main regulatory region and the only major non-coding area in mtDNA. It is also called the "A-T rich region" for invertebrates or the "D-loop region" for vertebrates. It contains the heavy-strand origin of replication (Desjardins & Morais 1990) and the promoters for heavy and light strand transcription (L'Abbè *et al*., 1991). In mammals, the length of the control region varies from 880 to 1400 bp (Sbisà *et al*., 1997). The variation in length has been attributed to variation in the tandem repeat number (Berg *et al*., 1995) and small insertion/deletion usually in the 5' and 3' ends of the control region. Despite its functional importance, control region is suggested to be the most variable part of the mtDNA. The variable blocks in control region evolve about 4-5 times faster than the entire mtDNA molecule (Horai & Hayasaka 1992; Brown *et al*., 1993). Thus, when studying closely related species or conspecific populations, the sequencing of this region can provide better resolution using less experimental efforts. Based on the distribution of the variable nucleotide positions and differential nucleotide frequencies in different parts of the control region, it is divided into three domains (Brown *et al*., 1986).

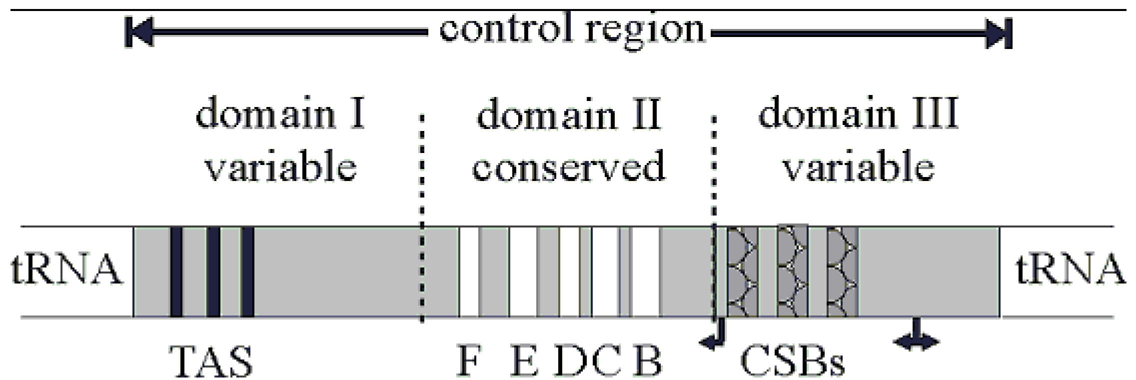


Figure 2.1- General structure of the vertebrate mitochondrial control region. The arrows indicate the location of the H-strand replication origin and the bidirectional promoter for L- and H-strand transcription. TAS, termination associated sequence; F through B, conserved sequence boxes in the central domain; CSBs, conserved sequence blocks

Domains I and III are rich in L-strand adenine, whereas the central domain II is low in adenine. Most of the variability, both nucleotide substitutions and deletions/insertions, is concentrated in domains I and III, whereas domain II is more conservative. The general structure of the control region and an overview of the sequence blocks are depicted in Figure 2.1.

II.4.2 Cytochrome b (Cytb)

The Cytochrome b (Cytb) is the most widely used gene for phylogenetic work for several reasons. Although it evolves slowly in terms of non-synonymous substitution, the rate of evolution in silent position is relatively fast (Irwin *et al.*, 1991). The wide use of cytb has created a status as a universal metric, in the sense that studies can be easily compared. Cytochrome b is thought to be variable enough for population level questions, and conserved enough for clarifying deeper phylogenetic relationships. However, the cytb gene is under strong evolutionary constraints because some parts of the gene are more conserved than others due to functional restrictions (Meyer 1994).

II.5 Microsatellites

Microsatellites have now become a tool of choice for population genetic studies, as they are abundant and fairly evenly distributed throughout eukaryotic genome (Gaypay *et al.*, 1994). Microsatellites are simple sequence tandem repeats (SSTRs) of 1-10 bp (Litt & Luty 1989). The repeat units are generally di-, tri-, tetra- or pentanucleotides. They tend to occur in non-coding regions of the DNA (although a few human genetic disorders are caused by microsatellite regions in coding regions). On each side of the repeat unit are flanking regions that consist of "unordered" DNA. The flanking regions are critical because they allow us to develop locus-specific microsatellites. In contrast, a given repeat unit may occur in thousands of places in the genome. This combination of widely occurring repeat units and locus-specific flanking regions are used for finding and developing microsatellite primers. Microsatellites are useful genetic markers because they tend to be highly polymorphic (Amos *et al.*, 1993). This variability is mainly due to mutation in microsatellites which occurs through slippage strand mispairing. They are also co-dominant markers as heterozygotes can be discriminated from homozygotes because alleles at a particular microsatellite locus vary in the number of tandem repeats and can be differentiated on the basis of the resulting differences in sequence length (Figure 2.2).

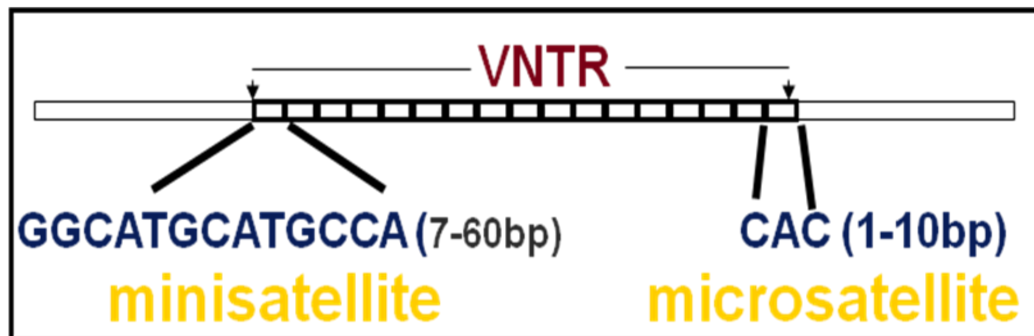


Figure 2.2- Microsatellites: Short Tandem repeats (2-8 nucleotides) present in hundreds or thousands of loci in eukaryotic genomes

The first use of microsatellites in natural populations was reported more than 20 years ago (Ellegren 1991, 1992; Schlotterer *et al*., 1991), while they were first isolated and amplified in humans in 1989 (Litt & Luty 1989; Tautz 1989; Weber & May 1989). There is extensive knowledge on general aspects of their use (Selkoe & Toonen 2006), pitfalls (Pompanon *et al*., 2005), development (Zane *et al*., 2002), evolution (Ellegren 2004), and transfer across species (Barbara *et al*., 2007). Being polymorphic, microsatellites have become the molecular markers of choice in many biological fields including conservation genetics. They have been used in a variety of studies to show population structuring (Forbes & Hogg 1999; Nesje *et al*., 2000), establish paternities and relatedness (Houlden *et al*., 1997; Marshall *et al*., 1999), assay genetic diversity (Brown & Houlden 1999; Mundy *et al*., 1997) and to identify species and individuals from small amounts of non-invasively collected tissue (Ernest *et al*., 2000; Sloane *et al*., 2000). Microsatellites provide data suitable for phylogeographic studies that seek to explain the concordant biogeographic and genetic histories of floras and faunas of large-scale regions. They are also useful for fine-scale phylogenies upto the level of closely related species.

SYSTEMATICS OF GOLDEN JACKAL, *Canis aureus*

III.1 Introduction

For hundreds of years, naturalists have looked at the world and attempted to describe and explain biological diversity. This attempt to examine and classify is called systematics- a system for imposing order on the seeming chaos of nature. Thus, biological systematics is the study of the diversification of life on earth, both past and present, and the relationships among living things through time. In 1758 Swedish naturalist Carolus Linnaeus devised a hierarchical classification system using two-part Latin names to categorize plants and animals. This system is still used today. Linnaeus was opposed to the theory of evolution, and his system was originally based on morphological features of structure and form. However, evolutionists rapidly adopted the Linnaean system and developed it into a classification based on phylogenetics, the evolutionary development of species. By 1866, German zoologist Ernst Haeckel had published a collection of detailed phylogenetic "trees" depicting what was then known about the evolutionary history of life. Interest in phylogeny waned over much of the nineteenth century, replaced by an emphasis on genetics, physiology, and geographic variances. That began to change with the work of botanist Walter Zimmerman in the 1940s, and German zoologist Willi Hennig, in the 1950s and 1960s. These scientists pioneered the definition of objective criteria for determining the shared genetic attributes of living and fossil organisms. A revolution in molecular biology took place in the 1960s. Methods for determining the molecular structure of proteins and amino acids allowed biologists to begin to estimate phylogenetic relationships. The exponential growth of systematics in the late twentieth century is due to a combination of increased sophistication in molecular biology techniques, and computer advances in hardware and software that allow scientists to model large and complex data sets.

Molecular Systematics uses a variety of techniques to derive phylogenetic trees. Polymerase chain reaction (PCR) is used to investigate variations of DNA on a large scale. Gene amplification is also fundamental to new approaches to DNA fingerprinting. Scientists can use “molecular clocks" to predict both past and future molecular divergences in genes. This theory claims that molecular change is sufficiently constant to determine how current genetic lineages branch off from a common ancestor and to determine when the branching occurred. Genetic markers are used to make inferences about relationships between environment and morphology, as well as physiology and behavior. The importance of phylogenetic trees, or estimates of evolutionary history, is that they allow biology to be predictive. Much as a chemist can use the periodic table of elements to predict chemical reactions, biologists can use phylogenetic trees to analyze biological variation and make predictions about behavior, morphology, and physiology, as well as biomolecular structure and other biological attributes.

Systematics is thus one of the oldest areas of biological research, a primary field of biology for as long as there have been biologists. Up until the second half of the twentieth century, however, almost all systematic biological research was based on morphological (or, less commonly, behavioral) data. Specimens were collected, and their morphological traits were used to determine species boundaries, assess phylogenetic relationships, study hybridization, and analyze geographic variation. Although morphology and behavior continue to be important for systematic studies today, there is now increasing reliance on molecular characteristics to study biodiversity. The use of protein and nucleic acid data to study variation among biological organisms comprises a field known as molecular systematics.

Although the earliest efforts to use protein data in systematic biology date back to at least 1904 (Nutall 1904), the field of molecular systematics did not begin to develop in any significant way until the 1960s. Until then, the only techniques that were widely used to compare homologous proteins in different species were

based on immunological comparisons (Maxson & Maxson 1990). These techniques were relatively expensive and technically demanding, and they produced only indirect estimates of amino acid differences in the proteins, they were one of the principal means for making comparisons among relatively distantly related species before the advent of DNA sequencing. Various immunological techniques were used through the 1980s, but these have rarely been used for systematic purposes since then. During the late 1950s and 1960s, techniques of protein electrophoresis were developed and popularized (Murphy *et al*., 1996). Amino acid replacements in proteins often produce changes in the net charge and/or shape of the protein. Thus, allelic variants of proteins move at different rates through an electric field in a porous medium (typically a gel). The development of histological staining techniques for many enzymes allowed biologists to separate thousands of proteins in crude tissue extracts simultaneously in an electrophoretic field, and then to visualize the location of upto 100 or more specific enzymes, using enzyme-specific staining. Using these techniques, allelic variants of enzymes (called allozymes) can be identified within and among individual organisms, and variation at dozens of different genetic loci can be examined. Early studies of allozyme electrophoresis revealed a vast and initially unexpected level of genetic variation in many populations and species (e.g., Hubby & Lewontin 1966). This technique remains one of the fastest and least expensive methods for comparing genetic variation across large number of independent loci, although use of the method has dropped precipitously since the early 1990s in favour of nucleic-acid-based methods. Although the structure of DNA was deduced in the early 1950s and its importance as the genetic basis of all biological variation was understood soon thereafter, it was not until the 1960s that the first systematic studies of variation at the DNA level were undertaken. The first method used to assess variation in homologous regions of DNA was DNA hybridization (Wetmur & Davidson 1968). The technique was widely applied to systematic problems in the 1970s and 1980s (Werman *et al*., 1996), but DNA hybridization was largely replaced by DNA sequencing in systematic investigations by the 1990s. The advantage of DNA hybridization is that it can

provide a measure of divergence across the entire single-copy component of the genome, while the primary disadvantage is that all the variation is reduced to a single, average measure of divergence, and thus the level of possible resolution is minimal.

Despite the diversity of molecular techniques that are applied to systematic problems, today molecular systematics is dominated by studies of DNA sequence variation. Practical methods for sequencing long, specific regions of DNA were developed in 1977 (Sanger *et al*., 1977; Maxam & Gilbert 1977), and these were routinely used in systematic studies beginning in the early 1980s. However, at first most target sequences of DNA had to be cloned before they could be sequenced, which required considerable time, effort, and expense. The development of the polymerase chain reaction (PCR) to amplify specific regions of DNA in vitro using a thermally stable DNA polymerase removed this obstacle (Mullis & Faloona 1987). By the late 1980s, DNA sequencing was becoming the method of choice for many systematic studies (Hillis *et al*., 1996), and new applications of DNA sequencing led to an exponential increase in studies of molecular systematics. Today, DNA sequencing is the most widely used method in molecular systematic investigations. The primary advantages of DNA sequencing are that it provides a large number of characters (potentially equal to the total number of independent genetically determined characters in an organism, if whole genomes are sequenced), and that DNA sequences can now be collected rapidly and easily from virtually any organism. Thus, it is possible to make comparisons at almost any level of biological hierarchy, from recently evolved allelic variants within individuals to homologous genes that are conserved across the entire life.

Evolutionary relationships within the family Canidae have been reconstructed using morphological data (Berta 1987; Lyras & Van Der Geer 2003; Tedford *et al*., 1995; Zrzavy & Ricankova 2004) and molecular data including G-banded karyotypes (Wayne *et al*., 1987a, b), DNA-DNA hybridization (Wayne *et al*.,

1990), allozyme electrophoresis (Wayne & O'Brien 1987) and mitochondrial DNA protein coding sequence data (Wayne & O'Brien 1987; Wayne *et al.*, 1997, 1987a, b). Further relationships at the genus level have been studied with mtDNA control region sequencing (a non-coding, hypervariable segment of about 1200bp in the mitochondrial genome) and microsatellite loci (hypervariable single copy nuclear repeat loci) (Geffen *et al.*, 1992; Bruford & Wayne 1993; Girman *et al.*, 1993; Gottelli *et al.*, 1994; Vila *et al.*, 1997, 1999 and Bardeleben *et al.*, 2005)

Despite numerous systematic studies, the relationships among many canid species and genera remain unresolved (Clutton-Brock *et al.*, 1976; Nowak 1979, Wayne *et al.*, 1987a, 1987b; Tedford *et al.*, 1995 & Bardeleben *et al.*, 2005). Two problematic systematic issues have broader evolutionary significance of the family and these are **a) monophyly of South American canids**, and **b) origin of a complex modification of the meat-processing tooth, the carnassials blade.** Nine South American extant species are classified into seven genera and represent the most taxonomically rich canid fauna in the world. These taxa are morphologically very diverse (Langguth 1975; Clutton-Brock *et al.*, 1976; Wayne 1987a, 1987b, Berta 1987) and include three unusual monotypic genera: the long-legged maned wolf (*Chrysocyon brachyurus*); the nearly extinct small eared dog (*Atelocynus microtis*); and the diminutive bush dog (*Speothos venaticus*). The remaining taxa are dominantly fox-like, range in size from that of a kit fox to that of a coyote (Wayne *et al.*, 1989). Webb 1985 reported that the first appearance of South American canids followed the immigration of North American mammals into South America during the early Pleistocene after the geologic emergence of the Isthmus of Panama. Just prior to that time, the large carnivorous fauna in South America was limited and included only a few didelphid species and a single phorusrhachid bird (Marshall 1977). Consequently, an interesting evolutionary question is whether the extant endemic South American canids trace their origin to a single North American lineage or whether several evolutionary distinct lineages invaded South America. If the latter hypothesis is verified, it would suggest the presence of undiscovered fossils

closely allied to the recent South American canids in Central and North America. The resolution of this question may provide important insights into constraints on morphological evolution in carnivores: a single origin implies rapid morphological change from the common ancestor to produce the diversity seen today; multiple origins would suggest a less dramatic burst of innovation (Wayne *et al.*, 1989; Van Valkenburgh 1991). In case of carnassials blade, in three canid species, the Asiatic dhole (*Cuon alpines*), the African wild dog (*Lycaon pictus*), and the bush dog, the lower carnassials molar has a unicuspid talonid called trenchant heel. Simpson (1945) used this character to place these three species in separate subfamily. However, previous allozyme and morphological phylogenetic hypotheses suggest that the character may have evolved more than once (Clutton-Brock *et al.*, 1976; Tedford *et al.*, 1995). The trenchant heel increase the length of the cutting blade of the carnassials molar and represents an adaptation for increased carnivory (most canids are omnivores). The three species with trenchant heel are considered the most highly carnivorous of the Canidae (Ewer 1973; Van Valkenburgh 1994; Van Valkenburgh & Koepfli 1993).

Although systematic treatments of the Canidae have used a wide variety of morphological, karyological and molecular genetic techniques, several specific taxonomic issues remain unresolved. Morphological and molecular data conflict strongly over the relationships of the bush dog (*Speothos venaticus*). Phylogenetic analysis of discrete morphological character data indicates that the bush dog's nearest relative outside South America is the raccoon dog (*Nycteruetes procyonides*), a small omnivorous canid with native populations now found only in Southern China and Japan (Berta 1987; Tedford *et al.*, 1995). However the diploid number (74) and characteristic acrocentric morphology of the bush dog's karyotypes are very similar to those of wolves and jackals and other South American canids (Wayne *et al.*, 1987a). In contrast, the raccoon dog has a predominantly metacentric karyotype that appears plesiomorphic (Wayne *et al.*, 1987b). Bush dogs also have allozyme allele frequencies that are more similar to those of *Canis* than to those of the raccoon dog (Wayne & O'Brien

1987). The two canid phylogenies based on discrete morphological characters are also in conflict. Berta 1987 allied the maned wolf with *Canis*, whereas Tedford *et al.*, 1995 placed the maned wolf near the base of a clade consisting predominantly of the South American foxes and the bush dog and raccoon dog. Chromosomal and allozyme studies support an affinity of the maned wolf with South American foxes (Wayne *et al.*, 1987a; Wayne & O'Brien 1987). Similar disparities among morphological, karyological, and molecular data set also are apparent in the relationships of the wolflike canids (grey wolves, coyotes, jackals, the Asiatic dhole and the African wild dog). The reasons for these disparities are not clear.

To address these broad evolutionary issues and a variety of more specific taxonomic problems, a phylogenetic study was conducted by Wayne *et al.*, 1997. The objectives of the study was to resolve a) relationships of the raccoon dog, grey fox and bat-eared fox, b) monophyly of the South American foxes, c) relationships of the maned wolf and bush dog, d) evolution of the trenchant heel, e) monophyly of the wolflike canids and, f) status of the jackals. Representatives of all the genera were analyzed to determine the monophyly of South American canids to determine how many lineages invaded South America. Their estimated divergence time was also estimated to access whether these lineages diverged before or after formation of the Panamanian Isthmus. The estimated divergence times were based on a fossil record directly and on a fossil record calibrated molecular clock for the canidae. Similarly, the molecular phylogeny was used to determine whether the trenchant heel evolved multiple times in the canidae. The analysis resolved most of the objectives as; a) the lineages leading to the raccoon dog, bat–eared fox and grey fox diverged early in the history of the extant canidae. These taxa are not closely associated with any living canid. The hypothesis that the raccoon dog is a sister taxon to the South American crab-eating fox is not supported nor is the association of the gray fox with any other fox like taxa, b) South American foxes were found monophyletic, c) the bush dog and maned wolf define a well-supported monophyletic group. The bush dog is not associated with the small-eared dog, the crab-eating fox, or the raccoon dog.

Similarly, the maned wolf is not a sister taxon to *Canis* nor does it lie within the South American fox lineage, d) the gray wolf, coyote, and simien jackal are monophyletic, with the golden jackal as the most likely sister group to this clade, followed by the black-backed jackals and dhole in an undetermined order. The African wild dog, the bush dog/maned wolf clade, and the side-striped jackal are basal to the other wolflike canids, but their relationships are not well resolved and, e) the jackals are paraphyletic. The phylogenetic relationships of the trenchant-heeled dogs is still not well resolved, some of the molecular trees are consistent with a single evolution of the character whereas others suggest it evolved at least twice independently. Thus, Wayne *et al*., 1997 suggested four monophyletic groups within the canidae-

a) The wolf and jackal like canids
b) The red-fox like canids
c) The South American foxes, and
d) The maned wolf and bush dog

It was also concluded that the grey fox, raccoon dog, and bat-eared fox are basal canids not closely associated with any of these monophyletic group. However, several phylogenetic issues remain unresolved in the study including, a) the branching order among gray fox, raccoon dog and bat-eared fox, b) the monophyly of the crab-eating fox and small eared dog, c) the relationship of the wild dog and side-striped jackal, d) the monophyly of the maned wolf and gush dog, and e) the iterative or single appearance of the trenchant heel. To better resolve these issues and to test previous hypothesis based on morphogenetic and molecular data, Bardeleben *et al*., 2005 reconstructed the phylogenetic relationships of 23 species in the dog family, canidae using DNA sequence data from six nuclear loci. Phylogenetic analysis with a data set using MP, ML, and Bayesian approaches produced a more resolved tree with three well-defined clades including the red fox-like canids, the South American foxes, and the wolflike canids. They also included mitochondrial and morphological data in the analysis. Their findings were-

a) Placement of maned wolf and bush dog in the South American canids clade. Nuclear and mtDNA data grouped maned wolf and bush dog while morphological studies do not (Lyras & Van Der Geer 2003; Tedford *et al.*, 1995).

b) The grouping of side-striped jackal (*C. adustus*) and black-backed jackal (*C. mesomelas*) as sister taxa. More studies assign these taxa to the wolflike canids (Bininda Emonds *et al.*, 1999; Wayne *et al.*, 1997) while some evidence suggested that these too do not group with the rest of the canids (Zrzavy & Ricankova 2004).

c) The grouping of raccoon dog and bat-eared fox as sister taxa.

d) The nuclear data provides moderate support for the monophyly of crab-eating fox and small-eared dog whereas it decreased in the combined nuclear+mitochondrial tree.

Thus, relationships within the clades containing the red fox-like canids and South American canids are well resolved whereas, the relationships among the wolflike canids remain largely undetermined and it was suggested that the lacking may be due to their recent divergence and insufficient time for the accumulation of phylogenetically informative signal.

The recent discovery of a lineage of grey wolf, *Canis lupus* in North-East Africa suggests the presence of a cryptic *Canis* on the continent, the African wolf, *Canis lupus lupaster*. Earlier it was known as Egyptian jackal, *Canis aureus lupaster* having its range from North to South-East Africa. Although Egyptian jackal has been assembled to grey wolf, *Canis lupus* based on some morphological features, which is now supported with genetic analysis also (Naseef 2003; Rueness *et al.*, 2011). To assess the actual range of African wolf, to further estimate the genetic characteristics and demographic history of its lineage and to question its taxonomic delineation from golden jackal, *Canis aureus*, a recent genetic study was conducted by Gaubert *et al.*, 2012. They analyzed the mitochondrial DNA (mtDNA) diversity of a series of African *Canis* including

wolflike animals from North to West Africa. The results confirmed the existence of four distinct lineages with the grey wolf, including *Canis lupus/familiaris* (Holarctic wolves and dogs), *Canis lupus pallipes* (Indian wolf), *Canis lupus chanco* (Himalayan wolf) and *Canis lupus lupaster* (African wolf). They found that the African wolf lineage-

a) Had the highest level of genetic diversity within the *Canis lupus*,

b) Coalesced during the late Pleistocene, contemporaneously with Holarctic wolves and dogs,

c) Had an effective population size of c.80,000 females.

Thus, their results suggest that the African wolf is a relatively ancient grey wolf lineage expanding its known distribution c.6,000 km to the west and with a fairly large past effective population size (as also suggested by the Pleistocene fossil record).

In order to get better insight of systematics or position of golden jackal, *Canis aureus* among the wolflike canids, here, I attempt to study the phylogeny of golden jackals by using mitochondrial CR and Cytb genes. The use of mtDNA has become increasingly popular in phylogenetic and population genetic studies, first with the developments in methodology for mtDNA isolation and use of restriction enzymes to detect nucleotide differences (Lansman *et al,.* 1981), and further with the development of PCR methodology and applicability of 'universal' primers (Kocher *et al.,* 1989) for amplification of mtDNA. Its maternal inheritance, lack of recombination, high copy number, variable substitution rates across regions, high mutation rate compared to nuclear DNA, and role in energy production make it an attractive genome for research that aims to understand species relationships, evolutionary history, and demographic patterns within both contemporary and historic contexts (Avise 2004). The CR, the major non-coding region of the animal mtDNA molecule evolves rapidly because of the lack of coding constraints. Sequence variation in the CR consists not only of substitutions but also of indels of various lengths and of variation in number of

copies of tandem repeats (Sbisà *et al.*, 1997). CR has been suggested to have one of the highest substitution rates of all the mitochondrial genes (Brown 1986; Meyer 1993). Mutation rate of the CR can be two to five times higher than that of mitochondrial protein-coding genes (Meyer 1993). These features make CR suitable for resolving relatively recent relationships. On the other hand the cytb gene encodes a protein and evolves relatively slowly, enabling distant comparisons and resolution of deep phylogenetic branches.

MtDNA has been reported as a useful genetic marker to examine phylogeny, phylogeography, and population genetic analysis in birds (Gorman 2000; Zink *et al.*, 2000; Buehler & Baker 2003 & Jones *et al.*, 2005), reptiles (Kumazawa & Nishida 1999 & Leache & McGuire 2006), fish (Liu & Chen 2003; Peng *et al.*, 2004 & Perdices *et al.*, 2004), amphibians (Driscoll 1998 & Shaffer *et al.*, 2000), and a variety of canid species (Vila *et al.*, 1999; Sharma *et al.*, 2004; Pires *et al.*, 2006; Kirschning *et al.*, 2007; Zachos *et al.*, 2009; Weckworth *et al.*, 2010; Pilot *et al.*, 2010; Bozarth *et al.*, 2011; Rueness *et al.*, 2011, and Gaubert *et a*l., 2012).

III.2 Materials and Methods

III.2.1 Sampling

Fifty five samples of golden jackal have been collected from a variety of locations covering diverse parts of its distribution across the world including India. Among them, most of the tissue samples collected from road kills, hair and blood obtained from golden jackal trapped as non-target species on the "Ecology of the Indian wolf" project of the Wildlife Institute of India (WII) were used for this study. Additionally a radio telemetry study of the Wildlife Institute of India done in Bhal region in 1997 also provided 10 blood samples. Two samples from Israel were provided by Dr. Eli Geffen, Department of Zoology, Tel Aviv University, Israel, while five Bulgarian samples were made available by Ivan Nikolov, molecular Zoology Unit, Department of Zoology, Technical University of Munich, Germany. Samples were preserved in 95% Ethanol and stored at -20°C until DNA extraction.

Sampling distribution is shown in the map (Figure 3.1) where blue triangles are the area from where samples were collected while the no. of samples analyzed are detailed in Table 3.1.

Table 3.1- Description of sampling location and number of samples collected for the phylogenetic study of golden jackal, *Canis aureus*

SNo.	No. of samples collected	Location
1	55	India
2	02	Israel
3	05	Bulgaria
	Total= 62	

Figure 3.1- Worldwide sampling locations to study the systematics of golden jackal, *Canis aureus*. Red shaded area is its worldwide distribution while black circles are sampling location for the present study

III.2.2 DNA Extraction

A very small portion of tissue (around 50-80mg) was cut out carefully and chopped finely using separate sterile blades for every sample, taking care to avoid contamination. Total genomic DNA was extracted from tissue (ear pinna), blood, hair samples using traditional Phenol Chloroform extraction procedure (Sambrook *et al*., 2001) and QIA quick DNeasy Blood/Tissue kit (Qiagen) as per manufacturer's protocol. Compared to the Phenol Chloroform, a time consuming procedure, QIA quick DNeasy Blood/Tissue kit method was found quick and more efficient for extraction with less comfort. Almost 75% of the extraction was done with kit while Phenol Chloroform method was used for remaining 25% extraction. The methodology of the DNA extraction from blood and tissue with

DNeasy Blood/Tissue kit is summarized in Figure 3.2. In continuation, Figure 3.3 is showing the steps for Phenol-Chloroform method of DNA extraction.
Extraction was followed by amplification using Polymerase Chain Reaction (PCR) with universal primers available for mtDNA (CR and Cytb) for canids.

