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Deciphering evolutionary history of three Indian carnivore species: Phylogenetic analysis using cytochrome b gene sequence of mitochondrial DNA

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Abstract
Phylogenetic analysis fundamental in investigations related to origin, ancestory and evolutionary history of wild carnivores. It provides not only vital information regarding origin and evolution of genes, genomes and species but also aids in ecological and behavioural studies besides predicting quantum and direction of future evolutionary trends in living organisms. In the present study cytochrome b gene sequence of mitochondrial DNA obtained from non-invasive samples of Indian leopard (*Panthera pardus fusca*), domestic dog (*Canis lupus familiaris)* and domestic cat (*felis catus*). These study sequences were phylogenetic ally analysed with reference sequences of cytochrome b gene of nine species of order *carnivora* obtained from NCBI gen bank database belonging to both *caniformia* and feliformia monophyletic assemblages. The genetic relationships, genetic distance and ancestory, diversification and speciation events of these carnivore species were studied after interpretation of the phylogram.

Keywords: Phylogenetic analysis, cytochrome b, Indian leopard, domestic dog, domestic cat

1. Introduction
The genetic data highlighting evolutionary trends and phylogenetic relationships of animals is very important for formulation of conservation plans or for understanding their ecology and biology [3, 24]. In the past evolutionary history of wild carnivores has been investigated on various morphological and molecular grounds, particularly a spectrum of molecular techniques have been used to decipher evolutionary history of order *carnivora* including protein electrophoresis [1], allozyme data [5], karyology [13], endogenous retroviruses [16], sex chromosome linked genes [19, 20] and chemical signals [22]. However more recently phylogenetic studies have been focused using mitochondrial DNA genes like cytochrome b, 16s r RNA and 12s rRNA [27, 15, 8]. Since mitochondrial DNA contains some highly conserved regions, the primary use of mitochondrial DNA can be phylogeny, phylogeography and identification of genetically distinct units for conservation but it could also be used to identify species hybridization and differentiate patterns of male and female gene flows [26] further in samples containing very small amount of DNA such as faeces and hair materials, mitochondrial DNA might be the only source for analysis and one of the regions of mt DNA extensively used for phylogenetic links between various species was identified as a fragment of gene coding for cytochrome b which could be amplified using a single pair of universal primers in a standard PCR reaction [2].

The order *carnivora* is subdivided into two suborders *caniformia* and feliformia which are regarded as monophyletic assemblages. Caniformia includes family canidae, ursidae, procyonidae, mustelidae, phocidae, odobenidae and Otsheridae. The feliformia includes family viverridae, herpestidae, *hyanidae* and *felidae* [8, 28, 29]. However the phylogenetic relationships among different families of carnivores has been disputed and is not well established. There are many uncertainties regarding interfamilial affinities within the *caniformia*, although there is general consensus for the earliest divergence of the family canidae [31, 27, 10] conflicting phylogenetic hypothesis exists for other members of *caniformia* lineage that evolved subsequently. The evolutionary relationships among members of feliformia lineage also remains contentious partially due to recent and rapid radiations in the Pliocene, individual speciation events occurring less than 1 million years ago and probable introgression between lineages following their divergence [12]. The pantherine lineage is believed to have diverged from other lineages of family *felidae* only less than 11 million years ago and their closely

related medium sized cats of genus *neofelis* diverged from pantherines approximately 6million years ago [17, 29, 11, 13, 18].
India is one of the 12 mega biodiversity nations of the world, animals belonging to order *carnivora* are widely distributed in forest landscape ranging from subtropical areas of southern India to northern Himalayan temperate eco-climatic zone. In the present study DNA was extracted from non-invasive faecal samples of three species of order c*arnivora* belonging to different lineages Indian leopard (*Panthera pardus fusca*), domestic dog (*Canis lupus familiaris*) and domestic cat (*Felis catus*). A partial sequence of mitochondrial cytochrome b gene was amplified using specific primers. The study sequences of cytochrome b obtained in this study were analysed with *cytochrome b* gene sequences of nine species of order *carnivora* representing different families and lineages i.e coyote (*Canis latrans*), wild dog (*Cuon alpinus*), Hyena (*Hyaena hyaena*), red fox (*Vulpes vulpes*), cheetah (*Acinomyx jubatus*), caracal (*Caracal caracal*), ocelot (*Leopardus pardalis*), cougar (*Puma concolor*) and wild cat (*Felis silvestris*).

2. Materials and Methods

Scat samples were collected from leopards (*Panthera pardus*) at Arignar Anna Zoological Park (AAZP) Vandalur, Chennai. Similarly, faecal samples were collected from domestic dogs (n=15) and cats (n=15) from clinical block and inpatient ward of Madras Veterinary College, Chennai. Surface scrapings of scat samples were taken using sterile surgical blades (BP blades) in labelled sterile plastic containers and immediately transferred to laboratory in ice flask for storage at -20ºC until further processing.