III.2.3 PCR amplification of mitochondrial DNA

Two regions of mtDNA: an approximately 440 base pair (bp) fragment of the CR (CR) using universal primers ThrL15926 and DL-H16340 as in Vilà *et al*. (1999); and a 412 bp fragment of the Cytb gene using a canid specific light primer Canid L1 (Paxinos *et al*. 1997) and a universal heavy primer H15149 (Kocher *et al*. 1989) were amplified. Description of primers is listed in Table 3.2.

Table 3.2- Description of mitochondrial CR and Cytb primers used for phylogenetic analysis in golden jackal, *Canis aureus*

Primer Name	Primer sequence (5'-3')	Reference
Control Region (CR)		
Thr-L15926	GAATTCCCCGGTCTTGTAAACC	Vila *et al*., 1999
DL-H16340	CCTGAAGTAGGAACCAGATG	Vila *et al*., 1999
Cytochrome b (Cytb)		
Canid L1	AATGACCAACATTCGAAA	Paxinos *et al*., 1997
H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	Kocher *et al*., 1989

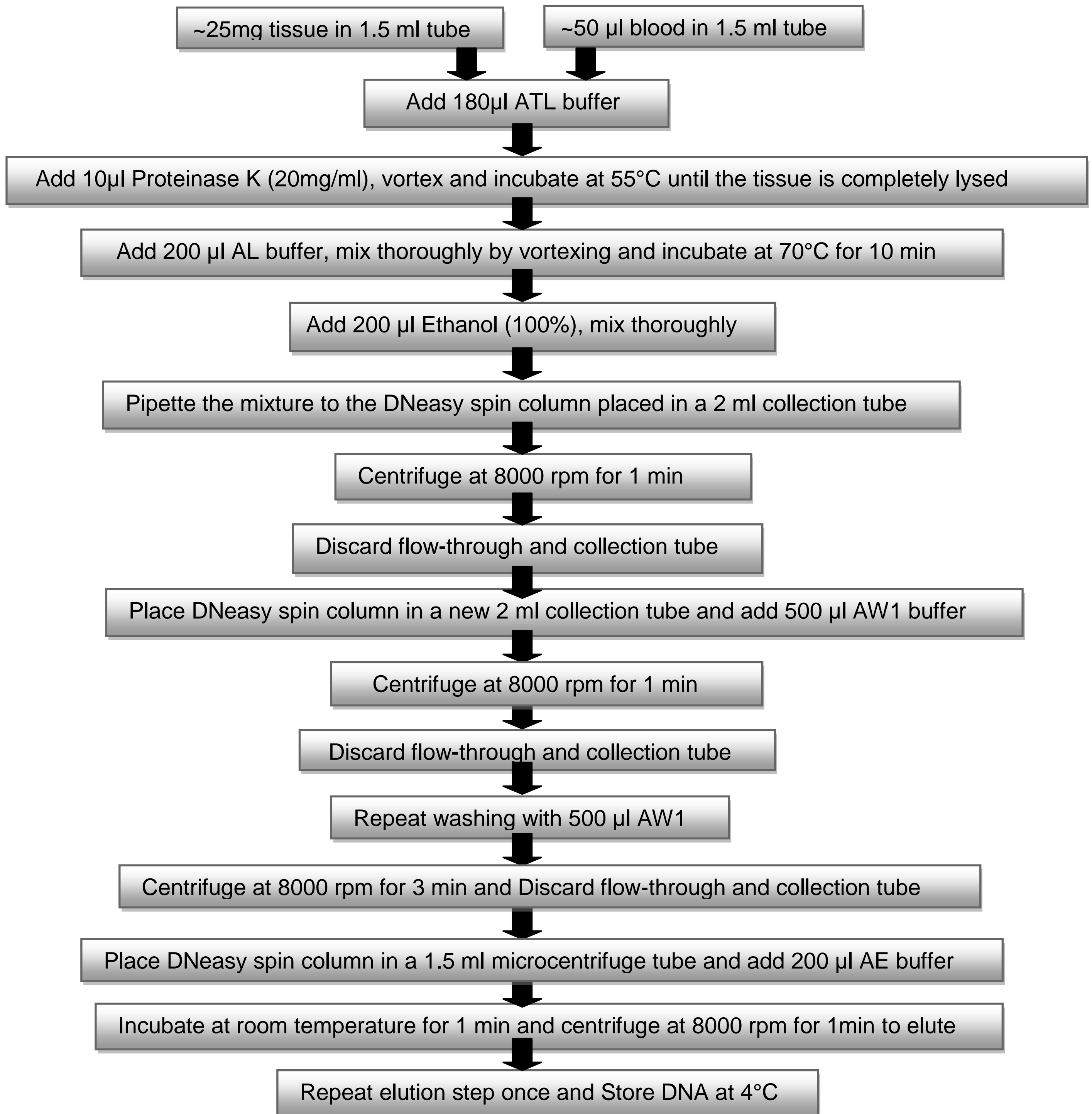


Figure 3.2- Methodology for DNeasy blood/tissue kit protocol DNA extraction from blood and tissue of golden jackal, *Canis aureus*

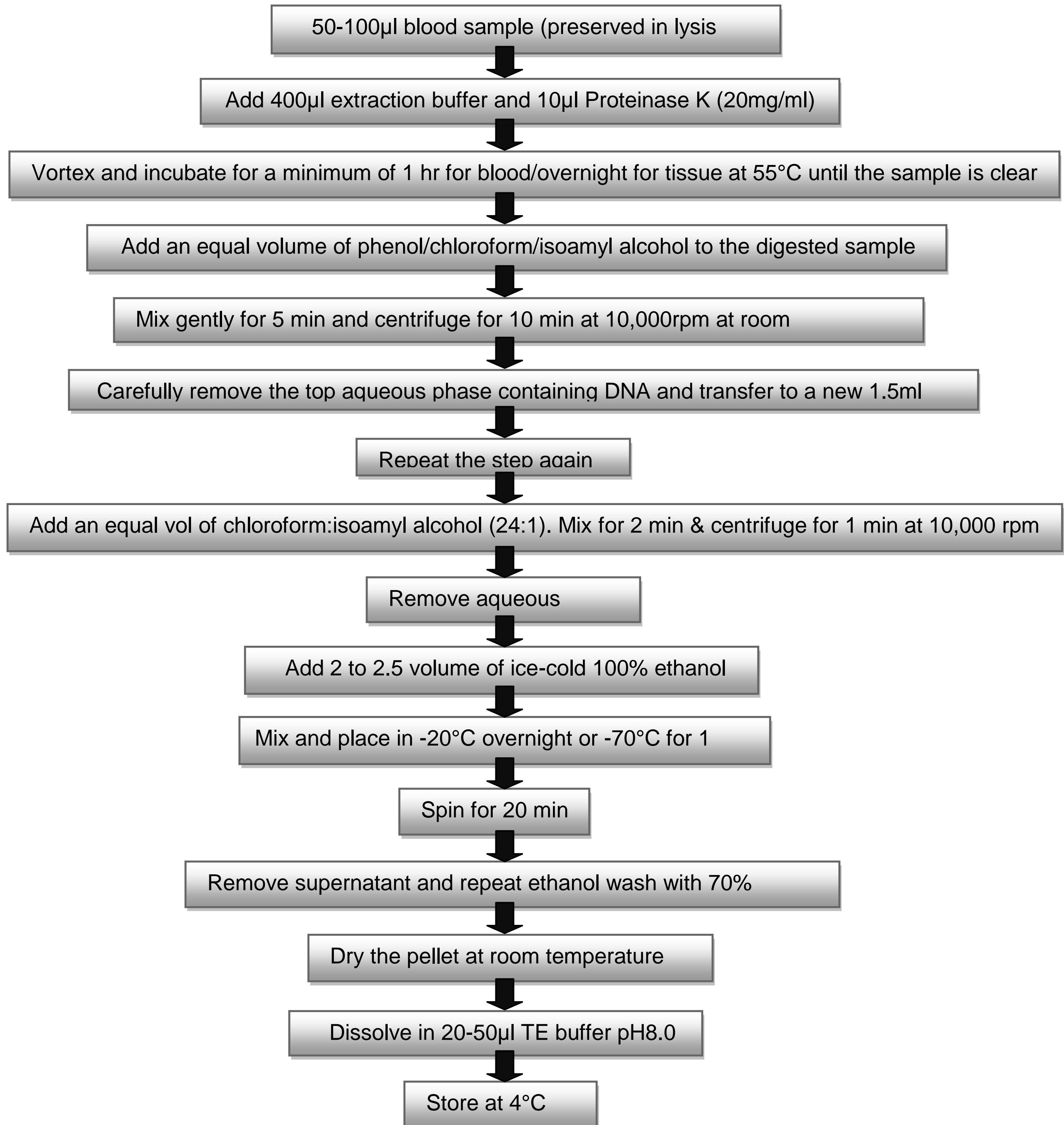


Figure 3.3- Methodology for Phenol-chloroform method for DNA extraction from blood of golden jackal, *Canis aureus*

Each PCR reaction was carried out in a 25µl volume including 0.5 units of AmpliTaq Gold (Applied Biosystems), 25 mM $MgCl_2$, 10X reaction buffer, 1X BSA, 2µM each dNTP, and 10µM each primer. Negative controls were run using blank extraction samples for each PCR run. Amplifications were performed in a PTC-200 (MJ Research) or GeneAmp PCR system 2700 (Applied Biosystems). The PCR conditions used by Wayne *et al*., 1997 were optimized by increasing the time for annealing and decreasing for extension. The protocol used was as below:

Initial denaturation	95° X 1’
35 cycles	94° X 45’’ denaturation
	50° X 35’’ annealing
	72° X 35’’ extension
Final extension	72° X 7’

A small aliquot of each amplification was electrophoresed on a 2% agarose gel to check for the correct fragment size and to ensure that only a single amplification product was obtained (Figure 3.4). Amplification products were cleaned with a Qiagen PCR purification kit or purified using 0.5 µl of a mixture of Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP) (GE Healthcare) that remove, respectively, unincorporated primers and dNTP. Steps for ExoSAP-IT PCR cleaning are shown in Figure 3.5 while Figure 3.6 is showing the steps for QIAquick PCR purification kit.

ExoSap-IT prepares PCR products for sequencing by either radioactive or fluorescent detection methods. Usually when PCR amplification is complete, any unconsumed dNTPs and primers remaining in the PCR product mixture will interfere with these methods. ExoSap-IT utilizes two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase, to remove these unwanted

dNTPs and primers.

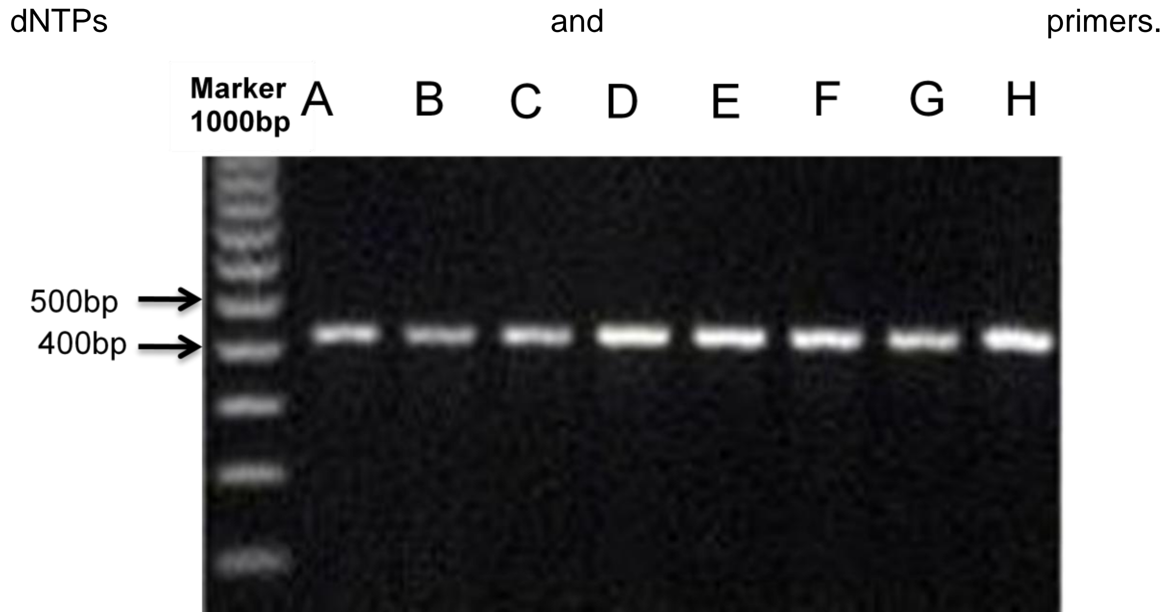


Figure 3.4- Agarose Gel showing the results of PCR amplification of mitochondrial Control region (CR) of golden jackal, *Canis aureus*. The PCR products are shown in the form of bands

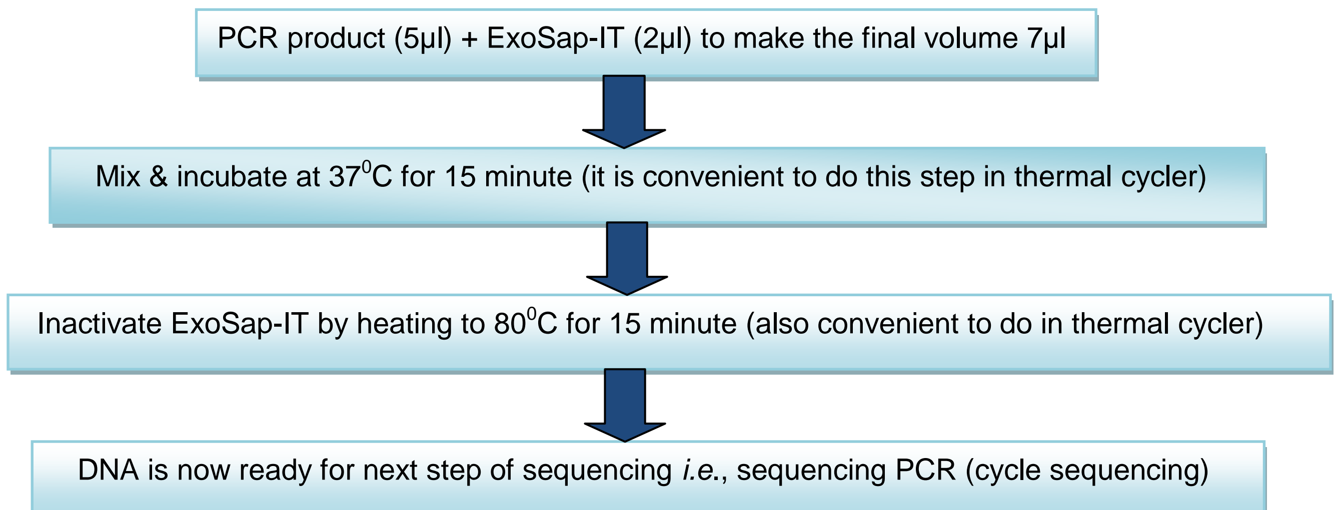


Figure 3.5- Steps for PCR cleanup using ExoSAP-IT

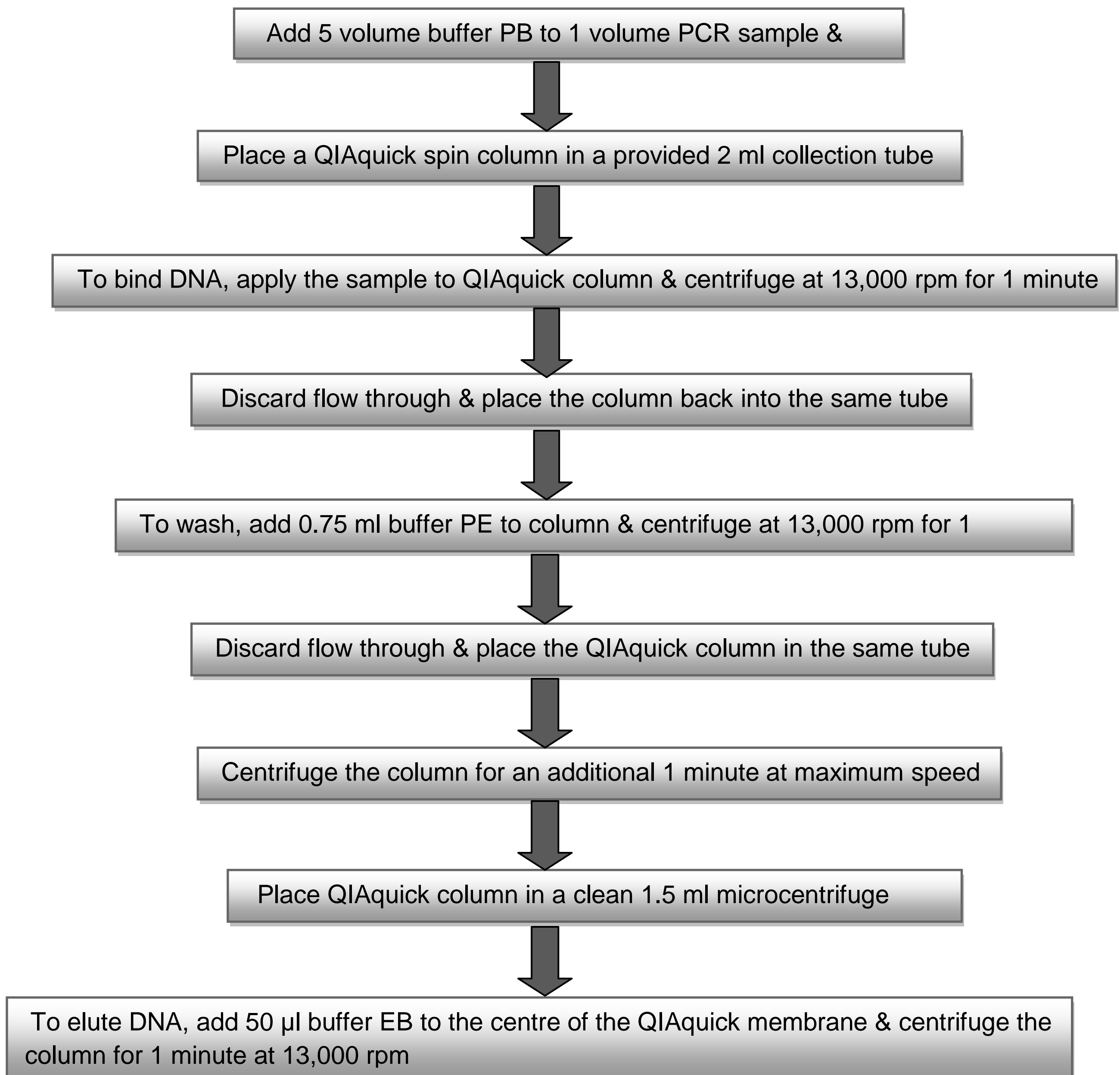


Figure 3.6- Steps for QIAquick PCR purification kit protocol

The Exonuclease I degrades residual single-stranded primers and any extraneous single-stranded DNA product by the PCR. The Shrimp Alkaline Phosphatase hydrolyzes remaining dNTPs from the PCR mixture which would interfere with the sequencing reaction. ExoSap-IT is added directly to the PCR product. The enzymes are active in the buffer used for the PCR, hence no buffer exchange is required. Products were cycle-sequenced using a Big Dye Terminator kit (Applied Biosystems), and all amplification primers listed above. Sequencing protocol is described below.

Initial denaturation	96° X 1'
24 cycles	96° X 10'' denaturation
	50° X 5'' annealing
	72° X 4' extension

Cycle sequencing reactions were ethanol-precipitated (Sambrook *et al*., 2001). Actually the sequence quality obtained from commercial purification kits or methods can be increased by performing a final ethanol precipitation of the purified DNA. Using this step can remove leftover salts and wash buffer that may not have been removed fully during the purification process. Steps are shown in Figure 3.7. Finally, the PCR products were run on an ABI 3300 Automated sequencer (Applied Biosystems).

The sequencer reads the DNA fragment and outputs the data as DNA sequence where each base pair is labeled with a particular color. The cleaned sequences show well-defined peaks while the non cleaned sequences show the ambiguous areas caused by dye blobs or noise *i.e.*, degradation of peak quality. Sequence data obtained for both strands of mitochondrial CR and Cytb for each individual and consistent sequences for all individuals were edited and aligned using Sequencher 4.6 (Gene Codes Corporation, Inc., Ann Arbor, Michigan) and rechecked visually. Figure 3.8 is showing the aligned CR sequences of golden jackal.

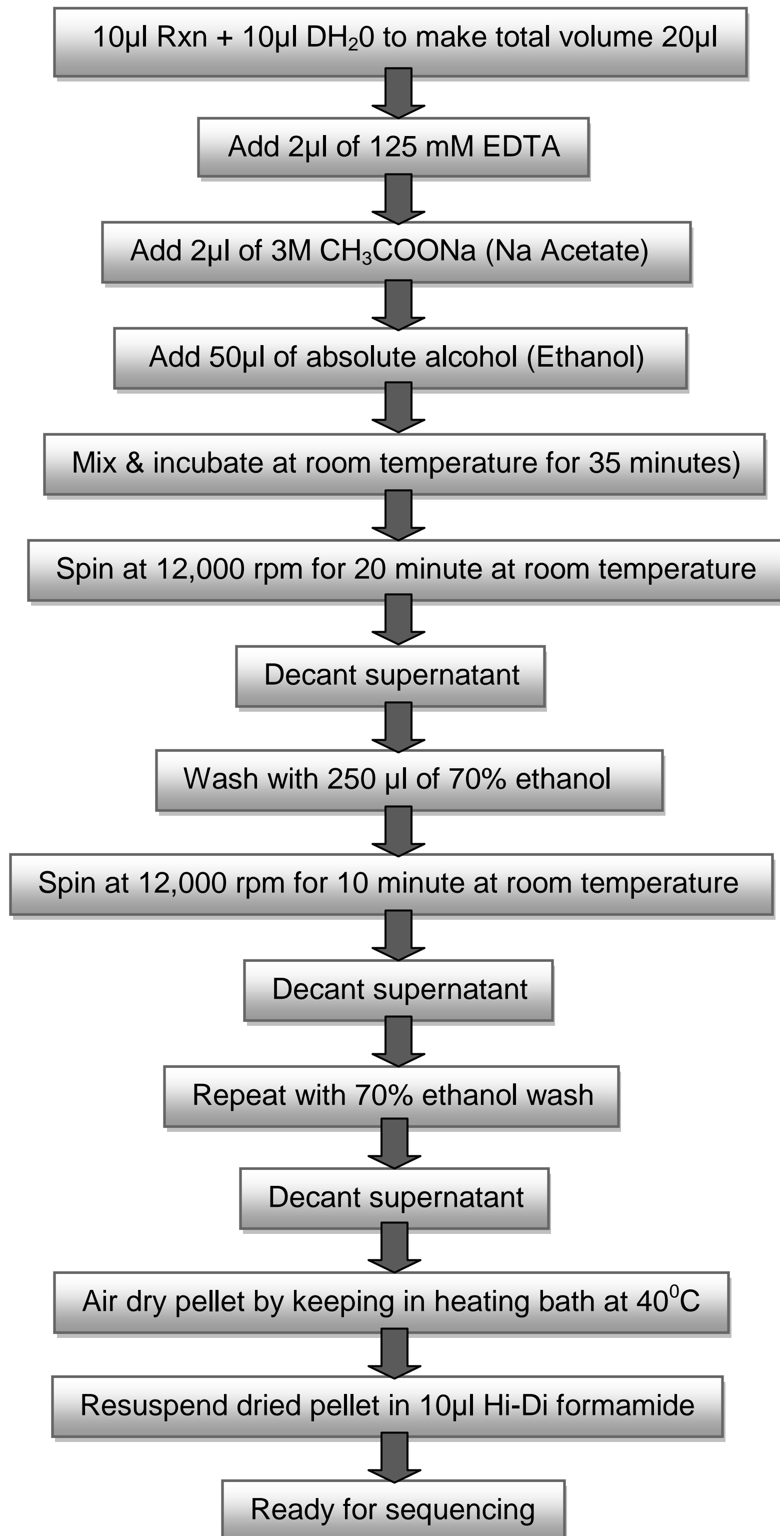


Figure 3.7- Steps for ethanol precipitation to make PCR products ready for sequencing

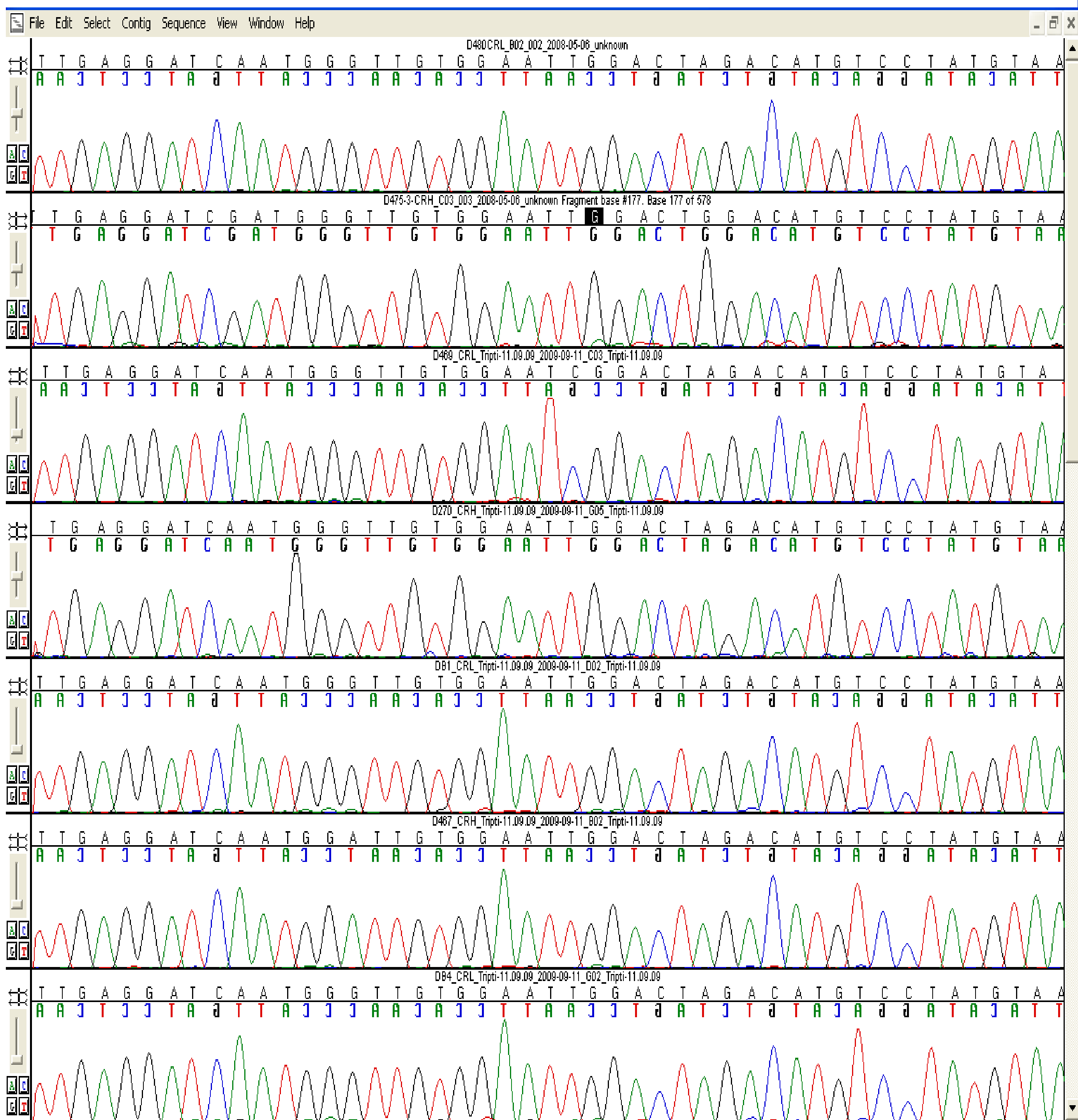


Figure 3.8- Aligned CR sequences of golden jackal, *C. aureus* using Sequencher 4.6

III.2.4 Statistical analysis of MtDNA sequences

The GenBank database (National Centre for Biotechnology Information, USA: NCBI Home page http://www.ncbi.nlm.nih.gov) was searched for similar sequences using BLAST search. Based on the search, reference mtDNA sequence described for jackals and other related canids species were retrieved from the database for each of the analyzed domain for phylogenetic comparison. The details of the reference sequences viz., GenBank accession number, source species, origin and their original contributors for mitochondrial CR and Cytb are given in Table 3.3 and 3.4 respectively.

Table 3.3- List of reference sequences for mitochondrial CR with their GenBank accession number and origin used in the present phylogenetic study

Sl. No.	Species	GenBank Accession No.	Origin	Reference
1	Grey wolf, *C. l. lupus*	FM201602.1	British Columbia	Violeta *et al*., 2009
2	Indian dog, *C. l. familiaris*	AY333727.1	Himanchal Pradesh, India	Sharma *et al*., 2004
3	Indian wolf, *C. l. pallipes*	AY333743.1	Gujarat, India	Sharma *et al*., 2004
4	Himalayan wolf, *C. l. chanco*	AY333738.1	Nepal	Sharma *et al*., 2004
5	Coyote, *C. latrans*	AC_008093.1	Nebraska, United States	Björnerfeldt *et al*., 2006
6	African wolf, *C. l. lupaster*	JQ088681.1	Algeria, Africa	Gaubert *et al*., 2012
7	Ethiopian wolf, *C. simensis*	HQ845261.1	Ethiopia, Africa	Rueness *et al*., 2011
8	Side-striped jackal, *C. adustus*	JQ088669.1	Guinea, Africa	Gaubert *et al*., 2012

Table 3.4- List of reference sequences for mitochondrial Cytb with their GenBank accession number and origin used in the present phylogenetic study

Sl. No.	Species	GenBank Accession No.	Origin	Reference
1	Grey wolf, *C. l. lupus*	NC009686	-	Arnason *et al.*, 2007
2	Indian wolf, *C. l. pallipes*	AY333749	India	Sharma *et al.*, 2004
3	Himalayan wolf, *C. l. chanco*	AY333748	India	Sharma *et al.*, 2004
4	Coyote, *C. latrans*	DQ840510	United States	Björnerfeldt *et al.*, 2006
5	African wolf, *C. l. lupaster*	HQ845258	Ethiopia, Africa	Rueness *et al.*, 2011
6	Ethiopian wolf, *C. simensis*	HQ845262	Ethiopia, Africa	Rueness *et al.*, 2011
7	Side-striped jackal, *C. adustus*	AF028136	Africa	Wayne *et al.*, 1997; Rueness *et al.*, 2011
8	Black-backed jackal, *C. mesomelas*	AF028142	Africa	Wayne *et al.*, 1997; Rueness *et al.*, 2011
9	Wild dog, *Lycaon pictus*	AF028147	Africa	Wayne *et al.*, 1997; Rueness *et al.*, 2011
10	Maned wolf, *Chrysocyon brachyurus*	AF028139	South America	Wayne *et al.*, 1997; Rueness *et al.*, 2011

III.2.4.1 Mitochondrial Control Region (CR) analysis

To infer the evolutionary history of golden jackal (*Canis aureus*), three standard methods named Minimum Evolution (ME), Maximum Likelihood (ML), and Neighbor-Joining (NJ) were used to construct the phylogenetic tree with mitochondrial Control Region, because no single approach has been shown to be always superior for finding the correct tree (Hillis & Huelsenbeck 1992; Hillis *et al.*, 1994; Hillis 1995; Hillis *et al.*, 1996). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-

Interchange (CNI) algorithm (Nei & Kumar 2000) at a search level of 0. The Neighbor-joining algorithm (Saitou & Nei 1987) was used to generate the initial tree. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 239 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

III.2.4.2 Mitochondrial Cytochrome b (Cytb) analysis

The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky & Nei 1992). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The bootstrap values were shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter=1). The ME tree was searched using the Close Neighbor- Interchange (CNI) algorithm (Nei & Kumar 2000) at a search level of 0. The Neighbor-Joining algorithm (Saitou & Nei 1987) was used to generate the initial tree. The analysis included 20 nucleotide sequences. Codon positions included 1st+2nd+3rd+Noncoding. All the ambiguous positions were removed for each sequence pair. There were a total of 280 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). Maned wolf, *Chrysocyon branchyurus* was used as out group to the rest of the species in constructing the phylogenetic tree.

III.3 Results

All the amplified PCR products gave good quantity of DNA resulted in cleaned sequences for both CR as well as Cytb. Sequences were aligned with known CR and Cytb sequences of a variety of jackal pieces from Genbank to confirm the sequence was mitochondrial.

III.3.1 Mitochondrial Control Region (CR) phylogeny

All the phylogenetic approaches (Minimum Evolution, Maximum Likelihood, and Neighbor Joining) used, produced remarkably similar topologies (with minor exception). All the species from the *Canis* form a monophyletic group called wolflike canids that also included the dhole or Asiatic wild dog (*Cuon alpinus*). This suggests that dhole should be included in the genus *Canis* (Vila *et al.*, 1999)(Figure 3.9; 3.10; 3.11). Wolves like grey wolf (*Canis l. lupus*), Indian peninsular wolf (*Canis l. pallipes*), and Himalayan wolf (*Canis l. chanco*), African wolf (*Canis. l. lupaster*), dog (*Canis l. familiaris*), coyote (*Canis latrans*), and Ethiopian wolf (*Canis simensis*) form a monophyletic group with golden jackal (*Canis aureus*) as the most likely sister taxon. This monophyly supports the grouping of grey wolf (*Canis l. lupus*) with dog (*Canis l. familiaris*), Indian peninsular wolf (*Canis l. pallipes*) with Himalayan wolf (*Canis l. chanco*), and Ethiopian wolf (*Canis simensis*) with golden jackal (*Canis aureus*). Additionally, *Canis l. lupaster*, recently designated as African wolf is grouped with coyote, *Canis latrans*. Interestingly, the constant grouping of Ethiopian wolf (*C. simensis*) with golden jackal (*C. aureus*) is showing in all the trees.

The only conflict between all tree topologies used is the position of side-striped jackal (*Canis adustus*) and dhole (*Cuon alpinus*). In Minimum Evolution (ME) tree (Figure 3.9), side-striped jackal is grouped with dhole while it is a sister group to rest of the canids in Maximum likelihood and Neighbor Joining trees (Figure 3.10 & Figure 3.11). Dhole is basal to all *Canis* in Maximum likelihood (ML) and Neighbor Joining (NJ) trees, while it

is replaced with side-striped jackal at a basal position in Minimum Evolution (ME) tree.

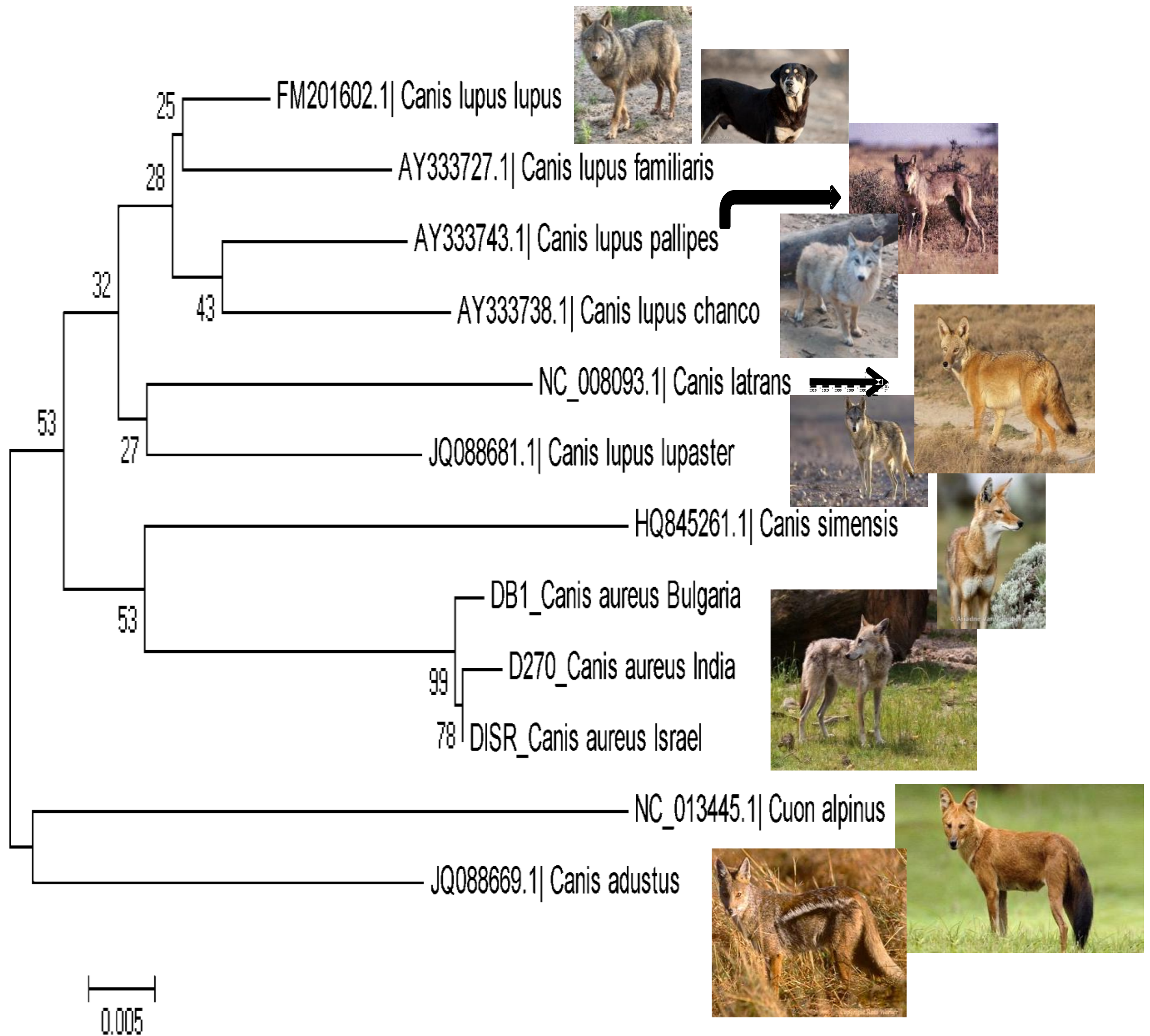


Figure 3.9- Minimum Evolution (ME) tree based on 239bp sequence of mitochondrial Control Region gene polymorphism showing phylogenetic relationship of golden jackal, *Canis aureus* with other canids from India and elsewhere. Bootstrap values from 1,000 replicates are given next to the branches. 0.005 is showing the rate of substitutions per site

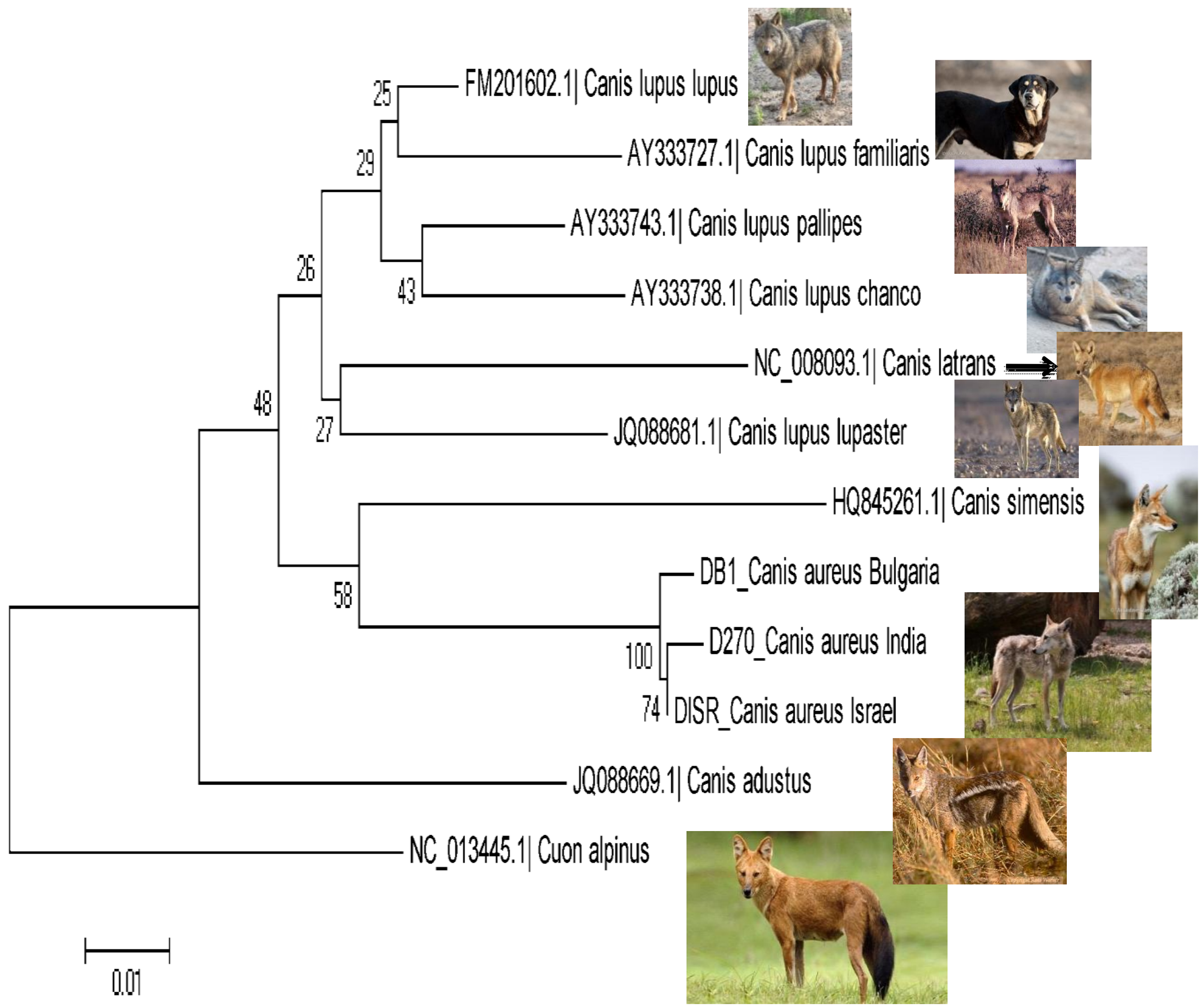


Figure 3.10- Maximum Likelihood (ML) tree based on 239bp sequence of mitochondrial Control Region gene polymorphism showing phylogenetic relationship of golden jackal, *Canis aureus* with other canids from India and elsewhere. Bootstrap values from 1,000 replicates are given next to the branches. 0.01 is showing the rate of substitutions per site

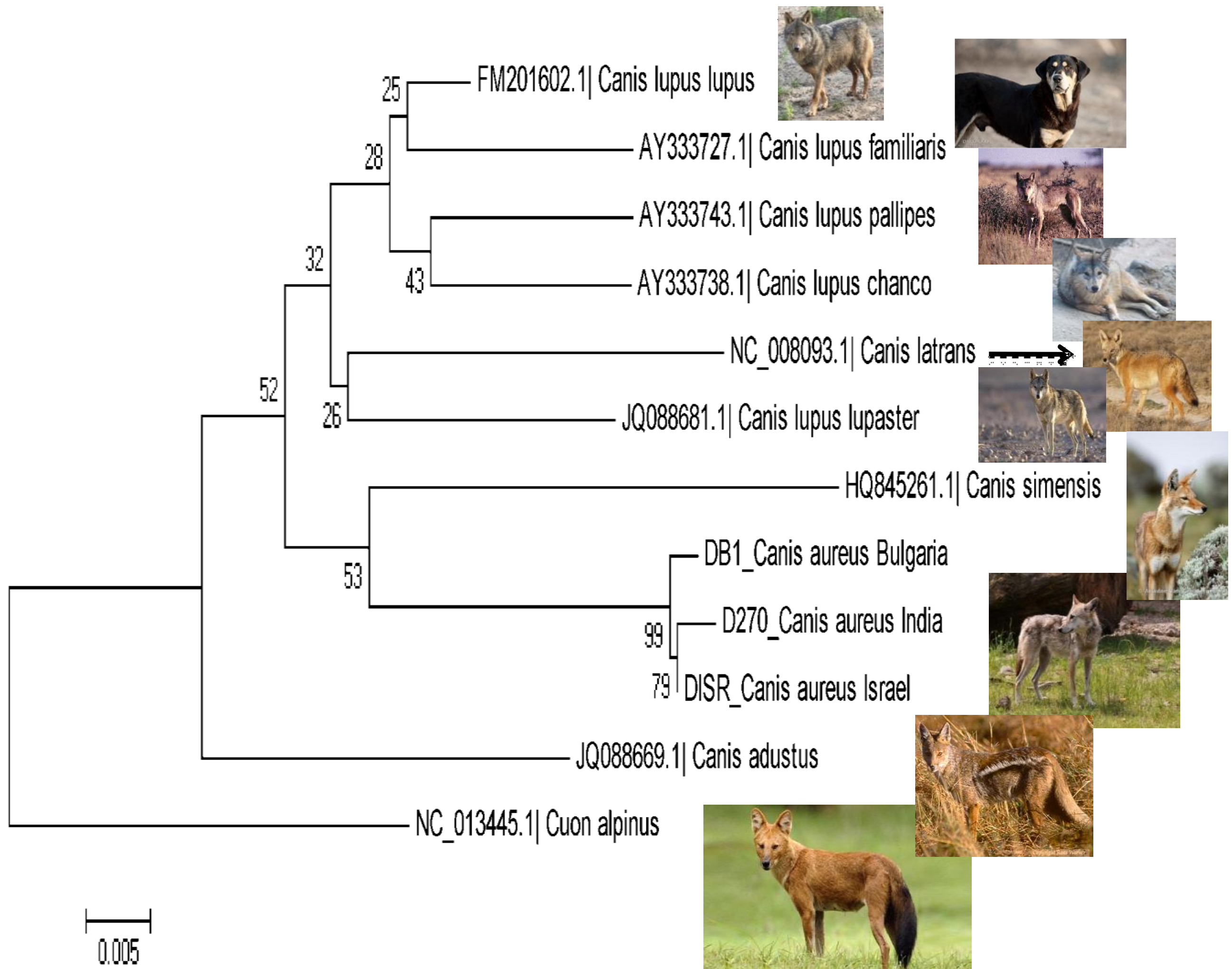


Figure 3.11- Neighbor Joining (NJ) tree based on 239bp sequence of mitochondrial Control Region gene polymorphism showing phylogenetic relationship of golden jackal, *Canis aureus* with other canids from India and elsewhere. Bootstrap values from 1,000 replicates are given next to the branches. 0.005 is showing the rate of substitutions per site

III.3.2 Mitochondrial Cytochrome b (Cytb) phylogeny

The Minimum Evolution (ME) tree also supports the monophyly of wolflike canids including dhole or Asiatic wild dog (*Cuon alpinus*) and African wild dog (*Lycaon pictus*). This also suggests that dhole and African wild dog should be included in genus *Canis* (Vila *et al*., 1999). Wolves (*C. l. lupus*, *C. l. pallipes*, *C. l. chanco*), dog (*C. l. familiaris*), coyote (*C. latrans*), Ethiopian wolf (*C. simensis*), and African wolf (*C. l. lupaster*) are sister taxa to golden jackal (*C. aureus*) and form a monophyletic group with it (Figure 3.12).

In this monophyletic clade all the golden jackal haplotypes are associated in one group with 98% bootstrap support. Coyote is grouped with Ethiopian wolf with 50% bootstrap support. Likewise, the African wolf is grouped with wolf-dog complex with 67% support. Side-striped and black-backed jackals are sister taxa but they don’t form a monophyletic group with golden jackal and Ethiopian wolf. Basal to all *Canis*, dhole and African wild dog are grouped together with 62% bootstrap support. The tree is rooted with maned wolf, *Chrysocyon brachyurus*, a South American canid.

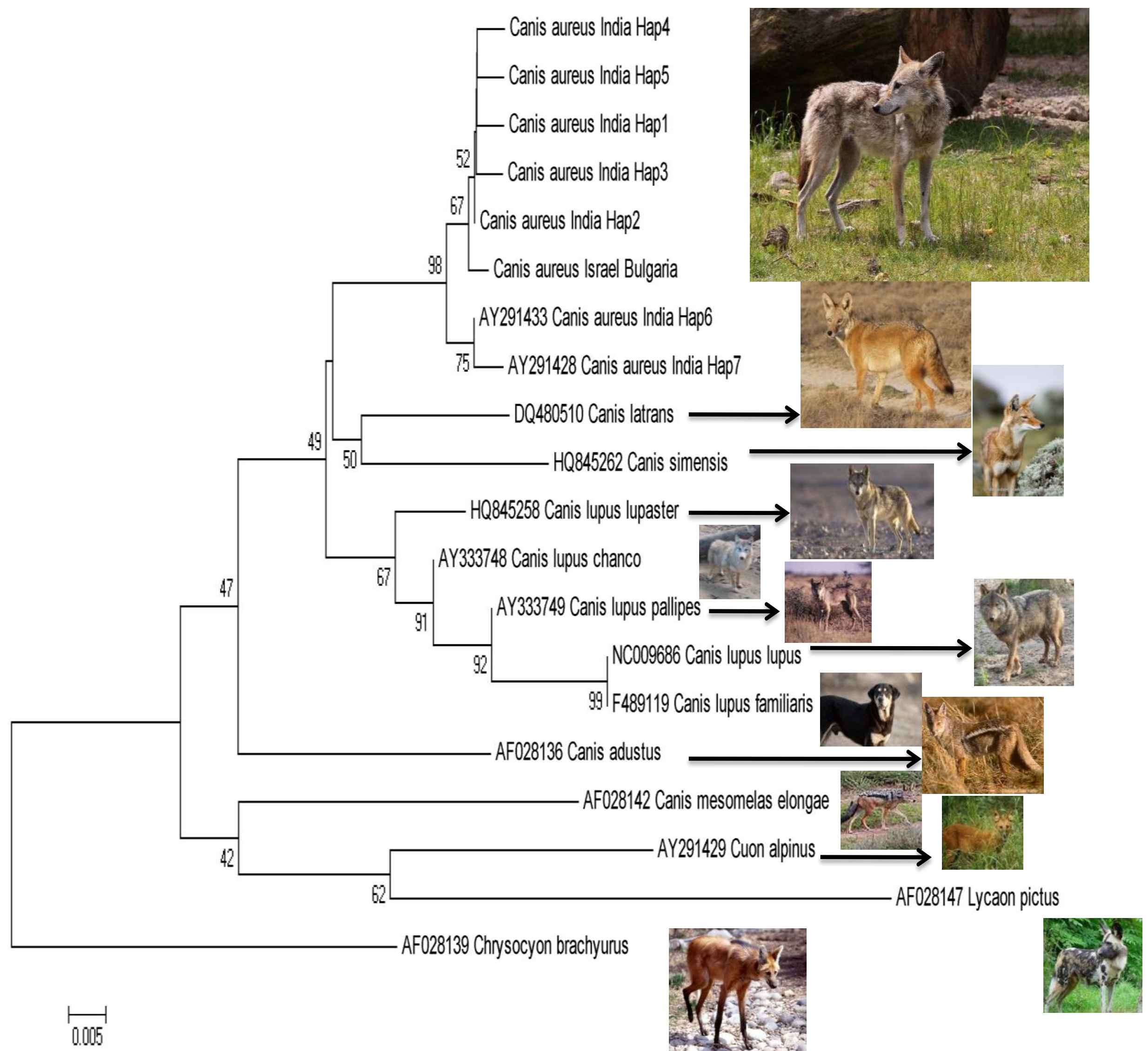


Figure 3.12- Minimum Evolution (ME) tree based on 280bp sequence of mitochondrial Cytochrome b (Cytb) gene polymorphism showing phylogenetic relationship of golden jackal, *Canis aureus* with other canids from India and elsewhere. Bootstrap values from 1,000 replicates are given next to the branches. 0.005 is showing the rate of substitutions per site

III.4 Discussion

Vertebrate mitochondrial DNA is composed of 13 protein coding genes (including Cytb), 22 tRNA genes, 2 rRNA genes, and a noncoding segment called CR. Among them Cytb and CR genes were used in the present study to understand the taxonomic affiliation of golden jackal, *Canis aureus* to other related jackals populations and to resolve their higher level phylogenetic status in the genus *Canis*. Being the most variable part of the mtDNA, CR provides better resolution when studying closely related species or conspecific population while the conserved nature of Cytb makes it a better marker for studying deeper phylogenetic relationships (Avise 2004). A large number of studies were reported showing remarkable divergence in mtDNA CR as well as Cytb sequences in different animal taxa (Liao *et al*., 2008; Li *et al*., 2007; Rhymer *et al*., 2001; Vila *et al*., 1999; Phillips 1994; Wayne *et al*., 1990; Reeb *et al*., 1990). Sequence variation in the mtDNA consists not only of substitutions but also of indels of various lengths and of variation in number of copies of tandem repeats leading mutation in the particular taxa (Sbisà *et al*., 1997). When compared, substitution rate was found higher in CR than Cytb in most of the species (Teacher *et al*., 2011; Bozarth *et al*., 2011; Salgueiro *et al*., 2006; Tang *et al*., 2006; Sharma *et al*., 2004). In some cases Cytb represented higher substitution than CR (Zheng *et al*., 2003; Taylor *et al*., 1993; Shedlock *et al*., 1992; Brown *et al*., 1986), while no or little variation was also reported in rainbow fish (Zhu *et al*., 1994) and wood turtle (Amato *et al*., 2008). In the present study both CR and Cytb yielded similar results with little variation.

The grouping of wolflike canids including wolves, dog, golden jackal, Ethiopian wolf, side-striped jackal, black-backed jackal, coyote, dhole, and African wild dog is well supported in both Control region as well as Cytb phylogenetic tree. The monophyly of all canids including maned wolf as out group supports the similar relationship among canids as were previously reported by Wayne *et al*., (1997); Vila *et al*., (1999); Zrzavy & Riconcova (2004); Rueness *et al*., (2011); and Gaubert *et al*., (2012) in their phylogenetic studies.

The internal cladistic structure of monophyletic clade shows three subclades in all the Control Region trees and they are *C. adustus*, Cuon and the other *Canis* species (Figure 3.9; 3.10; 3.11), while in Cytb tree four subclades named *C. adustus*, *C. mesomelas*, other *Canis* species, and Lycaon+Cuon subclades are present (Figure 3.9). Despite similarity of results, the position of *C. latrans* (coyote), *C. l. lupaster* (African wolf) and *C. adustus* (side-striped jackal) is found uncertain in both CR and Cytb trees. In CR tree *C. latrans* is grouped with *C. l. lupaster* while it is grouped with *C. simensis* (Ethiopian wolf) in Cytb tree. The grouping of *C. latrans* with *C. simensis* supports the finding of Wayne *et al*., (1997); Zrzavy & Riconcova (2004); and Rueness *et al*., (2011). Similarly, *C. l. lupaster* is grouped with *C. latrans* in CR tree while Cytb tree shows its grouping with wolf-dog complex.

Earlier, African wolf, *C. l. lupaster* was known a sub species of golden jackal, *C. aureus lupaster*. Studies done by Nassef (2003) and Rueness *et al*., 2011 found its similarity with wolves and placed it with wolves rather than golden jackal. Rueness *et al*., 2011 used only two sequences of Indian golden jackals in making the phylogenetic tree displaying the relationship among wild wolflike canids, while in the present study sequences of 55 golden jackals from India were used in phylogenetic analysis. The result showed that the golden jackal from India, Middle East, and Europe are not closely related to *C. l. lupaster* but are found close to wolf-dog complex. Thus, the findings of my study are in consonance with the results of Nassef (2003) and Rueness *et al*., 2011 (Figure 3.14). To answer the question of genetic identity, range expansion and coalescence history of African wolf Gaubert *et al*., (2012) analyzed mtDNA diversity with CR and Cytb primers of a series of African canids. Their results confirmed African wolf as a distinct lineage of wolf-dog complex which is closely related to Indian wolf lineages. Coalescence since late Pleistocene for African wolf was suggestive of an ancient wolf lineage in Africa. It can also be presumed that the presence of ancient lineages of wolves in India and Africa is suggestive of some Pleistocene

refuses within them where these lineages survived while they became extinct elsewhere. Alternatively it could be argued that the modern wolf-dog lineage evolved from a common ancestor of the African-Indian wolf lineage. Full genome sequence of these lineages would help resolve this understanding.

Further, *C. adustus* (side-striped jackal) grouped with *Cuon alpinus* (dhole) and placed at basal position in Minimum Evolution tree (ME) of Control Region while it is a separate clade in rest of the CR trees. Its position as a separate branch of *Canis* clade confirms the finding of most of the phylogenetic results of the wolflike canids (Vila *et al*., 1999; Zrzavy & Reconcova 2004; Rueness *et al*., 2011; and Gaubert *et al*., 2012), while its basal position in *Canis* clade supports the result of Maximum Parsimony tree (MP) based on 2,001 bp of canid mtDNA sequence showing the phylogeny of Family Canidae (Wayne *et al*., 1997) (Figure 3.13)

Furthermore, *Laycon pictus* (African wild dog) is placed at a basal position in *Canis* clade formed a monophyletic group with *Cuon alpinus* (dhole) in Cytb tree (Figure 3.12). This grouping also supports the results of previous studies showing the molecular phylogey of Family Canidae (Zrzavy & Reconcova 2004; Bardeleben *et al*., 2005; and Rueness *et al*., 2011), while the phylogenetic analysis done by Wayne *et al*., (1997); vila *et al*., (1999); and Gaubert *et al*., (2012) represents both Lycaon and Cuon as separate branch in *Canis* clade. The monophyly of Lycaon+Cuon clade is corroborated almost exclusively by the morphological evidence (Zrzavy & Reconcova 2004). The morphological synapomorphies of the Lycaon+Cuon clade include morphology of rostrum/palate part of skull, zigzag HunterSchreger bands of enamel, and similar hypercarnivorous teeth adaptations, high ratio of neocortex volume, a few developmental characteristics, and specialized hunting of large prey (Zrzavy & Reconcova 2004).