2.1 DNA extraction protocol

From each sample, 200mg of scat was weighed and transferred into labelled 2ml micro centrifuge tubes and 1 ml inhibitex buffer was added to each tube followed by vortexing, until the sample was thoroughly homogenized. The samples were centrifuged for a minute to pellet scat particles. Following this, new 1.5 ml labelled centrifuge tubes were taken and 25 µl of proteinase k was added to each tube. 600 µl of supernatant was pipetted out of 2ml tubes and was transferred into new 1.5 ml tubes containing proteinase k. Then 600 µl buffer AL was added to each tube followed by vortexing for 15 sec and incubated at 70 ºC for 40 minutes. After incubation, 600 µl of ethanol (96-100%) was added to lysate, mixed by vortexing and 600 µl of lysate from each tube was transferred into labeled QIAamp mini spin columns followed by centrifugation at 14000 rpm for 1 minute. The spin columns were then placed in new 2ml collection tubes and the previous tubes were discarded. Again 600 µl of lysate was charged on each column from respective micro centrifuge tubes followed by centrifugation at 14000 rpm for 1 minute. The procedure was repeated until all the lysate from tubes was transferred to spin columns. The QIA amp mini spin columns were then transferred into new collection tubes and 500 µl of buffer AW1 was added to each spin column and were then centrifuged for 1 min at 14000 rpm. The spin columns were again placed in new collection tubes and 500 µl of AW2 was added to each tube followed by centrifugation at 14000 rpm for 3 minutes. Finally the spin columns were placed in new labelled 1.5 ml micro centrifuge tubes and 100 µl of buffer ATE was directly added to membrane of each spin column and were incubated for 10 minutes at room temperature. The tubes containing spin columns were centrifuged at 14000 rpm for 2 minutes to elute the DNA and the extracted DNA was stored at -20 degree Celsius until further processing.

2.1.2 Amplification of target gene sequence

For the present study, mitochondrial DNA i.e Cytochrome b was chosen, primarily because of the presence of some highly conserved regions within these sequences that exhibit maximum inter-species variation and minimum variation between individuals of same species. The mitochondrial DNA also has higher number of copies per cell than nuclear DNA, resulting in higher amplification success rates than nuclear DNA. For the amplification of Cytochrome b gene, a universal forward primer developed by[25] and species specific reverse primers for all the three species developed by[23] were chosen for the present study.

Sequence of primers and expected PCR product size

Primer		Sequence (5'-3')	Product size
Reverse	Leopard	GCCACCAATTCACGTCAGGGCT	1067 (bp)
	Cat	TTTCCCTCAGATACATTCTACTAGTTCAGTC	550 (bp)
	Dog	GAGTAGGAGTAAGGCTCCTAGGATA	759 (bp)

The annealing temperature of each primer set was determined by carrying out gradient PCR, based on the melting temperature of the primer set. The temperature which provided a good and specific PCR product yield was chosen to be the annealing temperature of the primer set. The final reaction volume of 50 µl was made, with following composition:

PCR Reagents

S. No.	PCR Reagents	Quantity (µl)
1.	PCR assay buffer (10X)	4.4
2	$MgCl_2$ (1.5 mM - 3.0mM)	5
3	dNTPs (each at 200 µM)	3
4	Primers: Forward (10 p moles) and Reverse (10 p moles)	3
5	Taq DNA polymerase (1 unit)	0.6
6	Template DNA	9
7	Nuclease free water	25
	Total	50

2.2. PCR Protocol
2.2.1. Leopard

The amplification of Cytochrome b gene in leopards was carried out with following PCR conditions:

Step	Process	Temperature	Duration
1	Initial denaturation	95 °C	5 min
2	Denaturation	95 °C	45 sec
3	Annealing	57.5 °C	45 sec
4	Extension	72 °C	1 min
5	Back to steps 2 to 4	35 cycles	
6	Final extension	72 °C	7 min
7	Hold	4 °C	Until the samples were removed

2.2.2 Domestic cat

The amplification of Cytochrome b gene in cat was carried out with following PCR conditions:

Step	Process	Temperature	Duration
1	Initial denaturation	95 °C	5 min
2	Denaturation	95 °C	40 sec
3	Annealing	60.5 °C	35 sec
4	Extension	72 °C	35 sec
5	Back to steps 2 to 4	35 cycles	
6	Final extension	72 °C	5 min
7	Hold	4 °C	Until the samples were removed

2.2.3 Domestic dog

The amplification of cytochrome b gene in dog was carried out with following PCR conditions:

Step	Process	Temperature	Duration
1	Initial denaturation	95 °C	5 min
2	Denaturation	95 °C	40 sec
3	Annealing	59.5 °C	35 sec
4	Extension	72 °C	45 sec
5	Back to steps 2 to 4	35 cycles	
6	Final extension	72 °C	5 min
7	Hold	4 °C	Until the samples were removed

The PCR products were checked on 2 percent agarose gel. The electrophoresis was carried out at a voltage of 70 V and 500 mA current for 50 minutes. The bands developed were observed in a GelDoc (Bio-Rad, USA) system and upon confirmation of the size of the amplified products, PCR was performed for eight leopard, four dog and four cat samples for cytochrome b gene. The PCR products of cytochrome b gene were subsequently sequenced.