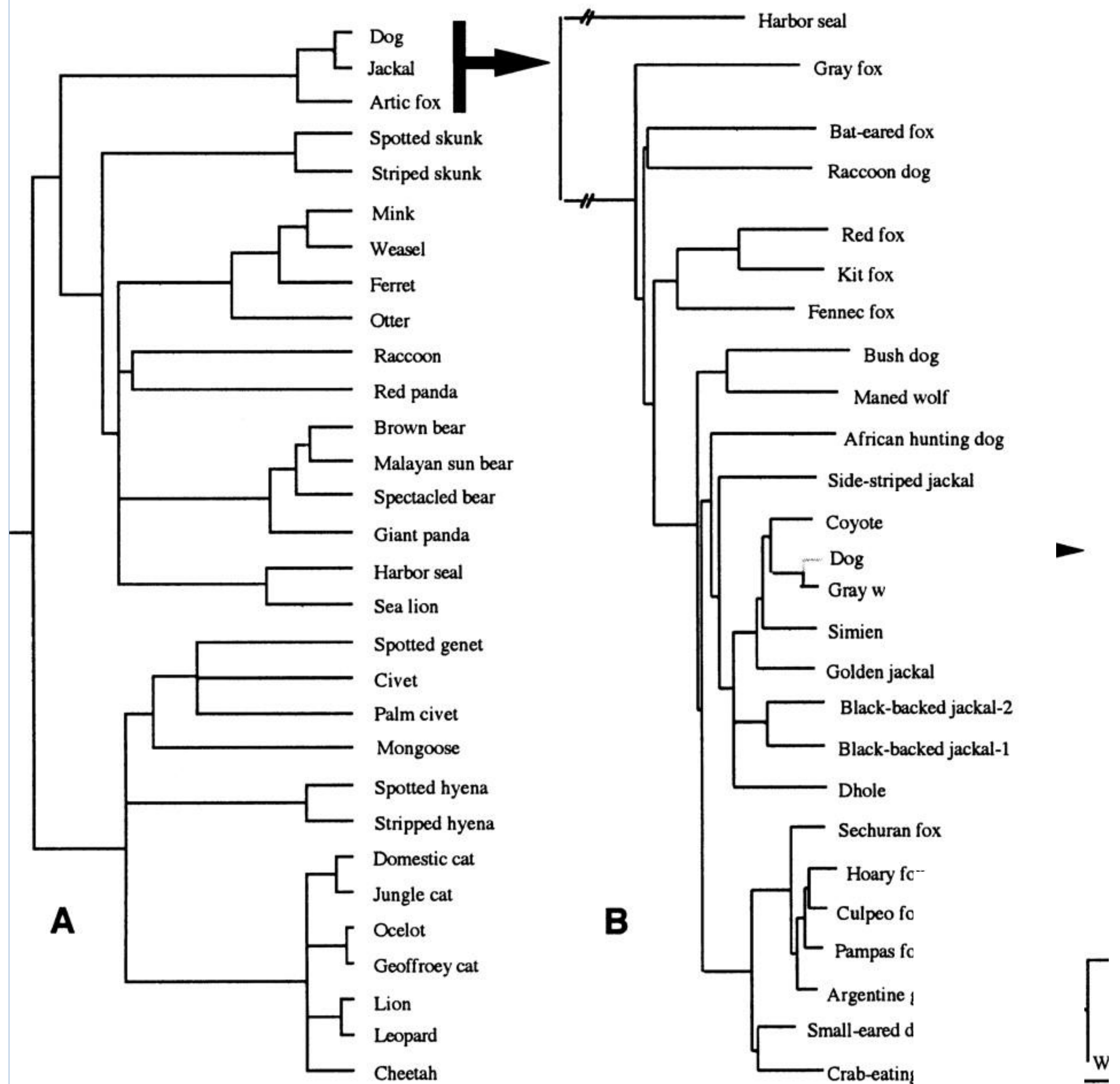


Figure 3.13- Neighbor Joining phylogenetic tree based on 2,001 bp from the mitochondrial DNA (Cytochrome b, Cytochrome c oxidase I and Cytochrome c oxidase II genes) showing the position of African wild dog, side-striped jackal and dhole as a separate branch of the tree (Wayne *et al.*, 1997) shown here in support of the present study. A is showing the relationships of carnivores, while B is showing the relationships of canids

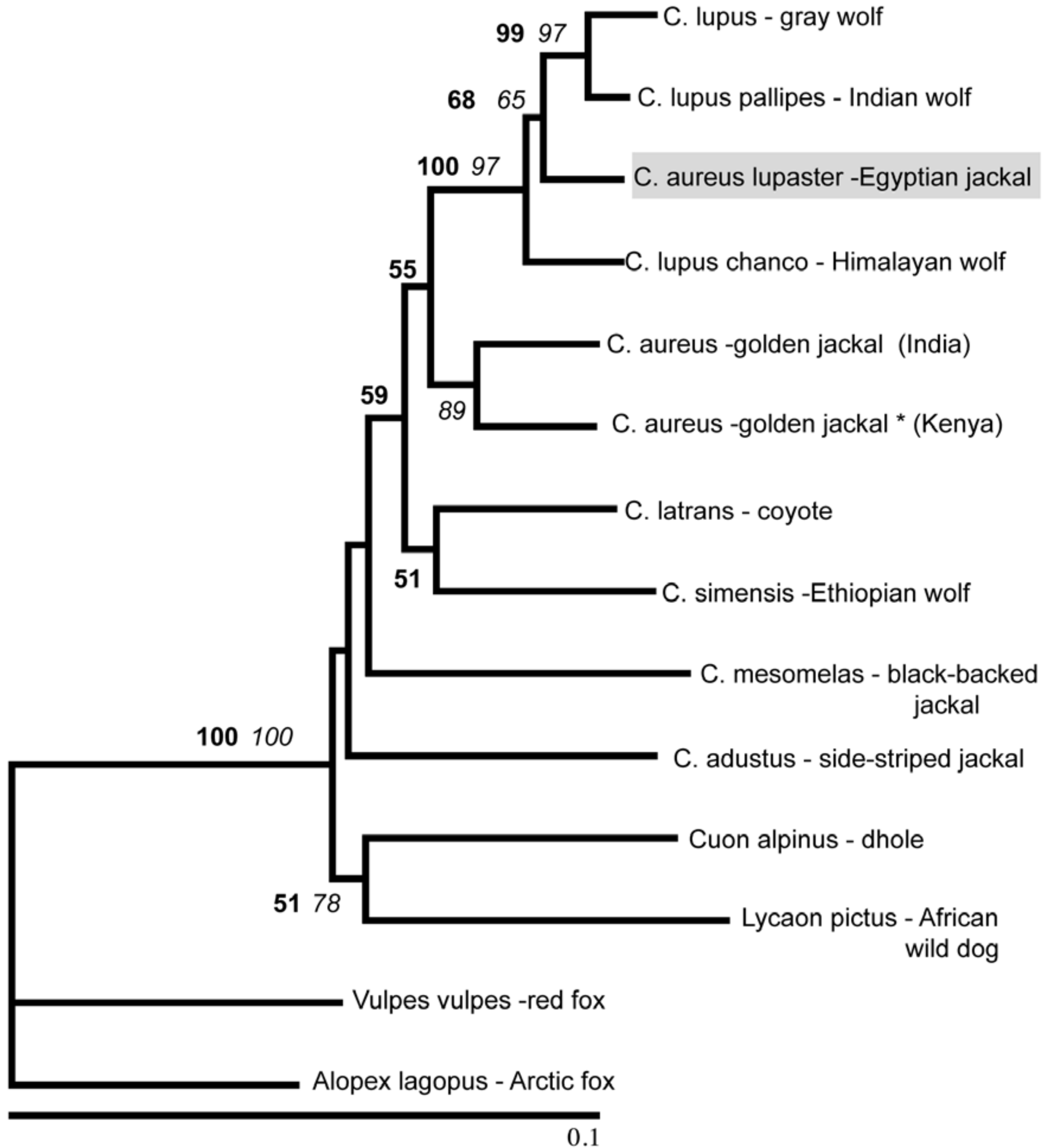


Figure 3.14- Maximum Likelihood phylogenetic tree based on 726 bp of Cytb genes displaying the relationship among all wild wolflike canids (Rueness *et al.*, 2011). Grouping of coyote with Ethiopian wolf; African wolf with other wolves; African wild dog with dhole and the position of side-striped jackal as a separate branch are in support of the present study

Status of Jackals

In both Control Region as well as Cytb analyses, the jackals are rendered paraphyletic by the inclusion of the wolf-dog complex within the clade consisting of golden jackal, Ethiopian wolf, and coyote. Additionally, the black-backed jackal and side-striped jackal lie outside the clade consisting of the other *Canis* species including wolf-dog complex, golden jackal, Ethiopian wolf, and coyote.

As far as golden jackal, *C. aureus* is concerned, its position is highly bootstrap supported (98-99%) in both the analysis. It is grouped with *C. simensis* (Ethiopian wolf) in CR trees while it represents a separate group in Cytb tree. It is also clear from the Cytb tree (Figure 3.9) that the haplotypes taken from GenBank (AY291433 & AY291428; Aggarwal *et al*., 2007) are not mixed with the haplotypes obtained from the present analysis, however, the haplotypes from Middle East and Europe are quite closer to them. The haplotype distribution pattern shows polytomy which indicates a lack of clear genetic structure between Indian, Israeli, Bulgarian and previously published haplotypes (See Chapter IV).

III.5 Conclusion

Molecular assessment of phylogenetic structure in concern with paleontological and geological information, are highly valuable for the reconstruction of evolutionary scenarios. Phylogeography has had significant influence upon historical ecology and population genetics by making it possible to assess the effects of historical events on the genetic composition and structure of modern population (Bernatchez & Wilson 1998). Determination of phylogeographic structure can also be important for the purpose of conservation management of the biological species. In agreement with the above statement, a phylogenetic study was conducted to understand the systematics of golden jackal (*C. aureus*) in context with other canids. All the steps for the genetic study (DNA extraction, PCR, sequencing) were carried out successfully. Both mitochondrial Control Region (CR) and Cytochrome b (Cytb) primers amplified the respective regions very well.

In sum, the grouping of wolflike canids is well supported in both CR as well as Cytb phylogenetic trees. In well bootstrap supported Cytb phylogenetic tree, within wolflike canids golden jackal, coyote, Ethiopian wolf, African wild dog, grey wolf, Indian Peninsular wolf, Himalayan wolf and Indian feral dogs form a monophyletic clade while side-striped jackal, black-backed jackal, dhole and African wild dog are separate clades.

The paraphyletic nature of jackals (golden jackal, side-striped jackal, black-backed jackal and Ethiopian wolf or simien jackal) is clear from the phylogenetic tree. Golden jackal is clearly associated with the larger wolflike canids, the wolves, coyote and simien jackal. It is also evident that golden jackal does not form monophyletic group with other two jackal species, the side-striped jackal and black-backed jackal. The findings are well supported by all the previous phylogenetic studies on wolflike canids.

PHYLOGEOGRAPHY OF GOLDEN JACKAL, *Canis aureus* ACROSS INDIA

IV.1 Introduction

Phylogeography is a field of study concerned with the principles and processes governing the geographical distributions of genealogical lineages, especially those at the intraspecific level (Avise 1998). The word 'phylogeography' itself was coined two decades ago (Avise *et al*., 1987a) and its use in the evolutionary genetics literature has grown exponentially since then. According to Ronquist (1997) this is an approach to historical biogeography on an ecological scale of time. As a sub discipline of biogeography, phylogeography emphasizes historical aspects of the contemporary spatial distributions of gene lineages (Avise 1996). The analysis and interpretation of lineage distributions usually requires input from molecular genetics, population genetics, phylogenetics, and demography. Thus, phylogeography is an integrative discipline.

Since phylogeography was first introduced, it has been used primarily to examine geographical structuring of gene lineage within single species. Typically, individuals are sampled from throughout the geographical range of a species, and a part of the mtDNA genome (or nuclear or cpDNA genomes) is characterized for each individual, either through restriction fragment analysis or direct sequencing. The resulting haplotypes are then used to infer a phylogeny, or gene tree, which reflects the evolutionary relationships of the individuals and population sampled. By combining the resulting gene trees, the geographical location from which each individual was sampled, one can elucidate the geographical distribution of major gene lineages (monophyletic clades) that comprise the gene tree, and also can examine phylogeographic patterns of mtDNA variation to evaluate the relative roles of the gene flow, bottlenecks, and historical or ecological barriers in effecting spatial patterns (Arbogast & Kenagy 2001).

Another refinement in the phylogeography with comparison of geographical patterns of genetic variation among multiple co-distributed (sympatric) species (Cracraft 1989) was called 'comparative phylogeography' (Avise 2000). This endeavor has strong parallels with historical biogeography (Wiley 1988; Riddle 1996; Zink 1996). In purest form, phylogeographic analyses deal with the spatial distributions within and among populations of alleles whose phylogenetic relationships are deduced. A phylogeny for spatially structured populations can be conceptualized as a statistical distribution of partially bundled allelic pathways of descent, each characterized by its own unique coalescent pattern (Maddison 1995; Avise & Wollenberg 1997). The many distinctions yet connections between notions of phylogeny at the levels of genes *vs* populations have made phylogeography a rich point of contact between the traditionally distinct field of population genetics and phylogenetic biology (Avise 1989a; Hey 1994).

Avise (1989a, Avise *et al*., 1987) has coined the term "intraspecific phylogeography" to describe the approach that 'taking advantage of the phylogenetic information contained in a DNA sequence, the genetic structure of a species can be clearly portrayed by superimposing a gene genealogy on a distribution map' and has emphasized the utility of animal mtDNA for analyses of population structure. Geographic surveys of mtDNA restriction site variation have revealed striking genetic structuring of populations in some animal species and absence of variation or homogeneity across vast distances in other taxa. For example, for afrotropical butterfly (Jong *et al*., 2011), Serbian jackals (Zachos *et al*., 2009), snowy owl (Marthinsen *et al*., 2009), red drum (Gold *et al*., 1999), greater amberjack (Gold & Richardson 1998), red-winged blackbirds (Ball *et al*., 1988), American eels (Avise 1986), crested newts (Wallis *et al*., 1989) and sea urchins (Palumbi *et al*., 1990), there is relatively little sequence divergence among the observed mtDNA haplotypes and no evidence of geographic distribution. In contrast, distinct phylogenetic assemblages (differing by 2-9% in mtDNA sequence) are geographically localized (often parapatrically distributed) in golden monkeys (Li *et al*., 2007), sandhill cranes (Rhymer *et al*., 2001),

spotted salamander (Phillips 1994), brown bear (Taberlet & Bouvet 1994), African black-backed jackals (Wayne *et al*., 1990), grasshopper mice (Riddle *et al*., 1990), American oysters (Reeb *et al*., 1990), desert tortoises (Lamb *et al*., 1989), field mice (Avise *et al*., 1983), and several species of fresh water fish (Bermingham & Avise 1986; Liao *et al*., 2008). The observed "phylogenetic discontinuities" often correspond to current barriers to gene flow or to historical barriers inferred from regional geology and paleoclimatic reconstruction. Rarely do species show large amounts of sequence divergence among mtDNA haplotypes in the absence of geographic structure.

Many studies have looked at the phylogeographic patterns of species, primarily using mitochondrial DNA sequence comparisons, and linked the patterns seen today to severe climatic changes in the past (Hewitt 2004). Pleistocene biogeographic events were likely a major influence in generating modern species diversity and determining community composition. The Pleistocene epoch was a time of dramatic oscillations in climate with an unprecedented cycle of global cooling about every 100,000 years that generated continental glaciations in boreal regions (Berger 1984). Climatic warming, with conditions more akin to those of the Holocene, periodically interrupted the cold glacial advances and profoundly influenced the evolutionary histories of organisms in the northern latitudes (Hewitt 1996, 2001). Among the family Canidae, a large number of studies have conducted to understand the consequences of geographical events (most likely Pleistocene glaciations) on the phylogenetic pattern of different canids.

The gray wolf, *Canis lupus,* is believed to be the most widely distributed terrestrial mammal, originally inhabiting major parts of the Northern hemisphere. From being omnipresent and abundant, it has assumed endangered species status in many countries. In most parts of Southern, Western and Northern Europe and Americas, major gray wolf populations are lost and their present distribution is highly fragmented (Mech 1970). In England and Japan, it has already become extinct while in many other parts it is on the verge of extinction

(Ellegren *et al.*, 1996). The wolves are now restricted to few large forested areas in Eastern Europe, a few isolated mountain ranges in the Mediterranean, mountains and semi-desert areas of middle-East and the wilderness areas of North America, Russia and China, with largest concentrations in Russia followed by Canada and Alaska. In Indian subcontinent, it is believed that the small numbers of wolves found in today's India are two different sub-species of *Canis lupus* which are represented by geographically isolated broadly non-overlapping (allopatric) populations. One of these wolf populations is found only in the upper Trans-Himalayan region of India across the two northernmost states of Himachal Pradesh and Jammu and Kashmir. This Himalayan wolf (HW) population, adapted to the cold environment, is considered to be representing the extant population (direct relatives) of the relatively better known Tibetan wolf, *Canis lupus chanco,* which is found distributed from eastern Kashmir into eastern Nepal and Tibet. On the other hand, a second wolf population is found throughout the arid/semi-arid plains of peninsular India called Indian peninsular wolf, *Canis lupus pallipes*.

During the Pleistocene (approximately 2.6 million to 12,000 years ago), Europe experienced cyclical glacial and interglacial periods, with the last glacial period ending approximately 10,000 years ago (Hewitt 2004). These fluctuations in climate had profound effects on species distributions, and during glacial periods temperate species in Europe are thought to have been forced south into warmer refugial areas, primarily in Iberia, Italy and the Balkans, although other smaller cryptic refugia have also been proposed (Hewitt 2000). Following the retreat of the glaciers towards the North, species were able to recolonise the new warmer and more habitable northern regions of Europe. These patterns of range contraction and expansion have shaped the genetic diversity in modern populations through a combination of genetic drift and gene flow. To address the question of genetic consequences of Pleistocene glaciations for European grey wolf, Pilot *et al.*, 2010 analyzed phylogenetic relationships and geographical distributions of mtDNA haplotypes for 947 contemporary European wolves. They also included sequences of ancient European wolves in the analysis. They found

that haplotypes representing two haplogroups, 1 and 2, overlap geographically, but substantially differ in frequency between populations from south-western and Eastern Europe. They also compared the haplotypes of grey wolves from its entire global range and concluded that both haplogroups are spread throughout Eurasia, while only haplogroup 1 occurs in contemporary North American wolves. All ancient wolf samples from Western Europe that dated from between 44,000 and 1,200 years B.P. belonged to haplogroup 2, suggesting the long-term predominance of this haplogroup in this region. Moreover, a comparison of current and past frequencies and distributions of the two haplogroups in Europe suggested that haplogroup 2 became outnumbered by haplogroup 1 during the last several thousand years.

Fossil and historical records indicate that gray foxes (*Urocyon cinereoargenteus*) were not present in the northeastern United States until well after the Pleistocene (ca. 900). To test the hypothesis that gray foxes experienced a post-Pleistocene range expansion Bozarth *et al.*, 2011 conducted a phylogeographic analysis of gray foxes from across the eastern United States. They sequenced a variable portion of the mitochondrial CR (411 base pairs) from 229 grey fox tissue samples from 15 states, representing the range of all 3 East Coast subspecies. Phylogeographic analyses indicated no clear pattern of genetic structuring of grey fox haplotypes across most of the eastern United States. However, when haplotype frequencies were subdivided into a northeastern and a southern region, they detected a strong signal of differentiation between the Northeast and the rest of the eastern United States. Indicators of molecular diversity and tests for demographic expansion confirmed this division and suggested a very recent expansion of gray foxes into the northeastern states. Thus, they summarized their results with the hypothesis that gray foxes 1st colonized the Northeast during a historical period of hemisphere-wide warming, which coincided with the range expansion of deciduous forest.

In Europe, golden jackals are found in the Caucasus, Greece, Bulgaria, Turkish Thrace, Romania and recently colonized in Serbia (Mitchell-Jones et al., 1999).

To understand the genetic characterization and to look for a sign of recent range expansion in Serbian population of golden jackal, Zachos *et al*., (2009) analyzed 121 golden jackals from Serbia with regard to genetic variability and differentiation as revealed by mitochondrial CR sequences and eight nuclear microsatellite loci. No variation in the mtDNA sequences and very low nuclear variability was found indicative of a strong founder effect in the recently established Serbian population.

Until now the Egyptian jackal (*Canis aureus lupuster*; Hepprich & Ehrenberg 1833) had been considered a rare sub-species of the golden jackal present in the highlands of North Africa, effectively expanding to taxon's range by at least 2,500 km to the Southeast. The evidences show that it is not a true jackal and considered to be a grey wolf. Although the similarity of the skull of certain North African jackals to that of the Indian wolf (*Canis lupus pallipes*) has already been noted by Thomas Huxley as early as 1880 (Huxley 1880). It also overlaps in size with the grey wolf (*Canis lupus*), being large and more long limbed than the holotype *C. aureus* and its cranial features differ from other golden jackals (Fergusen 1981). While investigating the relative relationship between Egyptian and Israeli jackals, Nassef (2003) found through phylogenetic analysis of the cytochrome b gene that the Egyptian jackal was more similar to the grey wolf. Very recent and strong evidence was found in the study of Rueness *et al*., 2011. They analyzed 2055bp of mtDNA from *Canis aureus lupaster* and investigated the similarity to golden jackal and grey wolf. Phylogenetic comparision based on 720bp of cytb gene with all wild wolf-like canids placed the *Canis aureus lupaster* within the grey wolf species complex, together with the holarctic wolf, the Indian wolf and the Himalayan wolf (Figure 4.2). They thus refer to *Canis aureus lupaster* as African wolf *Canis lupus lupaster*.

In the present chapter, I focus in reconstructing the phylogeographic patterns of mtDNA haplotypes recovered from jackals sampled throughout their range of distribution in India. In addition, the study will elucidate the levels of genetic

diversity of this species in India and will reveal if there are any signals of genetic structure between populations or biogeographic regions.

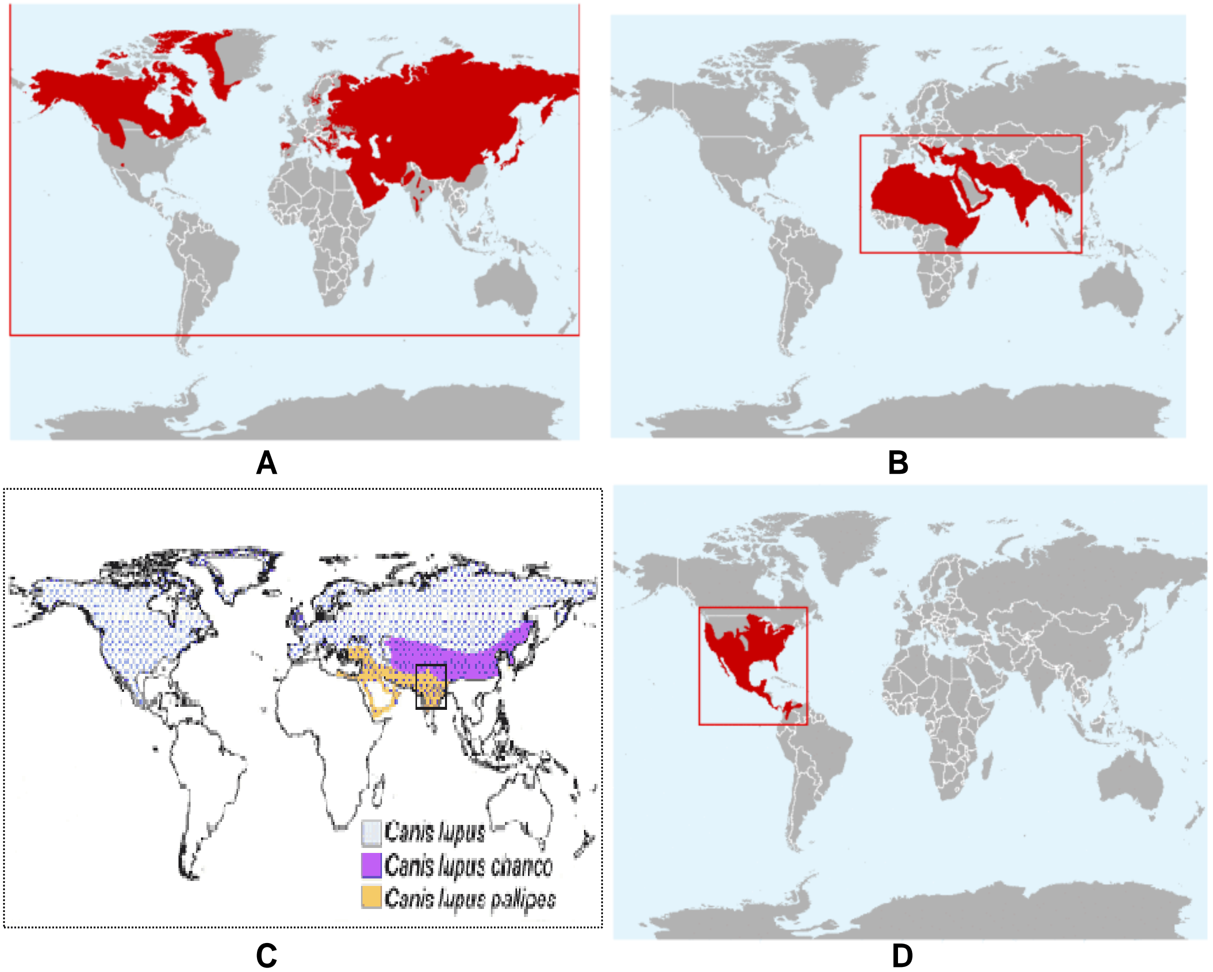


Figure 4.1- Worldwide map showing the geographical distribution of (A) grey wolf, *C. lupus* (B) golden jackal, *C. aureus* (C) wolf lineages, *C. lupus*., *C. l. pallipes*., *C. l. chanco* (Sharma *et al*., 2004), and (D) grey fox, *Urocyon cinereoargenteus*

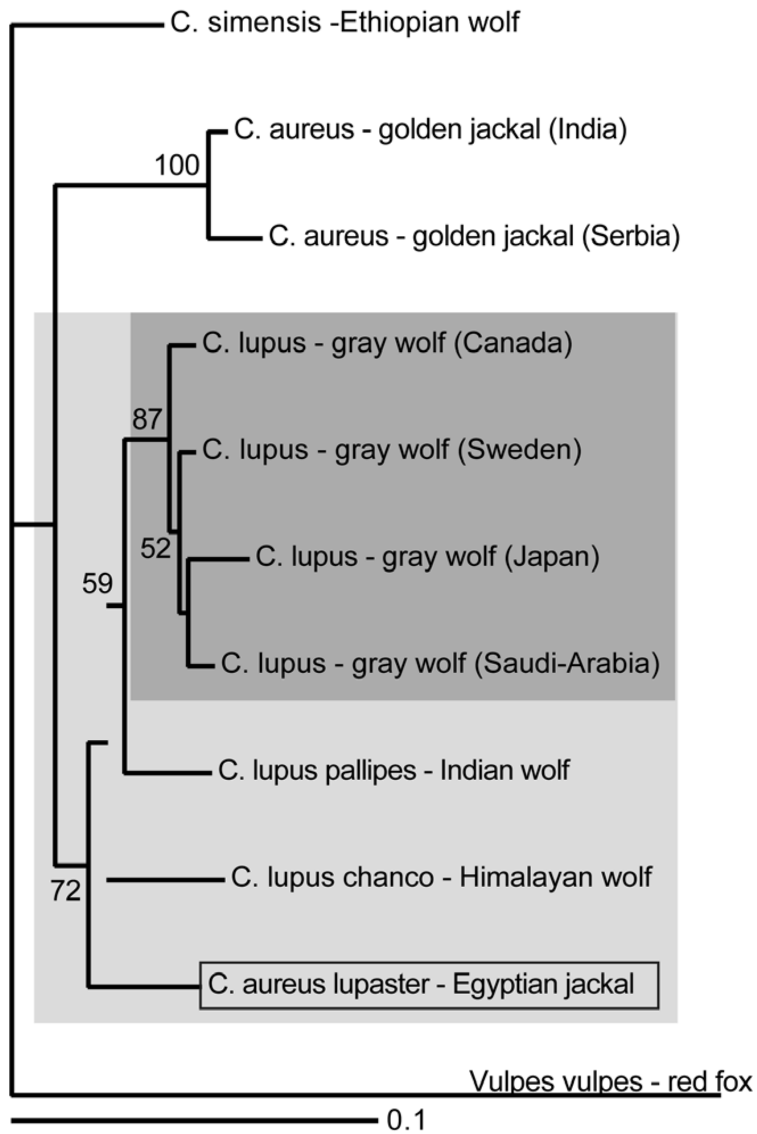


Figure 4.2- Neighbor Joining Phylogenetic tree based on 317 bp of the mtDNA D loop region showing the monophyletic grouping of Egyptian jackal, *C. a. lupaster* with grey wolf (*C. lupus*) species complex (shaded in light grey) (Rueness *et al.*, 2011)

IV.2 Materials and Methods

IV.2.1 Sampling

Fifty-five samples (blood, tissue, and hair) have been collected from a variety of locations covering most of the golden jackal's distribution across India. Samples were preserved in 95% Ethanol and stored at -20°C until DNA extraction.

Sampling distribution is shown in the map (Figure 4.3) where blue triangles are the sites from where samples were collected while the number of samples analyzed is detailed in Table 4.1.

Table 4.1- Description of sampling states and number of samples collected for the phylogenetic study of golden jackal, *Canis aureus*

Sl No.	No. of samples collected	Location
1	32	Gujarat
2	3	Rajasthan
3	5	Madhya Pradesh
4	8	Uttar Pradesh
5	2	Uttarakhand
6	2	Haryana
7	3	Karnataka
	Total= 55	

Figure 4.3- Map of India showing sampling locations to study the phylogeography of golden jackal, *Canis aureus*

IV.2.2 DNA Extraction

Methods for DNA extraction are detailed in **Chapter III**. Extraction was followed by amplification using Polymerase Chain Reaction (PCR) with universal primers available for mtDNA (Control Region and Cytochrome b) for canids.

IV.2.3 PCR amplification of mitochondrial DNA

Two regions of mitochondrial DNA: an approximately 440 base pair (bp) fragment of the CR using universal primers ThrL15926 and DL-H16340 as in Vilà *et al.* (1999); and a 412 bp fragment of the Cytb gene using a canid specific light primer Canid L1 (Paxinos *et al.* 1997) and a universal heavy primer H15149 (Kocher *et al.* 1989) were amplified. Protocol for PCR amplification and pre-sequencing steps are described in **Chapter III**.

IV.3 Statistical analysis of mitochondrial DNA sequences

Sequences were aligned with known CR and Cytb sequences of a variety of jackal sequences from Genbank. Forward and reverse sequencing was performed for each individual and consistent sequences for all individuals were edited and aligned using Sequencher 4.6 (Gene Codes Corporation, Inc., Ann Arbor, Michigan) and rechecked by eye.

IV.3.1 Phylogeographic pattern/Genetic structure

To assess the population structure by estimating the genealogy of haplotypes via phylogeographic reconstruction, a phylogenetic tree was constructed to show the relationship of golden jackal with other canids (wolf and dog) in India. The Neighbor-joining method (Saitou and Nei 1987) was used to construct the tree. The parameters used to construct the tree were same as described in **Chapter III**. The analysis involved 39 nucleotide sequences. All ambiguous positions were removed from each pair. Finally, there were a total of 289 positions in the final data set.

The evolutionary genetic distances between haplotypes were calculated under the Tamura-Nei distance method, which differentiate mutation rates for transition and transversions and between purines and pyrimidines (Tamura and Nei 1993). Heterogeneity of substitution rates per site was taken into account and the rate variation among sites was modeled with a gamma distribution where shape parameter was set to zero. All the evolutionary analysis was conducted in MEGA5 (Tamura *et al*., 2011).

Relationships between the observed haplotypes were assessed by constructing a median-joining network algorithm (Bandelt *et al*., 1999) in the program Network V.4.610 available at http://www.fluxux-engineering.com. Gaps were treated as evolutionary events, and data were analyzed with all characters weighed equally. The tolerance parameter (epsilon) was set to be zero. The distribution of haplotypes in the network was also found to be helpful in identifying the haplotypic origin of demographic expansion. In expanding populations ancestral haplotypes are expected to be at the origin of the expansion, with derived haplotypes more widespread (Templeton 1998). We inferred the relationships of ancestral and derived haplotypes by their position in the haplotype network, where interior haplotypes are more likely to be ancestral and haplotypes at the tips are likely to be derived (Templeton *et al*. 1992). Interior haplotypes will have multiple connections to the rest of the network, whereas more derived haplotypes will have only one connection to the network.

IV.3.2 Molecular diversity

To compare molecular diversity of golden jackals across India, program DnaSP V.5 (Liberado *et al*., 2009) was used to estimate haplotype diversity (h, Nei and Tajima 1983), the probability that two randomly chosen individuals have different haplotypes (Grant and Bowen 1998) and nucleotide diversity (Pi, Jukes and Cantor 1969; Nei 1987), the average pairwise nucleotide differences for

mitochondrial DNA (mtDNA) haplotypes varying from 0 for no divergence to >0.10 for deep divergences (Grant and Bowen 1998).

IV.3.3 Population demography

Three tests were performed to investigate expansion or contraction in the golden jackal populations: Tajima's D test of selective neutrality (Tajima's 1989), Fu's Fs statistics (Fu 1997), and the mismatch distribution (Roger and Harpending 1992). Tajima's D test and Fu's Fs statistics were used to test whether the data conformed to expectations of neutrality, considering that departure from neutrality could also be due to factors other than selection, such as a population bottleneck, a population expansion, or heterogeneity of the mutation rate. Tajima's D statistics was calculated in program DnaSP V5 (Liberado & Rozas 2009) and the significance of the D statistics was tested by simulating a distribution (1000 replicates) of D value under the null hypothesis of population stability. A population that remains static is expected to be close to zero. Fu's Fs statistics was calculated in program Arlequin V3.5 (Excoffier & Lischer 2010).

The Fs statistics use the observed mean number of nucleotide differences among samples to test whether a significant excess number of recent mutations or rare alleles exist compared to a random neutral sample. A significantly negative Fs value indicates recent demographic expansion. The significance of the Fs statistics is tested by generating random samples under the hypothesis of selective neutrality and population equilibrium based on a coalescent simulation algorithm adapted from Hudson (1990). The P-value is obtained from the proportion of random simulated Fs statistics less than or equal to the observed Fs statistics.

To further assess the demographic change indicated by mitochondrial DNA (mtDNA), a mismatch distribution (or the distribution of pairwise genetic differences) was constructed and the raggedness index of Harpending (1994)

was computed using DnaSP V.5 (Liberado & Rozas 2009). The shape of the observed distribution was tested against the expected distribution under population expansion using the sum of the squared deviations. The mismatch distribution is obtained by counting the number of nucleotide (or restriction) site differences between each pair of individuals, and assembling the resulting counts into a frequency histogram. The distribution is usually multimodal in samples drawn from population at demographic equilibrium, where as population which have gone through a recent demographic expansion are expected to be unimodal (Slatkin and Hudson 1991; Rogers and Harpending 1994; Rogers 1995; Rogers *et al.*, 1996).

The mismatch distribution is described by the formula $\theta_0 = 2N_0\mu$, $\theta_1 = 2N_1\mu$ and *Tau* = 2μt, where the initial effective population size N_0, suddenly changes in size to N_1 at *Tau* units of mutational time, calculated in terms of μ, the mutation rate per generation of the entire nucleotide sequence studied and t, the time of generations since expansion (coalescence time) (Schneider and Excoffier 1999). Further to calculate the time since coalescence t, parameter *Tau* was estimated from the mismatch distribution of pairwise differences among all Indian golden jackal haplotypes and the formula *Tau* = 2μt was rearranged to calculate t. A generation time of 1 year and a molecular clock estimate of 17.75% mutations per 10^6 generations were used as previously applied to CR sequences in red fox (Aubry *et al.*, 2009). Additionally, a mutation every 13,707 years in a 411bp sequence and $\mu=7.30\times10^{-5}$ bp^{-1} $year^{-1}$ was expected and used to calculate the coalescence time t (Bozarth *et al.*, 2011).

IV.4 Results

The primers ThrL15926 and DL-H16340 amplified 289bp sequence comprising part of the Control Region (CR) of mitochondrial DNA, while 280bp were successfully amplified with primers CanidL1 and H15149 for mitochondrial Cytochrome b (Cytb) region. The CR sequence contained 40 transitions, resulting in 19 haplotypes. No transversions were present in the sequences. Each CR haplotype differed from others by 1-8 variable sites (Table 4.2).

Among these haplotypes 15 were obtained from India, 1 each from Middle East (Israel) – Europe (Bulgaria), and 2 published haplotypes were from Central India (Aggarwal *et al*., 2007) and Europe (Randi *et al*., 2000). A BLAST search conducted against the sequences in GenBank revealed that all 15 haplotypes from India and 1 each from Middle East and Europe were new and were not previously described in GenBank. In the few instances where haplotypes were shared between localities, they were not always shared with golden jackals from the same geographic region, e.g., haplotype 1 is shared between Gujarat (GUJ), Uttar Pradesh (UP), and Rajasthan (RAJ); haplotype 3 and haplotype 6 between Gujarat (GUJ) and Madhya Pradesh (MP); haplotype 8 between Uttarakhand (UK) and Haryana (HAR), and haplotype 9 was shared between Gujarat (GUJ) and Uttarakhand (UK). The details of haplotype distribution in India are provided in Table 4.3 and shown in Figure 4.4.

The Cytb sequences contained 8 transitions and 2 transversions, resulting in 8 haplotypes that differed by 1-2 variable sites (Table 4.4). Among these, 7 haplotypes were obtained from India while 1 haplotype was shared between Israel and Bulgaria. Like CR all the haplotypes were Blasted in GenBank to find the similarity with submitted sequences. All the haplotypes were found to be novel and have not yet been reported for other golden jackals in GenBank. In addition, the haplotype found in Israel and Bulgaria was not found in India while in Bulgaria and Israel, the golden jackals share one haplotype in common.

In India, Cytb haplotypes are also shared between localities in few instances, *i.e.*, haplotype 1 is shared among Gujarat (GUJ), Rajasthan (RJ) and Uttarakhand (UK) while haplotype 2 is shared among Gujarat (GUJ), Madhya Pradesh (MP) and Uttar Pradesh (UP). The detail of haplotype distribution is shown in Table 4.5.

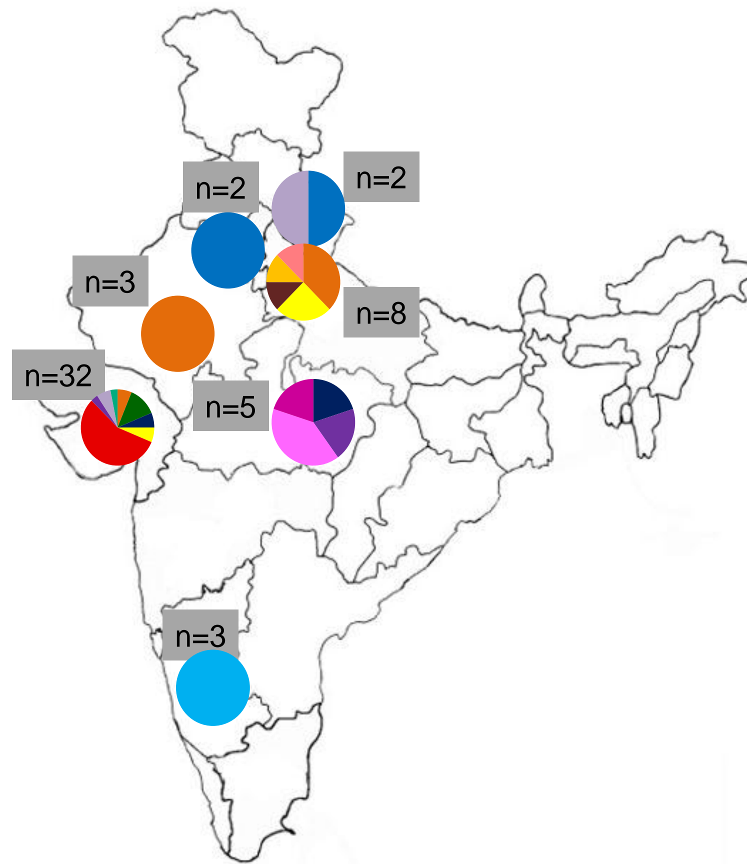


Figure 4.4- Map of golden jackal haplotype frequencies in India. Hap1 is orange, Hap2 is green, Hap3 is blue, Hap4 is yellow, Hap5 is red, Hap6 is purple, Hap7 is brown, Hap8 is royal blue, Hap9 is light purple, Hap10 is sky blue, Hap11 is pink, Hap12 is sea green, Hap13 is gold, Hap14 is peach, and Hap15 is Magenta. Sample sizes (=n) are shown for each population

Table 4.2- Nucleotide positions in golden jackal, *Canis aureus* with reference to the complete mtDNA genome of grey wolf, *Canis lupus lupus* (gb Accession ID – NC009686)

S. No	Hap ID	15,485	15,489	15,494	15,498	15,509	15,510	15,521	15,586	15,611	15,614	15,619	15,621	15,630	15,634	15,635	15,637	15,640	15,655	15,712	Locality
1	CauInd1	A	C	G	T	C	T	C	A	G	T	A	T	C	T	T	A	C	**A**	C	India
2	CauInd2	A	C	G	**C**	C	T	C	A	G	T	A	T	C	T	T	A	C	G	C	India
3	CauInd3	A	C	G	**C**	C	T	C	A	G	T	A	T	C	T	T	A	C	G	C	India
4	CauInd4	A	C	G	T	C	T	C	A	G	T	A	T	C	T	T	A	C	G	C	India – W & N
5	CauInd5	A	C	G	T	C	**C**	C	A	G	T	A	T	C	T	T	A	C	G	**T**	India – W
6	CauInd6	A	C	G	**C**	C	T	**T**	A	G	T	A	T	C	T	T	A	C	G	C	India – W
7	CauInd7	A	C	**A**	T	C	T	C	**G**	G	T	A	T	C	T	T	A	C	**A**	**T**	India – N
8	CauInd8	A	C	G	T	C	T	C	A	G	T	A	T	C	T	T	A	C	**A**	**T**	India – N
9	CauInd9	A	C	G	T	C	T	C	A	G	T	A	T	C	T	T	A	C	G	**T**	India – W & N
10	CauInd10	A	C	G	T	C	T	C	A	G	**C**	A	T	C	T	**C**	A	C	G	C	India – S
11	CauInd11	A	C	G	T	C	T	C	**G**	**A**	T	A	T	C	T	T	A	C	G	C	India – C
12	CauInd12	A	**T**	G	**C**	**T**	T	C	A	G	T	A	T	C	T	T	A	C	G	C	India – W
13	CauInd13	A	C	G	T	C	T	C	A	G	T	A	T	**T**	T	T	A	C	**A**	C	India – N
14	CauInd14	A	C	G	T	C	T	C	A	G	T	**G**	T	C	T	T	A	C	G	C	India – N
15	CauInd15	**G**	C	G	T	C	T	C	A	G	T	A	T	C	T	T	A	**T**	**A**	**T**	India – C
16	CauInd16	A	C	G	T	C	T	**T**	A	G	T	A	T	C	**C**	T	**G**	C	**A**	C	India – C
17	CauIsr1	A	C	G	T	C	T	C	**G**	G	T	A	**C**	C	T	T	A	C	**A**	**T**	Israel
18	CauEur1	A	C	G	T	C	T	**T**	**G**	G	T	A	T	C	T	T	A	C	G	**T**	Bulgaria & Serbia
19	CauEur2	A	C	G	T	C	T	C	**G**	G	T	A	T	C	T	T	A	C	G	**T**	Bulgaria

Table 4.3- Details of the states and corresponding haplotypes of Control region (CR) of mitochondrial DNA of golden jackal, *Canis aureus* in India including GenBank

Sl. No.	Haplotype	State to which haplotype belongs	Number of individuals
1	CauInd1	Uttar Pradesh (UP), Rajasthan (RAJ),	8
2	CauInd2	Gujarat (GUJ)	4
3	CauInd3	Gujarat (GUJ), Madhya Pradesh (MP)	3
4	CauInd4	Gujarat (GUJ), Uttar Pradesh (UP)	4
5	CauInd5	Gujarat (GUJ)	18
6	CauInd6	Gujarat (GUJ), Madhya Pradesh (MP)	2
7	CauInd7	Uttar Pradesh (UP)	1
8	CauInd8	Uttarakhand (UK), Haryana (HAR),	3
9	CauInd9	Uttarakhand (UK), Gujarat (GUJ)	3
10	CauInd10	Karnataka (KAR)	3
11	CauInd11	Madhya Pradesh (MP)	2
12	CauInd12	Gujarat (GUJ)	1
13	CauInd13	Uttar Pradesh (UP)	1
14	CauInd14	Madhya Pradesh (MP)	1
15	CauInd15	Uttar Pradesh (UP)	1
16	CauInd16	Hyderabad (HYD) *	

* Haplotypes submitted in GenBank (Aggarwal *et al.*, 2007)

Table 4.4- Nucleotide positions in Cytb haplotypes of golden jackal, *Canis aureus* with reference to grey wolf, *Canis lupus lupus* mitochondrial DNA (gb NC009686)

Haplotype ID	14,319	14,334	14,352	14,360	14,362	14,530	14,551	14,571	Reference	Locality
Ind_cytb1	C	T	T	G	C	G	C	**A**	This study	India
Ind_cytb2	C	T	T	G	C	G	C	G	This study	India
Ind_cytb3	C	T	**C**	G	C	G	C	G	This study	India
Ind_cytb4	C	T	T	G	C	G	**A**	G	This study	India
Ind_cytb5	C	T	T	G	C	**A**	C	G	This study	India
Ind_cytb6	**T**	T	T	**A**	C	G	C	G	Aggarwal *et al.*, 2007	India
Ind_cytb7	**T**	T	T	**A**	**G**	G	C	G	Aggarwal *et al.*, 2007	India
Isr_Blg	C	**C**	T	G	C	G	C	G	This study	Israel Bulgaria

Table 4.5- Details of the states and corresponding haplotypes of Cytochrome b (Cytb) region of mitochondrial DNA of golden jackal, *Canis aureus* in India

Sl. No.	Haplotype ID	State to which haplotype belongs
1	Ind_cytb1	Uttar Pradesh (UP), Rajasthan (RAJ), Haryana (HAR), Uttarakhand (UK)
2	Ind_cytb2	Gujarat (GUJ), Madhya Pradesh (MP), Uttar Pradesh (UP)
3	Ind_cytb3	Gujarat (GUJ)
4	Ind_cytb4	Karnataka (KAR)
5	Ind_cytb5	Uttarakhand (UK)
6	Ind_cytb6	Hyderabad (HYD) *
7	Ind_cytb7	Hyderabad (HYD) *

* Haplotypes submitted in GenBank (Aggarwal *et al.*, 2007)

IV.4.1 Phylogeographic pattern/Genetic structure

The Neighbor Joining (NJ) tree of Indian canids is shown in Figure 4.5. The tree contains four clades, each with bootstrap support greater than 90%. Indian feral dogs (*Canis lupus familiaris*) are in clade A. Clade B includes the Indian peninsular wolf (*Canis lupus pallipes*) which forms a well-supported sister clade to it (Sharma *et al*., 2004). Clade C consists of Himalayan wolf (*Canis lupus chanco*). Golden jackals (*Canis aureus*) are found in a well-supported (99%) clade D that is sister to the wolf-dog clade. The NJ tree does not resolve the golden jackal mtDNA haplotypes: the majority of branches lacked substantial bootstrap support. Furthermore, it indicates an absence of a clear geographical pattern in the distribution of golden jackal haplotypes.

The overall mean distance between all the canids was found to be 0.046 (Table 4.6). Estimates of average evolutionary divergence over sequence pairs within groups revealed that golden jackals (*C. aureus*) and Himalayan wolves (*C. l. chanco*) had an intermediate value (0.009) compared to Indian wolves (*C. l. pallipes*) (0.006) which is the lowest while the Indian feral dogs (*C. l. familiaris*) shared the highest value of 0.012 (Table 4.6). These values were estimated as the proportion of base substitutions per site averaging across all sequence pairs within each group.

The median-joining network tree depicted a tightly clustered haplotype network with shallow star-like radiation in both CR and Cytb regions. For CR, It was mostly composed of short branches, with individual haplotypes differing from one another by a single base substitution, or, by 2-3 substitutions in some cases (Figure 4.6). Haplotype Ind4 appeared to be the most interior in the network with six connections to other haplotypes. Assuming that ancestral haplotypes are internal and derived haplotypes are peripheral (Templeton *et al*. 1992), Ind4 is ancestral. The lineage interpretation was not clear because of conflicting homoplasy pattern across some haplotypes.

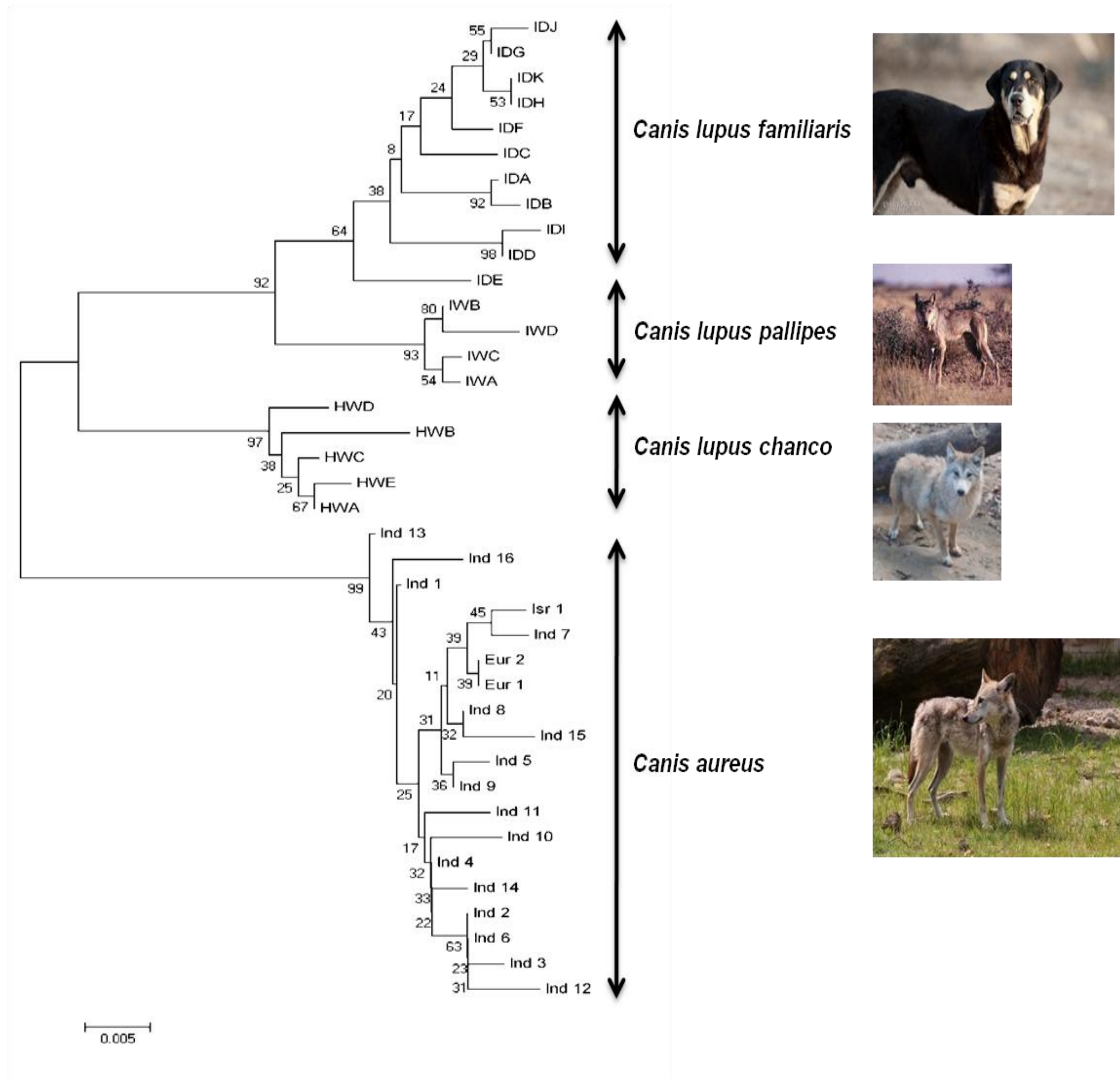


Figure 4.5- Neighbor Joining (NJ) tree constructed with mitochondrial Control Region sequences of canids in India. This includes sequences from Indian peninsular wolf, *C. l. pallipes* (n=45), Himalayan wolf, *C. l. chanco* (n=23), Indian feral dog, *C. l. familiaris* (n=24), and Indian golden jackal, *C. aureus* (n=55). Two golden jackals from Israel and five from Bulgaria were also included in the analysis. Bootstrap values from 1,000 replicates are given next to the branches. 0.005 is showing the rate of substitutions per site

Likewise, for Cytb all the haplotypes differ by one substitution except Ind2 and Ind6 where the substitution difference is of 2 (Figure 4.7). Haplotype Ind2 is found ancestral with six connections to other haplotypes. However, the tight clustering and shallow divergence seen in the golden jackal (*C. aureus*) haplotype network is indicative of a population that has recently undergone demographic expansion.

Table 4.6- mtDNA Control Region (CR) genetic distances across golden jackals and other canids

A) Overall mean distance between all groups- 0.046

B) Within group mean distances-

Group	Mean distance
Indian peninsular wolf, *C. l. pallipes*	0.006
Indian feral dog, *C. l. familiaris*	0.012
Himalayan wolf, *C. l. chanco*	0.009
Golden jackal, *C. aureus*	0.009

C) Mean distances between groups-

Group	**Indian peninsular wolf**	**Indian feral dog**	**Himalayan wolf**	**Golden jackal**
Indian peninsular wolf	—			
Indian feral dog	0.034	—		
Himalayan wolf	0.045	0.052	—	
Golden jackal	0.069	0.071	0.059	—

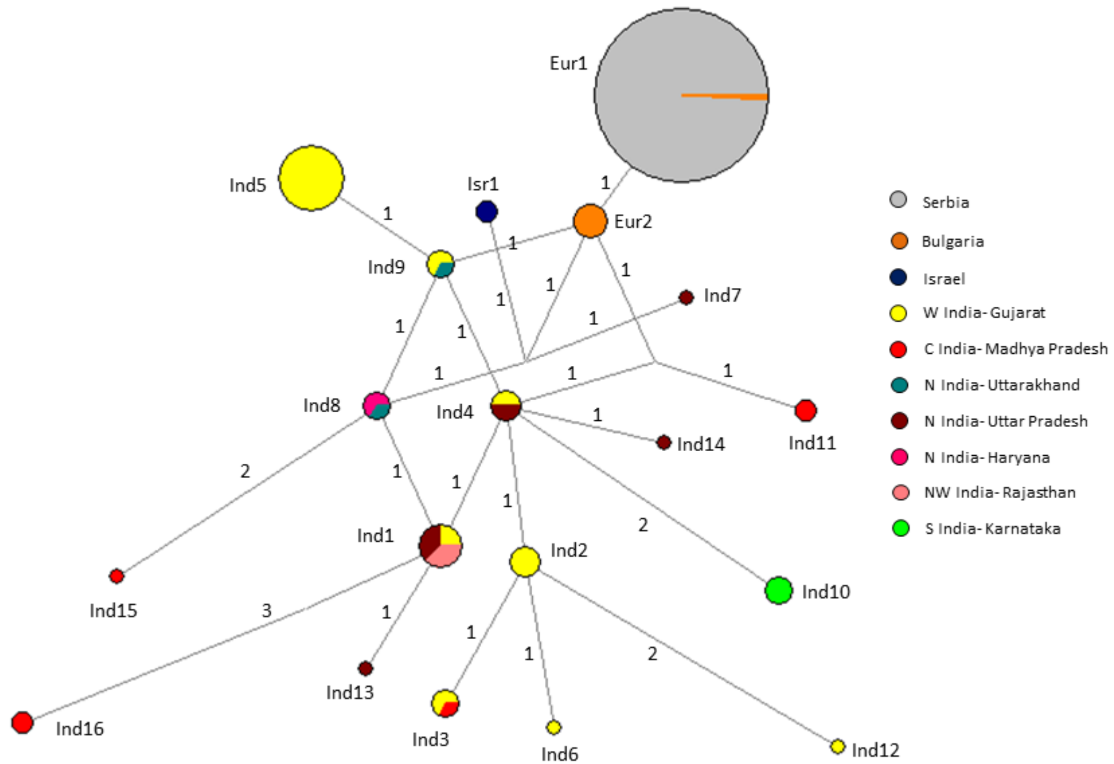


Figure 4.6- mtDNA Control Region Median joining haplotype network for golden Jackal, *Canis aureus* based on 289 base pairs (bp) showing the number of base substitutions between haplotypes on each branch. Circle sizes are proportional to the number of individuals with that haplotype. A total of 139 golden jackal individuals were included in the analysis

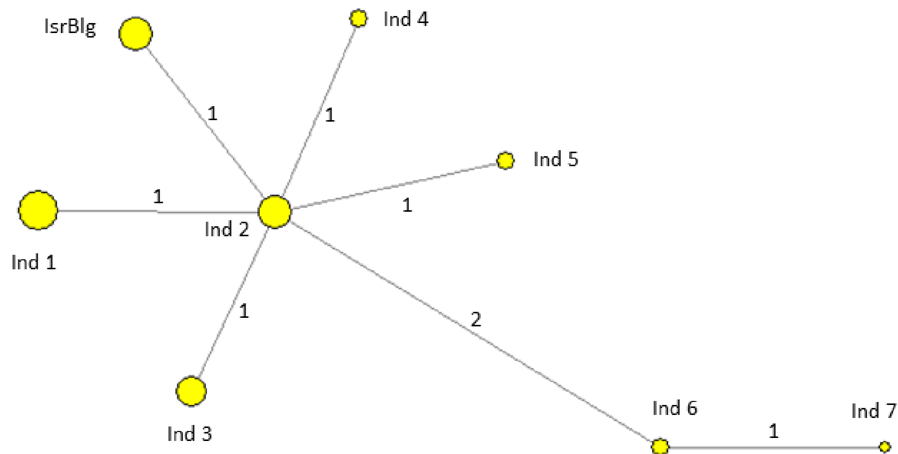


Figure 4.7- mtDNA cytb Median joining haplotype network for golden Jackal, *Canis aureus* based on 280 base pairs (bp) showing star-like radiation and polytomy of golden jackal haplotypes. Branch values depict base substation events. Circle sizes are proportional to the number of individuals represented. A total of 39 golden jackal individuals were included in the analysis

IV.4.2 Molecular diversity

Among all the *Canis* lineages in the phylogenetic tree, Indian feral dog (*C. l. familiaris*) showed the highest nucleotide as well as haplotype diversity (0.0147±0.0016; 0.899±0.037, respectively) followed by golden jackal (*C. aureus*) (0.0091±0.0007; 0.866±0.034, respectively) Himalayan wolf (*C. l. chanco*) (0.0042±0.0017; 0.533±0.142, respectively), and Indian peninsular wolf (*C. l. pallipes*) (0.0021±0.0004; 0.493±0.066, respectively) had the lowest genetic diversity estimates (Table 4.7).

Table 4.7- Mitochondrial Control Region (CR) molecular diversity table in golden jackal, *Canis aureus* and other canids in India

Species	Sample size	No of haplotypes	No of polymorphic (segregating) sites	Haplotype diversity (h)	hSD	Nucleotide diversity (Pi)	Pi SD	Av. no. of pairwise diff, k	Observed variance of k	Observed CV of k	Raggedness statistic , r	Mean Absolute Error, MAE
Golden Jackal	56	16	19	0.866	0.034	0.0091	0.00074	2.618	2.5079	0.6077	0.0523	0.7001
Indian Wolf	45	4	4	0.493	0.066	0.0021	0.00043	0.596	0.5201	1.2168	0.1582	0.3805
Himalayan Wolf	16	5	8	0.533	0.142	0.0042	0.00167	1.2	2.279	1.2777	0.1133	0.1339
Indian feral Dog	24	11	17	0.899	0.037	0.01465	0.00152	4.996	10.8691	0.6667	0.0415	0.6289

IV.4.3 Population Demography

Both Tajima's D statistics (Tajima's 1989) and Fu's Fs statistics (Fu 1997) showed negative values for golden jackal, Indian peninsular wolf and Himalayan wolf, however these values were significant only for Himalayan wolf. These values were found positive for Indian feral dogs (Table 4.8). Negative and significant value of Tajima's D statistics and Fu's Fs statistics rejects population stasis/neutrality, indicating an excess of recent mutations. Such populations support the hypothesis of population growth (Rogers *et al*., 1996) resulting in population expansion. Thus, golden jackal, Indian peninsular wolf and Himalayan wolf show the signal of demographic expansion.

The mismatch distribution of pairwise differences among all the states for jackals produced a right-skewed unimodal peak that is characteristic of demographic population expansion (Figure 4.8). The low value of raggedness index (r=0.0523) for all the sequences analyzed also indicates a smooth distribution and thus suggests a population expansion (Table 4.7) (Harpending 1994).

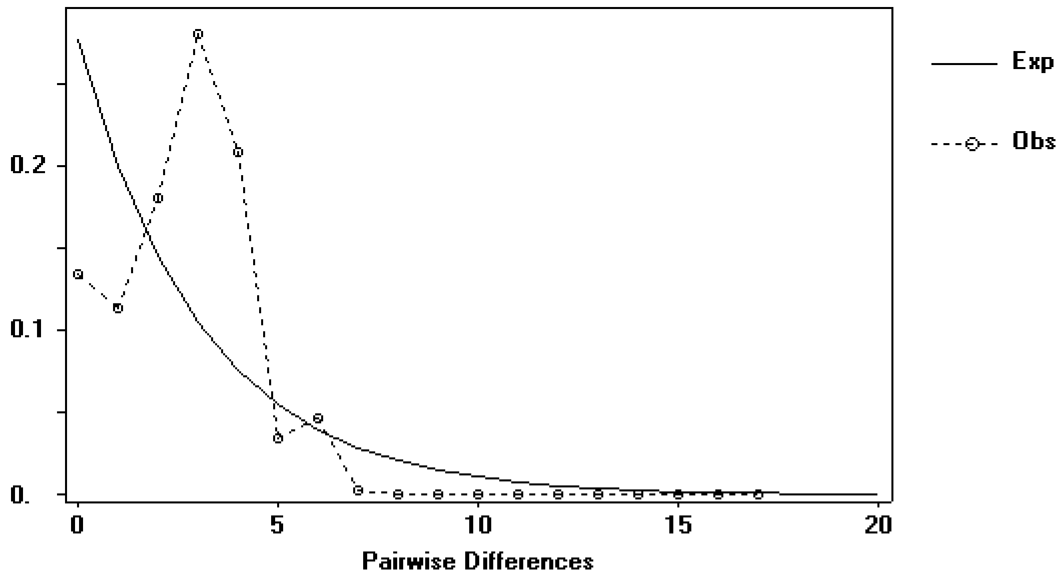
Further, the formula *Tau*=2μt was arrange to solve for the time since coalescence for golden jackal population in India. Using the mismatch distribution of pairwise differences among all haplotypes to estimate *Tau*, and a μ of 7.30×10^{-5} mutations bp^{-1} $year^{-1}$, a time since coalescence of 10,465 years ago was estimated for Indian golden jackal, *Canis aureus*.

Table 4.8- Fu's Fs and Tajima's D values for golden jackal, *C. aureus* and other canids in India

Species	Fu's Fs value	P value	Tau	time since expansion **	Tajima's D	P value
Golden Jackal	-5.616	NS, >0.10	2.618	10,465	-1.14323	NS, >0.10
Indian Wolf	-0.498	NS, >0.10	0.596	45,969	-0.80953	NS, >0.10
Himalayan Wolf	-0.967	*, <0.05	0.161	170,169	-1.81075	*, <0.05
Indian feral Dog	0.3228	NS, >0.10	2.573	10,648	0.13186	NS, >0.10

* significant P value, <0.05

** time since expansion calculated from Tau=2ut, where the mutation rate, u=0.000073 mutations per site per yr (Bozarth *et al.*, 2011)



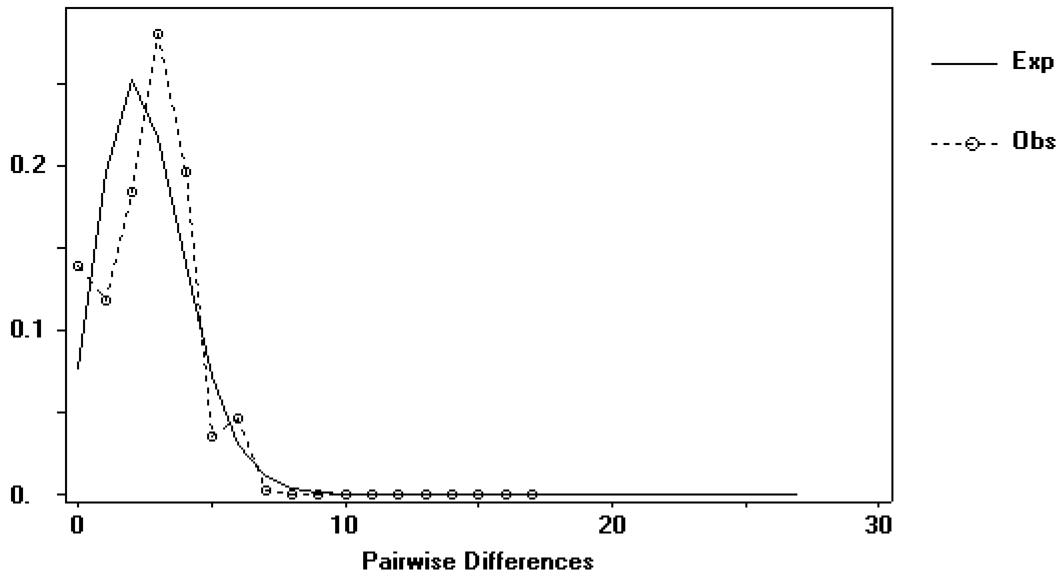


Figure 4.8- Mismatch distributions of pairwise differences of haplotypes for the Golden Jackal in India. Depicted are observed (hashed lines) and expected (solid lines) frequencies obtained under a model allowing for demographic expansion (bottom) and not allowing for expansion (top).

IV.5 Discussion

The analysis of mtDNA CR sequences for Indian golden jackals yielded 15 different haplotypes in 55 individuals, which means, on average, one distinct haplotype over 55/15=3.7 individuals, while Cytb yielded 7 haplotypes, or one haplotype per 6.9 individuals. These values are considerably lower than values for Serbian golden jackals (Zachos *et al.*, 2009), where only one haplotype was found in 121 individuals. This shows much higher variability in golden jackals in India than in Serbia.

When compared with other canids, our value is found in the middle. Highest value was were found in Russian grey wolf, *C. lupus* (one haplotype per individual; Weckworth *et al.*, 2010), while the smallest value was were found in European grey wolf, *C. lupus* (one haplotype over 35.07 individuals; Pilot *et al.*, 2010) (Table 4.9).

Among all 15 Control Region haplotypes, haplotype CauInd5 was the most common, being found in 18 (9.9%) individuals. Haplotype CauInd1 was found in 8 (4.4%) individuals. Haplotypes CauInd2 and CauInd4 were found in 4 (2.2%) individuals. Haplotypes CauInd3, CauInd8, CauInd9 and CauInd10 were found in 3 (1.65%) individuals each. Likewise, haplotypes CauInd6 and CauInd11 were found in 2 (1.1%) individuals each, while the haplotypes CauInd7, CauInd12, CauInd13, CauInd14, and CauInd15 were unique and found only in 1 (0.55%) individual each (Table 4.3).

In the phylogenetic tree, each of the canid taxa formed clades with no sharing of haplotypes of the respective species. Himalayan and Indian wolves and the wolf-dog clade were found to be sister to the golden jackal lineage. The level of resolution of the Neighbor-Joining tree did not reveal any clear phylogeographic pattern in Indian golden jackals as there was no significant association between haplotypes and geographic location of the sampling. The median-Joining (M-J) network also confirmed the result (Figure 4.6; 4.7).

Table 4.9- Comparative study of molecular diversity in canids

Species	Location	Sample size	Number of Haplotypes	Average haplotype per individual	Haplotype Diversity	Nucleotide Diversity	Reference
Golden jackal (*C. aureus*)	India	55	CR- 15	3.6	0.866	0.0091	This study
Indian peninsular wolf (*C. l. pallipes*)	India	45	CR- 4	11.25	0.493	0.0021	Sharma *et a*l., 2004
Himalayan wolf (*C. l. chanco*)	India	23	CR- 5	4.6	0.533	0.0042	Sharma *et al*., 2004
Indian feral Dog (*C. l. familiaris*)	India	24	CR- 11	2.18	0.899	0.0146	Sharma *et al*., 2004
Dog (*C. familiaris*)	Portugal	164	CR- 49	3.34	0.97	0.011	Pires *et al*., 2006
Grey fox (*Urocyon cinereoargenteus*)	Eastern United States	286	CR- 32	8.9	0.84	0.008	Bozarth *et al*., 2011
Grey wolf (*C. lupus*)	Worldwide	259	CR- 34	7.62	-	0.026	Vila *et al*., 1999
Grey wolf (*C. lupus*)	Europe	947	CR- 27	35.07	0.88	0.022	Pilot *et al*., 2010
Golden jackal (*C. aureus*)	Serbia, Europe	121	CR- 1	121	0	0	Zachos *et al*., 2009
Red fox (*Vulpes vulpes*)	Serbia, Europe	110	CR- 9	12.22	0.73	0.015	Kirschning *et al*., 2007
Grey wolf (*C. lupus*)	Southeast Alaska & Northern America-Continental group	173	CR- 11	15.7	0.60	0.0072	Weckworth *et al*., 2010
Grey wolf (*C. lupus*)	Southeast Alaska & Northern America-Coastal group	130	CR- 4	32	0.122	0.0003	Weckworth *et al*., 2010
Grey wolf (*C. lupus*)	Russia	4	CR- 4	1.0	1.0	0.0125	Weckworth *et al*., 2010
Grey wolf (*C. lupus*)	North & West Africa	292	Cytb- 31 CR- 150	Cytb- 9.42 CR- 1.95	0.424 0.923	0.00243 0.01769	Gaubert *et a*l., 2012

African wolf (*C. l. lupaster*)	North & West Africa	10	Cytb- 5 CR- 9	Cytb- 2 CR- 1.1	0.756 0.978	0.0492 0.02795	Gaubert *et al.*, 2012
Red fox (*Vulpes vulpes*)	North America	153	Cytb- 43	3.5	0.95	0.0232	Aubry *et al.*, 2009
Red fox (*Vulpes vulpes*)	Europe	8	Cytb- 6	1.3	0.89	0.0109	Aubry *et al.*, 2009
Red fox (*Vulpes vulpes*)	Asia	13	Cytb- 6	2.1	0.77	0.0136	Aubry *et al.*, 2009
Coyote (*C. latrans*)	North America	17	15	1.1	-	-	Vila *et al.*, 1999
Grey wolf (*C. lupus*)	Croatia	91	CR- 4	22.75	-	0.018	Gomercic *et al.*, 2010

Earlier phylogenetic studies conducted on wolves explained the presence of three lineages in India (Sharma *et al.*, 2004):

a) Wolf-dog clade

b) Indian peninsular wolf, *Canis lupus pallipes*, and

c) Himalayan wolf, *Canis lupus chanco*

Wolf-dog clade included all the worldwide wolf and dog haplotypes including Indian dogs and wolves from the Jammu-Kashmir region. Indian peninsular wolves were from across much of India and from the west of India and Pakistan (i.e. Iran, Israel, Afghanistan, Saudi Arabia and Turkey) while Himalayan wolves were found in Himachal Pradesh, Nepal and Tibet. The phylogenetic analysis of both CR and Cytb sequences showed both Indian peninsular wolves and Himalayan wolves to be genetically most divergent and forming a divergent separate clade with none of the haplotypes found in other wolves elsewhere (Sharma *et al.*, 2004, Aggarwal *et al.*, 2007). These clades appear to have diverged in the mid-Pleistocene (0.05-1.5 myr ago (Sharma *et al.*, 2004). The estimated time of the split of the Himalayan wolf from the other wolf lineages (0.8-1.5 myr ago) correlates with the period of the rapid uplift of the Tibetan plateau and associated habitat modification (0.9-1.1 myr ago; Sun & Liu 2000). In addition 24 Indian feral dogs had 11 CR haplotypes and all the haplotypes fall with the wolf-dog clade (Sharma *et al.*, 2004). In another study six haplotypes out of 13 Indian dogs fall in wolf-dog clade (Savolainen *et al.*, 2002). Thus, the presence of three distinct lineages of wolves in India suggested no mtDNA gene flow within them.

Mitochondrial DNA (mtDNA) diversity in Indian feral dogs was much higher than any other member of the genus *Canis* surveyed to date in India. When compared with 15 CR and 7 Cytb haplotypes in golden jackal, only 4 CR haplotypes were recovered for Indian peninsular wolves and 5 for Himalayan wolves while 11 haplotypes for Indian feral dogs explained one haplotype over 1.28 individuals (Sharma *et al.*, 2004). Moreover, the haplotype diversity as well as nucleotide

diversity was also higher in Indian feral dog to golden jackal, Indian peninsular wolf and Himalayan wolf. This indicates that Indian feral dogs have high levels of mitochondrial diversity compared to other canids inhabiting this area of world.

At the global scale, Indian golden jackal, *C. aureus* has much higher haplotype and nucleotide diversity than its Serbian conspecifics with zero haplotype and nucleotide diversity (Zachos *et al*., 2009). The haplotype diversity was found similar to grey wolf, *C. lupus* (0.88; Pilot *et al*., 2010), grey fox, *Urocyon* cinereoargenteus (0.94; Bozarth *et al.,* 2011) and red fox, *vulpes vulpes* (0.89; Aubry *et al*., 2009). Highest value was found in Russian grey wolf, *C. lupus* (1.0; Weckworth *et al*., 2010), while the lowest value was found in Serbian golden jackal, *C. aureus* (0.0, Zachos *et al*., 2009). Likewise, African wolf, *C. l. lupaster* yielded highest nucleotide diversity 0.049; (Gaubert *et al.,* 2012), while it was lowest for Serbian golden jackal, *C. aureus* (0.0; Zachos *et al*., 2009). A comparison of molecular diversity estimates in canids is shown in Table 4.9.

Based on the sequence divergence observed, coalescence was estimated at just over 10,465 years ago for golden jackals in India, which coincides with the end of Pleistocene and the onset of Holocene. The Pleistocene epoch was a time of dramatic oscillations in climate with 100,000 year old cycles interrupted by relatively warm interglacials. Much is known about the last glacial cycle (~135,000 years span) and the full ice age conditions of 20,000 BP to the present warm period (Hewitt 1996). These severe climatic oscillations of the late Pleistocene are believed to have shaped the distribution patterns of many species (Hewitt 2000). With regards to peninsular India, It is conceivable that the high extant diversity in golden jackals could be attributed to having survived as refugial populations during the Pleistocene glaciations and after the end of the glaciations event, underwent a dramatic range expansion from India westwards towards the Middle East and Europe.

IV.6 Conclusion

Now-a-days, the molecular genetic assessments have become the necessity of modern taxonomy in order to recognize phylogenetically distinct forms, misdirecting the conservation efforts for a species for protecting biological diversity. In line with this approach, a molecular study was conducted on golden jackal using the quickly evolving mitochondrial CR, and the more conserved mitochondrial Cytb gene to understand the phylogeographic and genetic structure of golden jackal in India and further to compare these results with other members of the genus *Canis* in India, *i.e.*, Himalayan wolf, Indian peninsular wolf and Indian feral dog. The DNA analysis methods were straightforward and followed established procedures (e.g., Sharma *et al.*, 2004).

Relatively high variability in Indian golden jackal CR haplotypes suggests that the habitat and climatic conditions after the estimated coalescence time did not result in the fixation of a single mtDNA haplotypes. Thus, extant population has retained more than one haplotype (15 Control Region and 7 Cytochrome b haplotypes) upto recently. Higher numbers of haplotypes as well as nucleotide diversity suggests that Indian golden jackal populations have relatively high levels of mtDNA diversity compared to conspecifics in other regions of world. It also suggests that they have historically had large effective population size and a potentially a longer evolutionary history in India than in other parts of the Middle East and Europe. Although further sampling throughout Europe, Africa and the Middle East is needed to confirm this hypothesis.

A weak phylogeographic structure was detected in Indian golden jackals, which also supports the finding of microsatellite analyses of golden jackal in Western India (See chapter V). Very interestingly, large divergences and lack of haplotypes overlapping between golden jackal and other Indian *Canis* (Indian feral dogs, Indian Peninsular wolves and Himalayan wolves) suggests no mtDNA introgression and gene flow between these canids. Thus, there does not seem to

be threats of hybridization with the large population of feral dogs and other endangered canids in India.

Furthermore, high nucleotide diversity and a star-shaped polytomy of CR haplotypes suggest that they may have undergone dramatic demographic change in the recent past and India may be the centre of radiation of golden jackals if the diversity is confirmed to be higher in India than in other regions of the world. This high CR diversity for Indian golden jackal contrasts with the extremely low genetic diversity at the western most limits of their range in Eastern Europe. This demographic expansion of golden jackals in India needs to be investigated in the light of the diversity and demographic changes with other *Canis* species in the Indian subcontinent. Indian peninsular wolf, *Canis lupus pallipes* is sympatric with golden jackal, *Canis aureus* throughout its range in India and Pakistan, while the wolves from the west of India and Pakistan (i.e. Iran, Israel, Afghanistan, Saudi Arabia and Turkey) represent mtDNA differences with Indian peninsular wolf and are classified as *C. l. pallipes* belong to the wolf-dog clade. The deep divergence and very low mtDNA diversity observed in the Indian peninsular wolf contrasts with the high genetic diversity and shallow divergence seen in the Indian golden jackal. Compared to the wolf, the jackal is a resource generalist with relatively solitary habits, and is tolerant to human-dominated landscapes. Indian wolves on the other hand have higher resource requirements owing to their larger body sizes, and gregarious social organization and also much smaller population sizes, especially given higher human persecution of wolves. The higher dispersal ability across both sexes in Indian wolves could result in the reduced genetic structuring and prevalence of few haplotypes spread over large landscape as reported in Himalayan wolves (Sharma *et al*., 2004).

To explain the higher mtDNA diversity in golden jackal as compared to the Indian peninsular wolf in India, a hypothesis is proposed here, according to which same geological or climatic phenomena likely structured both canid taxa. It is presumed

that the time period between 10,000-20,000 is too short for mutational diversity to occur. Thus, the original diversity of golden jackals even before the population bottleneck seems to have been preserved it as several different refugial population within India while for the Indian wolf, the refugial pockets seem to be limited and few, thereby only a fraction of original haplotypes were preserved. The appropriate explanation for this hypothesis is that jackals are opportunists and generalists inhabiting habitats ranging from tropical forests to scrublands and even semi-arid grasslands while wolves are specialists and limited in their habitat choice to semi-arid scrubland habitats (Jhala 2003). Therefore, a larger refugial pocket probably retained golden jackals while wolves were limited to a few pockets during the last glacial period. After climatic or geological events, both the species spread rapidly and mixing of haplotypes occurred resulting in lack of geographic structure with more haplotypes (mtDNA diversity) in golden jackal and few in Indian wolf. It is also presumed that golden jackal made their movement out of India as there is no similar size canid in the region between Pakistan and Eastern Europe, while Indian wolves, unlike other wolves (of wolf-dog clade), did not make it beyond Pakistan.

POPULATION GENETIC STRUCTURE OF GOLDEN JACKAL, *Canis aureus* IN GUJARAT, INDIA

V.1 Introduction

Understanding the variables that affect a population's ability to adapt and survive in a changing environment is a critical issue in evolutionary biology, conservation biology, and ecology (Bradshaw 1991; Peters & Lovejoy 1992; Kareiva *et al*., 1993; Gomulkiewicz & Holt 1995; Hoffman & Parsons 1997; Charlesworth & Hughes 2000). Genetic Diversity is a key factor enabling adaptation, and therefore survival of natural populations in changing environments. As genetic diversity is the basis of evolutionary potential of species to respond to environmental changes. This becomes an essential pillar in conservation genetics (Toro & Caballero 2005).

Genetic diversity has been defined as the variety of alleles and genotypes present in a population that is reflected in morphological, physiological and behavioural differences between individuals and populations (Frankham *et al*., 2002). From the functional point of view, genetic diversity can be classified as neutral, deleterious or adaptive (Hedrick 2001). Generally, neutral variants are used for conservation applications, but deleterious and adaptive variations are also important in the contexts of population survival and economically important traits in domestic plants and animals. Since the beginning of the 1990s, the developments of appropriate tools have resulted in a leading role for molecular markers in the characterization of genetic diversity. At this level, genetic diversity is usually measured by the frequencies of genotypes and alleles, the proportion of polymorphic loci, the observed and expected heterozygosity or the allelic diversity. In the context of structured populations, molecular measures of differentiations are based on genetic distances in allele frequencies among populations (Nei 1987; Laval *et al*., 2002) as well as on the popular Wright's (1969) fixation index, FST. The most widely used parameter to measure diversity within populations is the

expected heterozygosity, or gene diversity, defined by Nei (1973) as the probability that two alleles chosen at random from the populations are different. Allelic diversity is an alternative criterion to measure genetic diversity, and some authors (Petit *et al.*, 1988; Barker 2001) consider that this parameter is the most relevant in conservation programmes, as a high number of alleles imply a source of single-locus variation for important traits such as the major histocompatibility complex (MHC), which is responsible for the recognition of pathogens. It is also important from a long term perspective, because the limit of selection response is determined by the initial number of alleles (Hill & Rasbash 1986) and, because it is more sensitive to bottlenecks than expected heterozygosity, it reflects better past fluctuations in population size.

Studies of population genetic structure provide windows to the role that the fundamental evolutionary forces of selection, gene flow, and genetic drift play in processes such as local adaptation and speciation (Barton & Clark 1990; Avise 1994; Slatkin 1994; Foster *et al.*, 1998). Recent empirical and theoretical advances have led to the increased availability of an assortment of molecular markers and new methods for analyzing data derived from such markers, raising hope for gleaning more comprehensive pictures of genetic structure and deeper insights into its evolutionary causes and consequences (Avise 1994, Mitton 1994; Roderick 1996). Patterns of structure may differ among loci depending on the type of mutation process generating variation, the magnitude of variation, the mode of inheritance, the nature of genetic information obtained (genotypic or not), the effect of selection, and stochastic variation (Mitton 1994; Palumbi & Baker 1994; Pogson *et al.*, 1995; Neigel 1997). For instance, concordance in patterns of structure among nuclear markers of different classes can be inferred to signal that gene flow and drift, which affect all neutral nuclear elements similarly, are the major causes of the observed structure (Lewontin & Krakauer 1973; Mitton 1994; Scribner et al., 1994 and Burke 1994; Lehmann *et al.*, 1996; Estoup *et al.*, 1998).

Discordance between presumed neutral nuclear markers and markers for which other evidence suggests a role for selection (e.g., important physiological or behavioural genes) provides strong ancillary evidence that selection on the latter markers is a primary force molding their unique patterns of structure (Koehn *et al*., 1976; Chevillon *et al*., 1995; Long & Singh 1996; Bonnin *et al*., 1996; Lawson & King 1996; Yang *et al*., 1996). Finally, discordance between nuclear markers and various organellar markers can implicate differences in the strength of maternal, peternal, and biparental components of gene flow (FitzSimmons *et al*., 1997; Latta & Mitton 1997; Rassmann *et al*., 1997; Latta *et al*., 1998 & MacCauley 1998).

Data on heterozygosity, allelic diversity and gene flow have also led to the causes and consequences of genetic variation among individuals as well as population level of a species (Avise 1994; Mitton 1994, Roderick 1996). According to Gomulkiewicz & Holt 1995, evolution in a changing environment requires genetic variation for fitness (Reed *et al*., 2003). The maintenance of genetic variation for reproductive fitness, and fitness levels themselves, may be influenced by many variables. Critical parameters affecting the levels of genetic variation maintained in a population are i) the long term effective population size (Crow & Kimura 1970; Lei 1978; Franklin 1980; Kimura 1983; Falconer & Mackay 1996), ii) the rate at which spontaneous mutations occur and the distribution of their effects (reviewed in Lynch & Walsh 1998; Elena & Moya 1999; Fry *et al*., 1999; Schultz *et al*., 1999; Shaw *et al*., 2000; Zeyl *et al*., 2001), iii) the patterns of dominance, epistasis, and pleiotropy of mutations (Johnston & Schoen 1995; Falconer & Mackay 1996; Roff 1997), and iv) the strength and nature of selection activity on those mutations (Lynch *et al*., 1998; Charlesworth & Hughes 2000). Estimating genetic variation with both individuals and population is crucial to many population level studies. The key factor in determining a population's viability is its effective population size (Frankham 1995b). The calculated effective population size is often much lower than simply the number of individuals alive because many

individuals are not reproducing. This may be due to an unequal sex-ratio, variation among individuals in number of offsprings produced causing large fluctuation in population overtime. In other words, when the effective size of a population is reduced, inbreeding is increased causing genetic variation which reduces a species' capability to adopt to changing environments (Carson & Tempteon 1984; Leberg & Firman 2008). This phenomenon is referred to as a genetic bottleneck. When a population is greatly reduced in size, rare allele in the population will be lost since no individuals possessing those alleles will survive, with fewer alleles present and a decline in the heterozygosity, the overall fitness of individuals in the population declines (Frankham 1995a). Thus, population size is expected to have significant effects on both genetic variation for fitness and on fitness itself. Smaller populations are expected to have lower fitness for three major reasons i) increased inbreeding depression (Wright 1977; Charlesworth & Charlesworth 1987; Falconer & Makay 1996; Reed *et al.*, 2003), ii) selection is less efficient at eliminating deleterious alleles (Crow & Kimura 1970; Lei 1978) and, iii) fewer beneficial mutations occur (Kimura 1983) and they are more likely to be lost due to drift. Random genetic drift also leads to smaller population having less genetic variation than larger populations and, therefore, reduces evolutionary potential in those populations.

Thus, genetic divergence and gene flow among closely related populations are difficult to measure because mutation rates of most nuclear loci are so low that new mutations have not had sufficient time to appear and become fix. Ten microsatellites were analyzed to quantify genetic differentiation in three species of North American wolf-like canids (grey wolf, *Canis lupus*, coyote, *Canis latrans*, and red wolf, *Canis rufus*) by Roy *et al.*, (1994). They found that wolves and coyote do not show any pattern of genetic differentiation by distance. Their results show that genetic subdivision in coyote reflects persistent gene flow among newly established population, however, grey wolves show significant subdivision that may be due to drift in past Ice age

refugia populations. Kenyan golden jackals were also included in the same study, which were found to be less diverse than the grey wolves and coyote (Roy *et al*., 1994). The lack of differentiation between Portuguese village dogs and dogs from outside Portugal (Spanish mastiff, Aidi, Sloughi, and Tunisia dogs) is probably a consequence of high diversity found in all these breeds and/or populations (Pires *et al*., 2006). Zachos *et al*., (2009) used both mitochondrial DNA and nuclear microsatellite loci to reveal genetic variability in golden jackals (*Canis aureus*) from Serbia. They found no variation at all in the mtDNA sequences, and nuclear variability was very low as compared to the Kenyan golden jackals (Ho 0.29 vs. 0.41., He 0.34 vs. 0.52). This shows a considerable recent range expansion of golden jackals in the Balkans and indicates a strong founder effect in the recently established Serbian population. While studying the molecular genetics of pre-1940 red wolves, Roy *et al*., (1996) found a similar pattern of levels of genetic variation in recent and pre-1940 populations of red wolves to that of wild population of grey wolves and coyotes and one population of golden jackal. In a recent study, Kirschning *et al*., (2007) analyzed mitochondrial control region sequences in a similarly large sample of Serbian red foxes (*Vulpes vulpes*). They found nine different haplotypes as compared to one in Serbian jackals (Zachos *et al*., 2009), which reflects much longer history and the larger effective population size of fox in this region. European grey wolves, although having undergone severe persecution, also show considerably higher genetic variability, and this also holds for the strongly bottlenecked Italian population, which based on 18 microsatellite loci still yielded the observed and expected heterozygosity of 0.44 and 0.49, respectively (Lucchini *et al*., 2004). The lack of mitochondrial control region variability has also been observed in a range of population from different taxa, comprising Italian wolves (Lucchini *et al*., 2004), brown bears from the Apennines (Zachos *et al*., 2008), and the relict red deer from Mesola in Italy (Hmwe *et al*., 2006).

Inbreeding occurs when offsprings are produced from the mating of individuals who are related by descent. In general, such inbreed individuals show reduced levels of fitness compared to the mean fitness of the population, a phenomenon known as inbreeding depression. Two genetic mechanisms have been proposed as the cause of inbreeding depression. First, inbreeding will result in the expression of genetic load due to deleterious recessive alleles throughout the genome becoming homozygous (the dominance hypothesis: Charlesworth & Charlesworth 1987; Crow 1952). Second, if heterozygotes at particular loci have a higher fitness compared to both types of homozygote, the increase in homozygosity throughout the genome will result in the reduction in any such heterozygote advantage (the over dominance hypothesis: Crow 1948; Crow 1952; Mitton 1993). Most studies suggest that deleterious recessive alleles account for a large proportion of the inbreeding depression observed (Johnston & Schoen 1995; Lande 1994; Simmons & Crow 1977). As inbreeding leads to reduced average fitness, the degree to which populations suffer from inbreeding depression varies widely (Cavalli-Sforza & Bodmer 1971; Shields 1982; Ralls *et al*., 1988; Thornhill 1993; Pray & Goodnight 1995; Bijlsma *et al*., 1999). This variability makes inbreeding depression one of the major themes in conservation genetics (Frankham *et al*., 2002). The wolf could be useful as a model species because there are several studies of inbreeding in captive populations of this species. A captive Swedish wolf population expressed severe inbreeding effects (Laikre 1999), while in two American captive populations of red and Mexican wolf, no effects were noted on demographic parameters (Kalinowski *et al*., 1999), although effects on body size were noted in the Mexican wolves (Fredrickson & Hedrick 2002). A study was conducted on wild population of wolf in Scandinavia (Liberg *et al*., 2005), where a severe inbreeding depression was recorded. The genetic load was substantially heavier (6.04±3.44, 95% CI) than that for the red and Mexican wolves (0.63 and 0.71 respectively), and also clearly higher than the average estimate of 3.14 in a study of 40 captive mammal populations (Ralls *et al*.,

1998). This indicates that impact of inbreeding can vary substantially, even within the same species, depending on the random subset of genes from the source population drawn by the founders, and succeeding random drift.

I order to assess the population genetic parameter *i.e.*, intrapopulation allelic diversity, heterozygosity and gene flow between the golden jackal populations in Gujarat, here I use a panel of 10 nuclear microsatellite markers derived from the most polymorphic loci in domestic dog, *Canis familiaris*.

V.2 Materials and Methods

V.2.1 Sampling Area

The Kachchh Region

Covering an area of 45,612 Km, Kachchh (historically also referred as Kutch) is the largest district in the state of Gujarat and the second largest district of India after Leh in Jammu and Kashmir. It lies in the extreme west of India between 22°44'8'' to 24°41'30'' North latitude and 68°7'23'' to 71°46'45'' East longitude. Kachchh is geographically shaped like a tortoise. Topographically it is divided into five distinct regions i) The Great Rann and Little Rann or uninhabited wasteland in the North and East, ii) The Grassland of Banni, iii) Mainland, consisting of planes, hills and dry river beds, iv) The coastline along the Arabian Sea in the South and, v) Creeks and mangroves in the West. More loosely, the Southern portion of Rann is considered an Island, with seawater inundating the land for most of the year. The mainland is plane but has some hill ranged and isolated hills Kachchh literally means something which intermittently becomes wet and dry, a large part of this district is known as Rann of Kachchh which is shallow wetland and submerges in water during other season. The Great Rann of Kachchh (GRK) and the little Rann of Kachchh are the World's only saline desert spread in 25,000 sq. km. Seasonal, inundation of the entire area by rain water and diurnal inundation of

Western half of the GRK by sea water coupled with a high residual salinity level provide a rare and unique type of ecosystem.

The tropical thorn forest and Littoral or Mangrove forests are distributed in Kachchh. Major species in tropical thorn forests include *Acacia nilotica, A. senegal, A. catechu, A. leucophiila, Zizyphus spp.* etc. The animals inhabiting these forests are wild ass, chinkara, chital, etc. Regarding wildlife, Kachchh Desert Wildlife Sanctuary is present in the North-Eastern part that borders Pakistan and Wild Ass Sanctuary. It is the largest sanctuary of India occupying an area of 7505.22 sq. km. It houses some of the rare wild animals like striped hyena, porcupine, fox, small Indian civet, Indian pangolin and some of the rare species of birds. Jackals, lizards, snakes, nilgai, wild boar, chinkara, sambhar and chittal are some common animals share this sanctuary.

The Bhal Region

The arid and saline area North of Gulf of Khambhat that covers an area of 1,420 sq. km. under the talukas of Dhilka, Dhandhuka, and Vallabhipur is the Bhal Region. Bhal is spread across political boundries of two districts of Gujarat namely, Bhavnagar and Ahmadabad. Bhal in Gujarati means forehead, denoting barren soil where nothing grows.

Velavadar Blackbuck National Park (VNP), Pipli wetland and, wetland between Naari and Bawaliyali villages are major reservoir of animals in Bhal. VNP, the only tropical grassland in India recognized as a National Park is located in Bhavnagar district. The park now spread over an area of 34.08 sq. km. It is declared as NP in 1976. The main attraction of the park located seventy-two km away from the city of Bhavnagar is the massive population of blackbuck, antelope, wolf and other endangered species of birds. On the southern border of the park lies the high tidal zone of the Gulf of Khambhat and as a result that portion of the park is flooded with water. The other sides

are surrounded by wastelands and agriculture fields. Its semi-arid conditions, together with overflowing of sea water during monsoon create habitats for the varied fauna. The Park has areas of dense grasslands, sparse grasslands, Prosopis shrubland, Saline lands and high tidal mudflats. Thirty-nine species of grasses and 46 species of sedges, shrubs and trees represent the diversity of flora. *Dichanthium annulatum*, *Sporobolus virginicus*, *Sporobolus coromandelianus*, *Sporobolus madernspatensis* are the dominant grasses. *Prosopis juliflora* growing in the form of shrub covers large area of the Park. Among the medium sized trees, Salvadora, *Acacia nilotica*, Zizyphus, Capparis and Suaeda are common. The fauna of the park comprises mainly of blackbuck, antelope, blue bull, wolf, jackal, hyena, jungle cat, fox, wild Boars and birds as well. All these animals can be viewed on the open Flat grassland from very close range. Endangered birds like houbara bustard, lesser florican, sarus white storks, white pelican, montagu, pallid harriers, marsh and other raptors including the greater spotted eagle, juvenile imperial eagle, Bonelli's eagle, short-toed snake eagle and long-legged buzzard are also seen in the park. The climate in Velavdar makes it one of the best places for the migrating birds to breed.

V.2.2 Sampling

Twenty four samples collected from the Kachchh and Bhal regions of Gujarat were analyzed. Samples were preserved in 95% Ethanol and stored at -20°C until DNA extraction. Sampling distribution is shown in the map (Figure 5.1) where blue triangles are the area from where samples were collected while the no. of samples analyzed are detailed in Table 5.1.

Table 5.1- Description of sampling location and number of samples collected for the population genetic study of golden jackal, *Canis aureus* in Western India.

S. No.	No. of samples collected	Location
1	17	VNP & Bhal
2	07	Kachchh
	Total= 24	

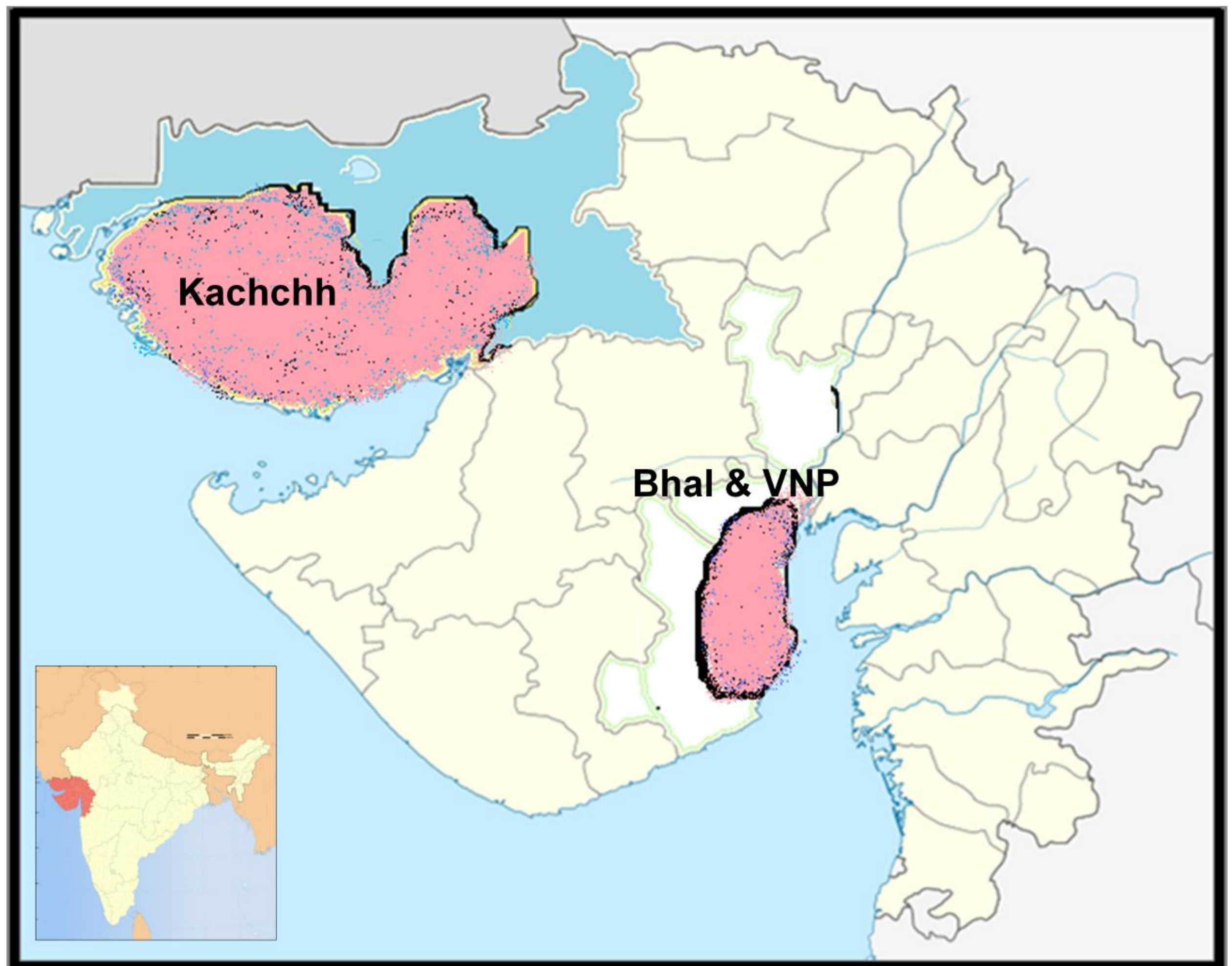


Figure 5.1 Map of golden jackal, *Canis* aureus sampling locations in Western India.

V.2.3 DNA Extraction

Methods for DNA extraction are detailed in **Chapter III**. Extraction was followed by amplification using Polymerase Chain Reaction (PCR) with universal primers available for mtDNA (Control Region and Cytochrome b) for canids.

V.2.4 PCR amplification of microsatellite DNA

Extraction was followed by amplification using Polymerase Chain Reaction (PCR) with universal primers available for mtDNA (CR and Cytb) for canids. Genetic analyses were completed using ten microsatellites selected from the 2006 International Society for Animal Genetics domestic dog (*Canis familiaris*) panel that were consistent with wild dog genetic studies in South Africa (Moueix 2006). All individuals were genotyped at eight dinucleotide microsatellite loci (INRA21, AHTk253, REN54P11, REN105L03, INU030, INU005, LEI004 and FH2412) and two tetranucleotide loci (FH2328 and FH2079). The main characteristics of these primers are shown in table 5.2.

As all the markers were labeled with fluorescent dye FAM, multiplexing was done with the caution of size range e.g., INRA21 was multiplexed with REN54P11, AHTk253 with INU030, and LEI004 with REN105L03 while other loci were amplified individually. Each PCR reaction was carried out in a 25µl volume including 0.2 units of AmpliTaq Gold (Applied Biosystems), 25 mM $MgCl_2$, 10X reaction buffer, 1X BSA, 2µM each dNTP, and 10µM each primer. Amplifications were performed in a PTC-200 (MJ Research) or GeneAmp PCR system 2700 (Applied Biosystems) using following protocol:

Table 5.2- Description of microsatellite loci used for population genetic analysis in golden jackal, *Canis aureus*

S.No.		Locus	Fluorescent Label	5'-3' Sequence	Size range
1	F	INRA21	FAM	ATGTAGTTGAGATTTCTCCTACGG	87-111
	R			TAATGGCTGATTTATTTGGTGG	
2	F	AHTk253	FAM	ACATTTGTGGGCATTGGGGCTG	277-297
	R			TGCACATGGAGGACAAGCACGC	
3	F	FH2328	FAM	ACCAGGTAGTTTTCAGAAATGC	171-213
	R			AGTTATGGGACTTGAGGCTG	
4	F	REN54P11	FAM	GGGGGAATTAACAAAGCCTGAG	224-242
	R			TGCAAATTCTGAGCCCCACTG	
5	F	REN105L03	FAM	GGAATCAAAAGCTGGCTCTCT	231-249
	R			GAGATTGCTGCCCTTTTTACC	
6	F	INU030	FAM	GGCTCCATGCTCAAGTCTGT	143-157
	R			CATTGAAAGGGAATGCTGGT	
7	F	INU055	FAM	CCAGGCGTCCCTATCCATCT	204-220
	R			GCACCACTTTGGGCTCCTTC	
8	F	LEI004	FAM	CATCATGCATCAAGCAGAGC	86-112
	R			TCATGTAAGCAGAGACTGAC	
9	F	FH2412	FAM	GCTGGGGATTTATTCTGACC	162-186
	R			AAATTAACCAAATGTTTGCAACA	
10	F	FH2079	FAM	CAGCCGAGCACATGGTTT	266-286
	R			ATTGATTCTGATATGCCCAGC	

Initial denaturation	96° X 10’
35 cycles	95° X 1’ denaturation
	50° X 1.30’ annealing
	72° X 1.30’ extension
Final extension	72° X 7’

A small aliquot of each amplification was electrophoresed on a 2% agarose gel to check for the correct fragment size and to ensure that only a single amplification product was obtained. PCR products were denatured at 95° for 5 min before genotyping. The steps for plate formation for genotyping are shown in Figure 5.2.

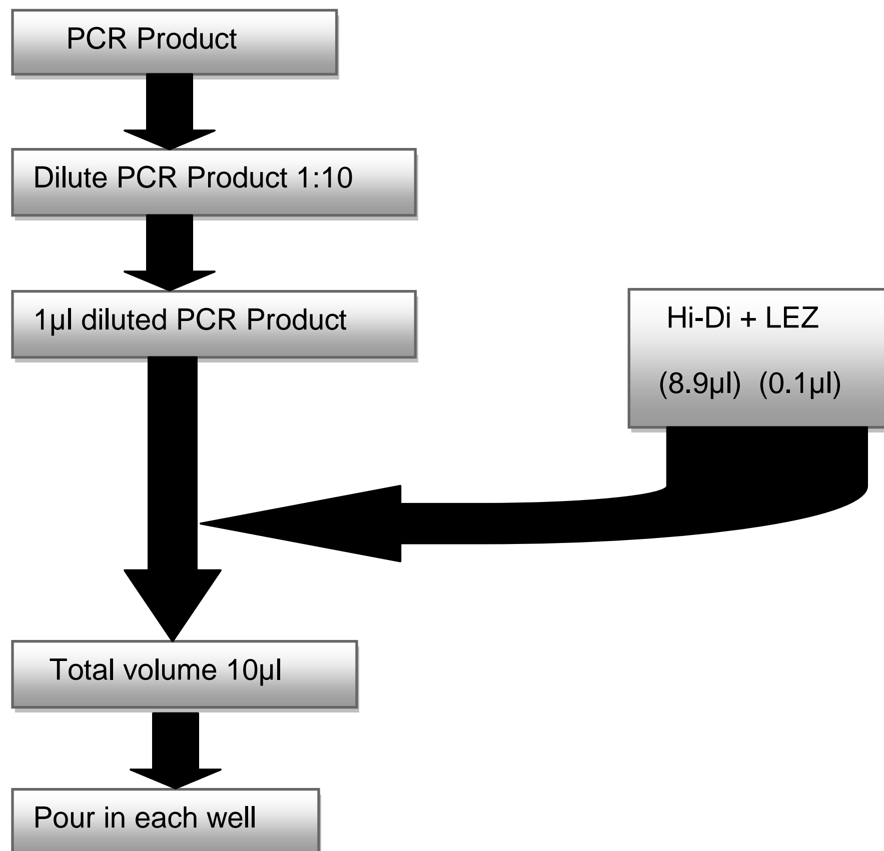


Figure 5.2- Steps for plate formation before genotyping

V.2.5 Statistical analysis of microsatellites

Microsatellite genotyping was carried out using ABI 3130 Genetic Analyzer (Applied Biosystems) with GeneScan-500 LIZ as the internal lane size standard. The first step of analysis is allele calling i.e. identifying peaks that correspond to alleles and measuring the size of the corresponding fragments. Commercial software provided by constructors of capillary electrophoresis systems decreases analysis set-up time through automated correction of common genotyping problems, including saturated peaks, excessive baseline noise, voltage spikes caused by micro-air bubbles or debris in the laser path, and stutter peaks. Genemapper v.3.7 (Applied Biosystems) and Peak Scanner 1.0 softwares were used for allele identification and sizing. Different individuals were genotyped as either homozygote or heterozygote on the basis of band pattern, *i.e.* the presence of one or two different alleles respectively, shown for each microsatellite loci. Scoring was done manually.

V.2.5.1 Genetic diversity

Genetic diversity was quantified in terms of number of alleles (N), effective number of alleles (Ne), observed (Ho) and expected (He) heterozygosities and polymorphism information content (PIC) across the 10 loci using the computer programme Cervus version 3.0.3 (Marshall *et al*., 1998). The observed and expected heterozygosity were calculated per locus and in each population. GENEPOP version 4.1 (Raymond & Rousset 1995b, Rousset 2008) was employed to test deviations from the Hardy–Weinberg equilibrium (Weir & Cockerham 1984). For the Hardy–Weinberg equilibrium estimation, we followed the probability test approach (Guo & Thomson 1992). Exact probability (*P*) values were calculated using a Markov chain algorithm with 10000 dememorisation steps for 20 batches and 5000 iterations per batch. To know whether the deviations from Hardy–Weinberg equilibrium were in the direction of heterozygote excess or deficit, Hardy–Weinberg tests for each locus in each population and a global test over all populations were performed. Corrections for multiple significance tests were performed using

Fisher's method and by applying a sequential Bonferroni correction (Rice 1989). The Chisquare ($\chi 2$) test with significant levels of probability (P) for knowing the Hardy–Weinberg equilibrium was performed in software GeneAlEx 6.0 (Peakall & Smouse 2006). The presence of pairwise linkage disequilibrium between loci over sampling locations was tested using exact test with Genpop 4.1. The program was also used to calculate the Frequency of null alleles at each locus.

V.2.5.2 Genetic differentiation

Wright's F-statistics was used to analyze differentiation within and between population structure. Genetic differences between populations were estimated with *Fst* (Weir & Cockerham, 1984), based on differences in allele frequencies using GENEPOP version 4.1 (Raymond & Rousset 1995b, Rousset 2008). The *Rst* statistic, based on differences in the allele size was also used. Estimator of heterozygote deficiency or coefficient of inbreeding (*Fis*) was calculated across all populations and loci (global *Fis*), and for populations and loci individually. Similarly the *Fst* calculations were performed over all populations (global *Fst*) and within population (pairwise). Wright's guidelines, as referenced by Conner and Hartl (2004), were used to classify *Fst* values as low, moderate, or high.

V.2.5.3 Genetic structure

The Bayesian clustering procedure implemented in computer program STRUCTURE version 2.3.3 (Pritchard *et al*., 2000, Falush *et al*., 2003, 2007) was used to simultaneously infer the number of distinct genetic clusters suggested by the microsatellite data, and likewise probabilistically assign each analyzed individual to one of the inferred clusters. Admixture model was assumed and analysis was performed considering correlated allele frequency models. STRUCTURE analyses were performed using values of K (the assumed number of clusters) ranging from 1 to 2. Analyses were performed

using an initial burn-in of 4×10^4 steps, followed by 1.0×10^5 Markov-Chain Monte Carlo (MCMC) analysis sweeps. Number of iteration was set to 10.

V.3 Results

V.3.1 Genetic diversity

All 24 Jackal samples were successfully amplified and 78 distinct alleles were distinguished over the ten microsatellite loci used. Linkage disequilibrium was not detected between the investigated loci, therefore, all the loci were retained for the further analysis. All ten loci were highly polymorphic (100%). The minimum number of observed alleles per locus ranged from 5 for loci FH2412 and maximum 13 for locus FH2328 while the overall mean number of alleles per locus was reported 8.8 (±2.33).

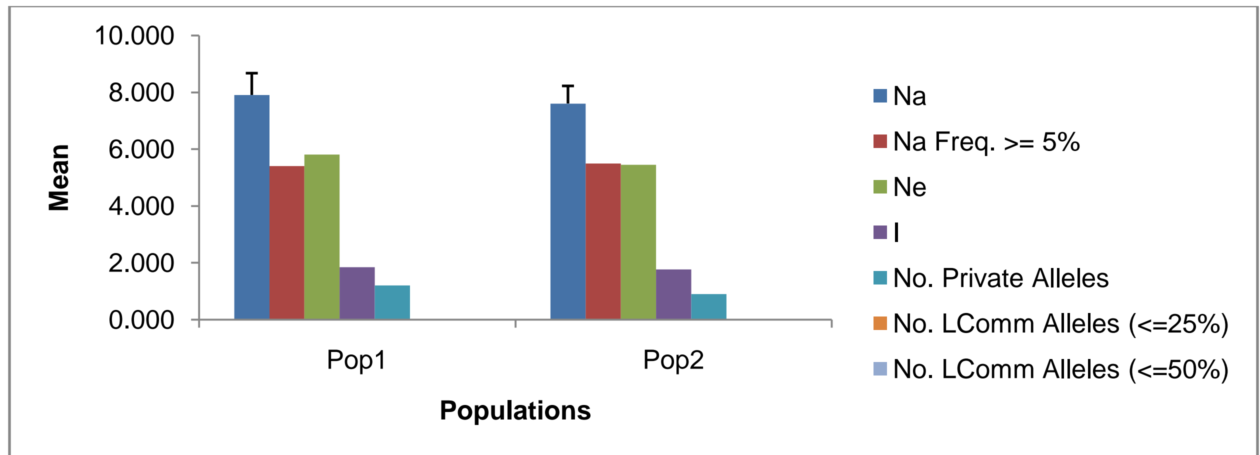


Figure 5.3- Alleleic patterns across the total population of golden jackal, *Canis aureus* in Western India. Na is number of alleles and Ne is effective number of alleles.

The effective number of alleles (Ne) is the number of alleles that can be present in a population. For golden jackal population Ne per locus ranged from 2.49 for locus FH2412 to 8.93 for locus FH2328 with mean (±) of

6.15±1.91 (Table 5.3). Allelic patterns across total population of Golden Jackal is shown in figure 5.3. Average number of allele per locus in Bhal population was 7.6 (±0.618) while it was 7.8 (±0.629) in Kachchh population. 5.4±0.34 alleles with frequency less than 5% were present in Bhal alongwith 5.7±0.559 in Kachchh. Average effective allele ranges from 5.67 (±0.522) for Bhal to 5.69±0.66) for Kachchh population of Jackal.

All the microsatellite loci showed PIC (Polymorphism Information Content) value higher than 0.5 except FH2412 (PIC-0.370) which is normally considered as informative in population genetic analyses (Botstein *et al.*, 1980). The mean PIC was 0.798. The observed heterozygosity (Ho) per locus ranged from 0.250 (FH2412) to 1.0 (INRA21, INU005 and LEI004) with a mean value of 0.812±0.233, while the expected heterozygosity (He) per locus varied from 0.599 (FH2412) to 0.888 (FH2328) with an average value of 0.815±0.083. From 20 instances (2 populations, 10 loci), 5 deviations significant at $P<0.05$ from the Hardy-Weinberg equlilibrium were detected (Table 5.4). Inetrestingly, all locus specific genotype proportions of the Bhal population were congruent with the Hardy-Weinberg expectations (Table 5.5). Observed Hardy-Weinberg deviations were not consistent over loci, but generally occurred with different microsatellite in different populations and most likely due to presence of null allele (Shaw *et al.*, 1999). The Kachchh sample size was small and therefore these test results should be viewed with caution.

Overall, both Bhal and Kachchh populations showed almost the same allelic variability with an average of 7.6 alleles per locus for the Bhal and 7.8 for the Kachchh population. The loci with the highest number of alleles in the Bhal population was FH2328 with 11 alleles while in Kachchh it was FH2328 and LEI004 with 10 alleles each (Table 5.6). The value for observed heterozygosity (Ho) for each locus ranged from 0.417 for FH2412 at Bhal population to 0.917 for AHTk253 and FH2328 at Kachchh population. The

expected heterozygosity (He) value ranged from 0.462 for FH2412 at Kachchh population to 0.868 for AHTk253 and FH2328 at Bhal population. Value for observed heterozygosity (Ho) for each population for all loci examined were 6.417<Ho<1.0 for Bhal population and 0.083<Ho<1.0 for Kachchh population while valuse for expected heterozygosity (He) were 0.681<He<0.868 and 0.462<He<0.885 for Bhal and Kachchh population respectively (Table 5.6).

Table 5.3- Measures of genetic variability for each locus analyzed for golden jackal population in Gujarat, Western India

Locus	**Number of alleles (Na)**	**Effective Number of alleles (Ne)**	**Polymorphic Information content (PIC)**	**Observed heterozygosity (Ho)**	**Expected heterozygosity (He)**	**FIS**	**FST**
INRA21	8.000	6.545	0.829	1.000	0.847	-0.180	0.018
AHTk253	10.000	7.024	0.842	0.875	0.858	-0.020	0.022
FH2328	13.000	8.930	0.925	0.833	0.888	0.062	0.015
REN54P11	7.000	5.908	0.808	0.875	0.831	-0.053	0.005
REN105L03	10.000	6.621	0.832	0.917	0.849	-0.080	0.027
INU030	10.000	6.545	0.831	0.708	0.847	0.164	0.027
INU055	7.000	4.251	0.73	1.000	0.765	-0.308	0.001
LEI004	11.000	8.662	0.874	1.000	0.885	-0.131	0.015
FH2412	5.000	2.494	0.37	0.250	0.599	0.583	0.046
FH2079	7.000	4.608	0.505	0.667	0.783	0.149	0.035
Mean	8.800	6.1587	0.7976	0.8125	0.8151	0.0184	0.021
SE	2.3305	1.9125	0.1722	0.2231	0.0831	0.2389	0.0125

Table 5.4- Result of the Chi-square test (χ2) for Hardy-Weinberg equilibrium (HWE) for all loci among the golden jackal population in Bhal and Kachchh in Gujarat

Locus	No. of alleles (Na)	ChiSq (χ2)	Degree of freedom (DF)	Probability (P)
INRA21	8.000	33.876	28	0.205
AHTk253	10.000	61.342	45	0.053
FH2328	13.000	132.106	78	0.000***
REN54P11	7.000	29.567	21	0.101
REN105L03	10.000	54.622	45	0.154
INU030	10.000	57.919	45	0.094
INU055	7.000	50.571	21	0.000***
LEI004	11.000	75.413	55	0.035*
FH2412	5.000	33.615	10	0.000***
FH2079	7.000	36.034	21	0.022*
Mean	**8.8**	**56.507**	**36.9**	**0.066**

Four loci at Kachchh population showed significant deviation from the genotype proportion expected according to Hardy-Weinberg equilibrium whereas the Bhal population has all the loci in H-W equilibrium (Table 5.6). The FIS value estimated according to Weir & Cockerham (1984) showed a lower value for Kachchh population (Mean FIS=-0.006) than for Bhal population (Mean FIS=0.015).

Table 5.5- Result of Chi-square test (χ2) for Hardy-Weinberg equilibrium (HWE) for golden jackal population in Bhal and Kachchh

Loci & pop	Number of alleles (Na)	ChiSq (χ2)	Degree of freedom (DF)	Probability (P)
BHAL				
INRA21	7.000	16.762	21	0.725
AHTk253	9.000	45.833	36	0.126
FH2328	11.000	45.920	55	0.803
REN54P11	7.000	26.040	21	0.205
REN105L03	8.000	35.167	28	0.165
INU030	9.000	30.720	36	0.718
INU055	6.000	23.452	15	0.075
LEI004	9.000	33.227	36	0.601
FH2412	5.000	15.651	10	0.110
FH2079	5.000	14.815	10	0.139
KACHCHH				
INRA21	8.000	37.000	28	0.119
AHTk253	9.000	50.400	36	0.056
FH2328	10.000	71.500	45	0.007**
REN54P11	7.000	24.567	21	0.266
REN105L03	9.000	40.500	36	0.278
INU030	9.000	68.037	36	0.001***
INU055	6.000	29.738	15	0.013*
LEI004	10.000	48.333	45	0.340
FH2412	4.000	24.042	6	0.001***
FH2079	6.000	21.261	15	0.129

Table 5.6- Measures of genetic variability for each locus analyzed for golden jackal in Bhal and Kachchh population

Locus	Population											
	Bhal				**Kachchh**				**Mean across population**			
	Na	Ho	He	FIS	Na	Ho	He	FIS	Na	Ho	He	FIS
INRA21	7	1.0	0.799	-0.252	8	1.0	0.865	-0.157	7.5	1.0	0.832	-0.203
AHTk253	9	0.833	0.868	0.040	9	0.917	0.809	-0.133	9	0.875	0.839	-0.043
FH2328	11	0.750	0.868	0.136	10	0.917	0.882	-0.039	10.5	0.833	0.875	0.048
REN54P11	7	0.750	0.813	0.077	7	1.0	0.840	-0.190	7	0.875	0.826	-0.059
REN105L03	8	0.833	0.840	0.008	9	1.0	0.813	-0.231	8.5	0.917	0.826	0.0109
INU030	9	0.833	0.858	0.028	9	0.583	0.792	0.263	9	0.708	0.825	0.141
INU055	6	1.0	0.764	-0.309	6	1.0	0.764	-0.309	6	1.0	0.764	-0.309
LEI004	9	1.0	0.858	-0.166	10	1.0	0.885	-0.129	9.5	1.0	0.872	-0.147
FH2412	5	0.417	0.681	0.388	4	0.083	0.462	0.820	4.5	0.250	0.571	0.562
FH2079	5	0.583	0.726	0.196	6	0.750	0.785	0.044	5.5	0.667	0.755	0.117

V.3.2 Genetic differentiation

At individual loci, *Fis* estimates ranged between -0.308 (locus INU005) to 0.563 (locus FH2328). Mean *Fis* value for ten microsatellites was 0.0184±0.238. Statistically significant departure from the Hardy-Weinberg equilibrium occurred consistently with positive *Fis* estimates, reflecting the deviation to be a result of heterozygote deficiency (Table 5.3). The *Fis* value estimated according to Weir & Cockerham (1984) showed a lower value for Kachchh population (Mean *Fis*=-0.006) than for Bhal population (Mean *Fis*=0.015). Across the ten microsatellite markers, the estimator of genetic differentiation (*Fst*) ranged from 0.001 for locus INU005 to 0.005 for locus REN54P11 with a mean value of 0.021±0.0125 (SE) (Table 5.3).

Low genetic differentiation was found among populations with almost similar *Fst* and *Rst* values. Pairwise *Fst* and *Rst* value for Bhal and Kachchh population was found to be 0.0182 and 0.026 respectively.

Table 5.7- Pairwise *Fst* (below diagonal) and *Rst* (above diagonal) values among two sampling locations of golden jackal, *Canis aureus* based on ten microsatellite loci

Pairwise *Fst*, *Rst*	Bhal	Kachchh
Bhal	—	0.026
Kachchh	0.0182	—

V.3.3 Genetic structure

The Bayesian clustering analysis using STRUCTURE showed no strong pattern of population differentiation and only one population cluster was identified (Figure 5.4). Using a Bayesian MCMC approach and considering a range of one to two potential populations, the probability value (mean value of ln likelihood) obtained for two populations (K=2) was -879.7±31.3 while for one population (K=1) it was found little higher -878.8±30.9

The results revealed that the maximal value of L (K) was attained at K=1 (Figure 5.5). These values decreased for each K>1 and became more variable among runs.

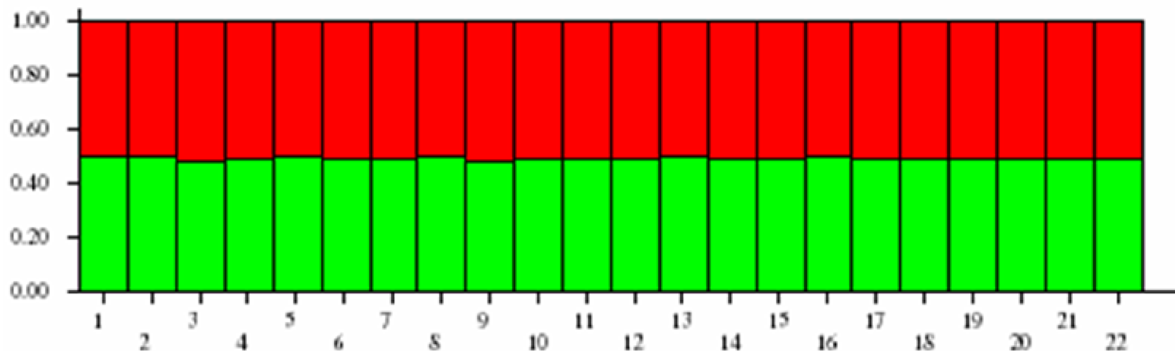


Figure 5.4- STRUCTURE analysis of microsatellite data showing a single clustered population of golden jackal, *Canis aureus* in Gujarat, Western India

The proportion of indiviuals in each inferred cluster for K>1 were evenly distributed e.g., for K=2, each cluster contained 50% of individuals. The maximum values of L (K) never reached a plateau, so there was no need to employ the rate of change matrics recommended by Evanno *et al.*, (2005) for identification of genetic clusters. Thus, it was concluded that STRUCTURE results supported only a single genetic cluster in jackal population across Gujarat.

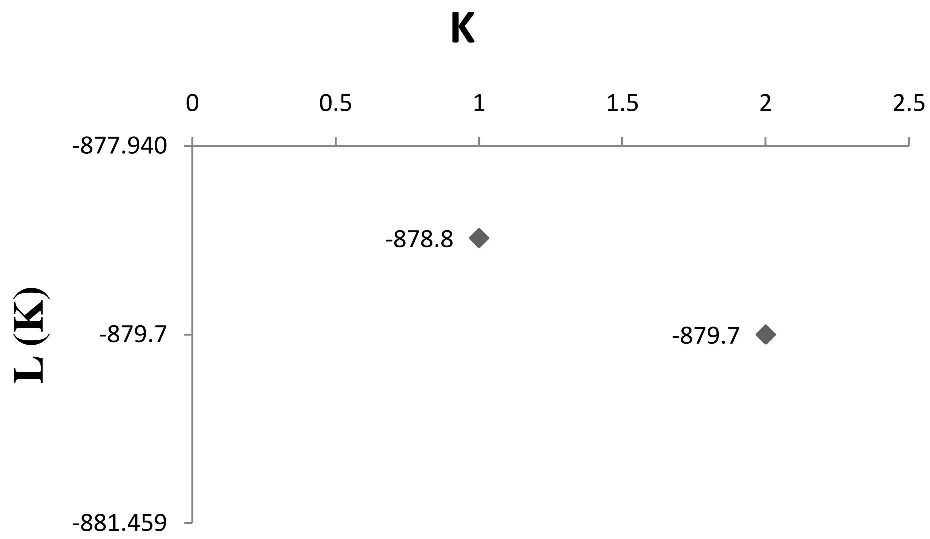


Figure 5.5- Log probability of data [L(K)] as a function of K averaged over 10 independent runs for golden jackal, *Canis aureus* derived using a Bayesian clustering algorithm implemented in the computer programe STRUCTURE

V.4 Discussion

The chance of a successful cross-species (heterologous) amplification of any DNA sequence is inversely related to the evolutionary distance between the two species as has been demonstrated in several other papers. Indeed, several studies have shown that microsatellites isolated from various species amplify the corresponding and polymorphic loci in closely related but not in more distant one (Moore *et al*., 1991; Primmer *et al*., 1996; Gemmel *et al*., 1997; Galbusera *et al*., 2000; Hille *et al*., 2002; Williamson *et al*., 2002; Nguyen *et al*., 2005). In the present study, highly polymorphic dinucleotide and tetranucleotide microsatellites characterized and developed for domestic dog, *Canis familiaris* were successfully transferred to golden jackal, *Canis aureus*. Microsateliite transferability was also obtained for other carnivores (including canids) species related evolutionarily (Primmer *et al*., 1996; Gemmel *et al*., 1997; Koskinen & Bredbacka 2000; Williamson *et al*., 2002; Ellegren *et al*., 1997; Lucchini *et al*., 2002 and Sacks *et al*., 2004).

Unfortunately, very few data are available for direct comparison with our results. To our knowledge, the study that includes golden jackal in a population genetic analysis is done by Roy *et al*., (1994), who genotyped 18 golden jackals from Kenya at ten microsatellite loci as outgroup for comparison with North American canids. Very recent study was done by Zachos *et al*., (2009) who genotyped 121 golden jackals at 8 microsatellite loci in order to reveal their genetic variability and differentiation in Serbia (Europe). They used four out of ten loci from Roy *et al*., (1994) in their study. We analyzed 24 golden jackals with ten microsatellite loci designed for domestic dog. All the loci were highly polymorphic with a mean of 8.8 (±2.33) alleles per locus. This value was 3.9 for Serbian jackal and 2.0 for Kenyan jackal. The population genetic study using same loci used here are also reported in other canids (Table 5.8).

Among the ten loci analysed here, nine were highly polymorphic with a PIC>0.5 (except FH2412, PIC-0.370). Except FH2079 (PIC-0.505), all the loci were most informative (PIC>0.7). The loci were found to be less informative in other canids as compared to the Indian jackals (Moueix 2006, Oberbauer *et al.*, 2003 & Spiering *et al.*, 2010), as the PIC value may vary considerably in different individuals with same markers used (Eggleston *et al.*, 2002).

Table 5.8- Studies on canids with markers same as in the present study

Canid Species	**Study**	**Marker/loci used**	**Reference**
African wild dog, *Lycaon pictus*	Multiple paternity	INRA21, AHTk253, FH2328, REN54P11, REN105L03, LEI004, INU030, INU055	Moueix 2006
African wild dog, *Lycaon pictus*	Cooperative breeding	INRA21, AHTk253, FH2328, REN54P11, REN105L03, LEI004, INU030, INU055	Spiering *et al.*, 2010
The Belgian Tervuren & Shepherd dog	Genetics of Epilepsy	INRA21, AHTk253, FH2328, LEI004, FH2079	Oberbauer *et al.*, 2003
The German Shepherd dog	Comparative population genetic study	INRA21, AHTk253, FH2328,	Coutts & Harley 2009
Coyote, *Canis latrans*	Population structure study	FH2328, FH2079	Sacks *et al.*, 2004

In line with this picture, the Indian jackal yielded observed and expected heterozygosity (Ho 0.81, He 0.82) much higher than those of their Serbian (Ho 0.29, He 0.34) and Kenyan conspecifics (Ho 0.41, He 0.52). The levels of He exceeded those observed in previous microsatellite studies of other canids while it was found similar to grey fox (*Urocyon cinereoargenteus*) (He 0.80, Deyoung *et al.*, 2009), Arctic fox (*Alopex lagopus*) (He 0.78 Carmichael *et al.*, 2007), coyote (*Canis latrans*) (He 0.73, Sacks *et al.*, 2004), Latvian Wolf from Europe (He 0.73, Lucchini *et al.*, 2004), Wirehaired Dachshund dog (He 0.72, Zajc *et al.*, 1997) and Kintamani Dog (He 0.70, Puja *et al.*, 2005). A comparative study of heterozygosity in different canids is shown in Table 5.9. Highest value for Indian jackal show highly diversed and harbor high genetic

variability than Kenyan, Serbian jackals and any other canid even with a limited sample of 24 jackals within a single state of India.

To test for Hardy-Weinberg equilibrium (HWE), an exact test (probability test), which is appropriate for a small sample size (Weir 1990) and a Chi-square ($\chi 2$) test were used. To test whether nonrandom mating was responsible for departure from HWE for five loci, *Fis* (Inbreeding coefficient) was calculated. Six estimates of *Fis* were negative including two loci that significantly differed from HWE and also had the highest negative values for *Fis*, substantiating that an excess of heterozygotes was responsible for departure from HWE (Rosewich *et al.*, 1999). However, three loci showing departure from HWE had positive *Fis* values. Overall positive and relatively small mean *Fis* value (*Fis* 0.018) approaches zero, which- in natural populations is indicative of random mating within a subpopulation (Nei 1977). The *Fis* estimates also conclude very less or no inbreeding and an indication of minimal barriers to gene flow between the populations studied. Overall, the inbreeding in the individuals relative to the total population is fairly small and this could also be an indication of little genetic drift among the populations.

Fixation index values also provide informative measures of population structure. The most important of these is the *Fst* value (Wright 1951), the proportion of total variation that is due to differences between subpopulations (If *Fst*=1, subpopulations have no allele in common; if *Fst*=0, allele frequencies in all subpopulations are identical) the mean *Fst* of 0.021 for the polymorphic loci (Table 5.3) indicate little or no genetic differentiation between populations resulting from genetic drift. The *Fst* value (0.021) for golden jackal in this study falls within the low range of differentiation of 0-0.05 (Hartl & Clark 1997). This level of differentiation is comparable to that among wolves (*Canis lupus*) from different regions with *Fst* value of 0.029 (Kennedy *et al.*, 1991) and for Arctic fox (*Alopex lagopus*) with *Fst* value of 0.02 (Carmichael *et al.*, 2007). A global value of 0.043 was found in Kit fox (*Vulpes*

macrotis) (Schwartz *et al*., 2005), which was similar to that of male Guiana Dolphin (*Satalia guianesis*) (*Fst* 0.04, Hollatz 2011).

With regard to differentiation, no evidence of significant difference between the Bhal and Kachchh population of golden jackal was found. With pairwise *Fst* and *Rs*t (microsatellite-specific analog of Fst based on the stepwise mutation model) values of 0.018 and 0.026 respectively, the Indian jackals within Gujarat are considered as genetically homogeneous population. Zachos *et al*., 2009 found significant genetic differentiation among the Serbian jackal (overall *Fst* 0.07 and *Rst* 0.089). To our knowledge, the smallest pairwise *Fst* observed in the arctic fox (*Alopex lagopus*) was 0.002 (Carmichael *et al*., 2007) which was followed by grey fox (Urocyon cinereoargenteus) (pairwise Fst 0.007), (Deyoung *et al*., 2009), red fox (*Vulpes vulpes*) (pairwise *Fst* 0.009), (Lade *et al*., 1996, Wandeler *et al*., 2003). Coyote (*Canis latrans*) were once considered genetically homogeneous (Roy *et al*., 1994), but recent work suggests the existence of previously undected genetic subdivisions (Sacks *et al*., 2004). The lowest level of differentiation in genetic structure of arctic fox populations considers them genetically homogeneous and unique among the studied canids till date (Carmichael *et al*., 2007).

Table 5.9- Heterozygosity values from microsatellite studies of different canids reported in literature used here to compare the results of golden jackals of Gujarat

Species	**Location**	**Sample size**	**Mean no. of alleles**	**Mean Expected heterozygosity (He)**	**Reference**
Golden jackal (*C. aureus*)	Gujarat, India	24	8.8	0.83	Present study
Cimarron Uruguayo dog	Uruguay, South America	30	-	0.65	Gagliaedi *et al*., 2011
Golden jackal (*C. aureus*)	Serbia, Europe	121	3.9	0.34	Zachos *et al*., 2009
Maned wolf (*Chrysocyon brachyurus*)	Brazil, South America	112	4.3	0.67	Salim *et al*., 2007
Bali street dog	Bali, Indonesia	40	7.7	0.58	Irion *et al*., 2005
Chow Chow dog breed	China	20	7.0	0.65	Puja *et al*., 2005
Kintamani Dog breed	Bali, Indonesia	40	7.0	0.70	Puja *et al*., 2005
Korean dog breed	Korea	183	12.73	0.66	Cho *et al*., 2004
Wild dog (*Lycaon pictus*)	East & South Africa	228	3.9	0.61	Girman *et al*., 2001
Asian dog	East Asia	213	4.34	0.58	Kim *et al*., 2001
German Shepherd dog breed	Finland, Europe	25	6.4	0.64	Koskinen & Bredbacka 2000
Bedlington Terrier dog breed	Finland, Europe	25	5.2	0.56	Koskinen & Bredbacka 2000
Wirehaired Dachshund dog bred	Finland, Europe	25	8.0	0.72	Koskinen & Bredbacka 2000
Australian Shepherd dog breed	Australia	20	5.5	0.66	Puja *et al*., 2005
Dingo (*C. lupus dingo*)	Australia	20	4.6	0.52	Puja *et al*., 2005
Red wolf (*C. rufus*)	North America	40	5.3	0.55	Roy *et al*., 1994
Grey wolf (*C. lupus*)	North America	103	4.5	0.62	Roy *et al*., 1994
Golden jackal (*C. aureus*)	Kenya, East Africa	20	2.0	0.52	Roy *et al*., 1994
Coyote (*C. latrans*)	California, USA	457	-	0.73	Sacks *et al*., 2004
Ethiopian wolf (*C. simensis*)	Ethiopia, East Africa	22	2.4	0.24	Gottelli *et al*., 1994
Grey wolf (*C. lupus*)	Italy	105	4.4	0.49	Luchhini *et al*., 2004
Grey wolf (*C. lupus*)	Latvia, Europe	47	6.8	0.73	Luchhini *et al*., 2004
Grey wolf (*C. lupus*)	North America	2025	-	0.74	Charmichael *et al*., 2007
Arctic fox (*Alopex lagopus*)	North America	1063	-	0.78	Charmichael *et al*., 2007
Grey fox (*Urocyon cinereoargenteus*)	Texas, USA	469	6.3	0.80	Deyoung *et al*., 2009

Geographical (spatial distance and topographic barriers), historical factors such as past colonization, range expansion or isolation in different glacial refugia (Hewitt 1996, 2000; Taberlet 1998) may explain the population genetic structure of a species. Indeed, an increasing number of species that cannot be explained by either geographical or historical factors concern large and medium-sized carnivoran mammals with extensive continuous distribution: grey wolf, *Canis lupus* (Carmichael *et al*., 2001; Geffen *et al*., 2004), coyote, *Canis latrans* (Sacks *et al*., 2004), lynx, *Lynx lynx* and *Lynx canadensi*s (Ruesness *et al*., 2003a,b), puma, *Puma concolor* (McRae *et al*., 2005), and for arctic fox, *Alopex lagopex* (Dalen *et al*., 2005). High mobility of these animals and their ability to cross most of potential topographic barriers (such as rivers or mountain ranges) minimize the influence of geographical factors on gene flow and reduce the effects of historical events, so that the effect of ecological factors may be more prominent (Pilot *et al*., 2006).

No genetic structure was detected for golden jackal in Western India. There was no evidence for unique genetic clusters within these two populations. A high rate of dispersal appears a likely explanation for the pattern of population structuring. The golden jackal is one of the most mobile terrestrial mammal that disperse rapidly over a large distance comparable to wolves that can disperses upto 900 km (Fritts 1983; Mech & Boitani 2003). Linear movement of golden jackal of >40 Km was recorded by Jhala (Pers. Comm.) Dispersing wolves were reported to successfully cross four-lane highways and circumvent large lakes and cities (Mech *et al*., 1995a; Merill & Mech 2000; Wabakken *et al*., 2001). Long-distance dispersal capabilities combined with the ability to occupy a variety of habitat imply high rates of gene flow that reduces genetic differentiation among local populations. Comparable studies of golden jackal population structure have not been reported from other parts of the species' range. Studies of widely distributed species of carnivores have revealed that long-distance dispersal can result in weak genetic structuring at

spatial scale (Deyoung *et al*., 2009; Carmichael *et al*., 2007; Hollatz *et al*., 2011).

The landscape of Kachchh is unique in the sense that it has evolved essentially as a result of several phases of tectonic movements since the Late Jurassic (Biswas 1987, 2005). Quaternary tectonics has primarily influenced the shaping of the present landscape, which shows an obvious conformity with the structural and tectonic set up. Based on the known data for Quaternary geological aspects, there are two main areas of Quaternary sedimentation in Kachchh, one is the vast saline tract of the Little Rann and Great Rann of Kachchh which is a product of drying up of a pre-existing shallow sea and the Banni plain which forms a sort of transition between the Kachchh mainland and the Ranns. The other is the southern coastal plain of mainland where recent studies have revealed thick Late Quaternary sequences which are exposed along the incise cliffs of the Southern flowing rivers. It has also been suggested that the Ranns were submerged under sea-water until ~2 ka which correlates with the termination of lacustrine deposition in Nal and estuarine terrace deposition in the Mahi and Narmada valleys resulting in drying up of Ranns providing a unique and rare ecosystem to golden jackal. Thus, taken together, the geologic and genomic results indicate that the Little Rann of Kachchh was not a barrier for the movement of golden jackal. Jackal populations occur all along the edge of the Little Rann as well as on many of the islands within the Rann itself (Jhala Pers Comm.). This suggests that after the drying up of the Rann, jackals have colonised the new habitats resulting in a high genetic exchange due to the high mobility of the species obliterating any geographic structure that may have existed between the Kachchh and mainland of Gujarat.

V.5 Conclusion

The study has presented the fundamental steps (sampling, DNA extraction and analysis) used to study the genetics of Indian golden jackal. The DNA extraction techniques were applied following a well described and validated protocol. All samples provide good quantities and quality of DNA. The use of ten domestic dog microsatellite markers on golden jackal genetic material was also successful. PCR amplification gave good results for all the markers used.

The heterozygosity was high and comparable to other studies on golden jackal and of other canids. Higher heterozygosity values make Indian jackal highly diverged and harbor high genetic variability than Kenyan, Serbian jackal and any other canid reported till date (see discussion). Being separated by a hostile Rann habitat, very low differentiation and inbreeding resulted in high gene flow between and among the Bhal and Kachchh populations. A weak genetic structure was detected for golden jackal population in western India with no evidence for unique genetic clusters which implies that landscape and habitat feature have a minimal effect on mixing of jackal populations and golden jackals were genetically homogeneous in Western part of India. Thus, genetically it can be stated that golden jackals have a single continuous population in Western India. Mitochondrial and nuclear marker study is suggestive of a large intermingling population capable of long distance dispersal in consonance with the known ecology of the species.

GENERAL CONCLUSIONS

This study laid down the ground for better understanding of molecular genetic aspects of Indian golden jackal in relation to golden jackal across their range and other wolflike canids. The results elucidate the global phylogenetic status of golden jackal in order to understand its systematic position in the genus *Canis*, its relationship with other canid species in India, as well as its genetic structure in Gujarat, a Western state of India. Molecular markers used for the study (mitochondrial CR and Cytb for phylogenetic study while microsatellites for population genetic study) amplified successfully their respective regions and were found suitable for molecular studies of the species.

Global systematic position of Indian golden jackal among canids was in agreement with the findings of previous studies. Results of phylogenetic study represented golden jackal as a member of the monophyletic clade including Indian peninsular wolf, Himalayan wolf, and Indian feral dog. No sign of mitochondrial introgression was found between any of these species. Thus, the possibility of hybridization was unlikely for these species in natural populations. Golden jackals from India were found having higher mtDNA diversity than its Serbian conspecifics. No clear genetic structure but an indication of demographic range expansion was found in golden jackal. A hypothesis was also proposed here to explain the reason behind higher mtDNA diversity and range expansion compared to larger sympatric canids of India i.e. Indian wolf lineages. Higher values of nucleotide diversity in golden jackal were indicative of demographic changes in recent past and I propose that India may be the centre of radiation of golden jackal if nucleotide diversity is confirmed to be higher in India than in other parts of the world. Thus, further sampling throughout Africa, Europe, Middle East and South East Asia is needed to confirm this hypothesis.

Furthermore, a weak genetic structure with no evidence for unique genetic clusters within the populations was detected for golden jackal in Gujarat, Western India.

Additionally, the golden jackal populations of Kachchh and Bhal regions were found to be contiguous across Gujarat.

Although golden jackals are slated Schedule III species and also declared "Species with least concern" in India, the present research contributes to the practical understanding of population genetic parameters of the species which will generate a baseline data for future studies and monitoring of golden jackal in India and elsewhere.

My study shows that Indian golden jackals show high genetic diversity both for mitochondrial and nuclear regions with no geographical structure. This finding is important for designing conservation strategies for the species in light of recent declines of all canid species within India caused by human population growth, habitat loss and infrastructural development.

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