2.3 Phylogenetic Analysis
The nucleotide sequences of cytochrome b gene of leopard (*Panthera pardus*), dog (*canis lupus familiaris*) and cat (*Felis catus*) were analysed using Basic Local Alignment Search Tool (BLAST) of NCBI [6] and phylogenetic relationship was determined among the cytochrome b gene sequences using "phylogeny. fr" in " A La Carte" mode [7] using 100 bootstrap value and a phylogenetic tree (phylogram) was constructed. The nucleotide sequences of cytochrome b gene used in the phylogenetic analysis were retrieved from NCBI Gen bank database and the sequences were *Panthera pardus* (NC_010641.1), *Felis catus* (NC_001700.1), *Canis lupus familiaris* (NC_002008.4), *Canis latrans* (KT4447698.1), *Cuon alpinus* (KT447690.1), *Hyaena* (AY048788.1), *vulpes* (AB 292765.1), *Acinomyx jubatus* (NC_005212.1), *Leopardus pardalis* (KU253484.1), *Puma concolor* (KU253489.1) and *felis silvestris* (KP202273.1).

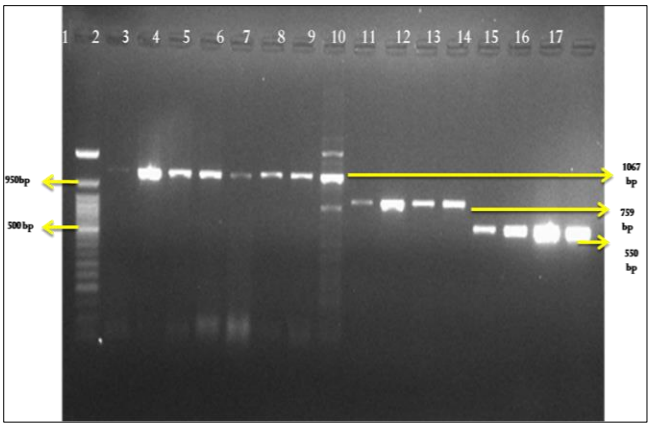

Fig 1: Agarose gel electrophoresis showing PCR products of *cyt b* gene of leopard, domestic cat and dog. Lane 1: 50 bp DNA ladder; Lanes 2-9: PCR amplicons of *cyt b* gene of leopards; Lanes 10-13: *cyt b* gene of domestic dog and Lanes 14-17: *cyt b* gene of domestic cat.

3. Results and Discussion
In the present study, sequence analysis of cytochrome b gene proved useful in delimiting the three species of Indian carnivores into separate clades. It is also evident from phylogram that *Canis lupus familiaris* belonging to family canidae is distantly related to *Felis catus* and *Panthera pardus fusca* of family *felidae*, however they have had a common ancestory before diversification. The high bootstrap values also indicate that *Felis catus and Panthera pardus* might have separated recently, this is in agreement with the findings of earlier studies conducted by various authors [17, 29, 11, 13, 18], who have reported that pantherine lineage of family felidae has diverged from other lineages of the family including genus *felis* only less than 11 million years ago in pilocene era. The phylogram further revealed formation of two distinctively separate clusters for the canine and feline species compared in this study showing occurrence of greater genetic distance between *caniformia* and feliformia suborders. Originating from a common ancestor *caniformia* have evolved earlier with subsequent diversification and speciation. On the other hand feliformia has evolved much later with events of recent diversification and speciation this is in concurrence with the analysis made by some authors who opined that feliformia lineage has had some recent and rapid radiations in the pilocene with speciation events having taken place less than 1 million years ago[12]. Hyena (Hyaena hyaena) which belongs to family *Hyanidae* has a closer genetic relationship with *caniformia* especially with canines belonging to vulpini tribe like red fox (*Vulpes vulpes*). The morphological, ecological and behavioural resemblance of hyena with canines can be attributed to this genetic relationship and common ancestory.
The phylogram also showed occurrence of sub-groups within a cluster which indicates existence of more genetic similarity and less genetic distance between the species of a sub-group than the other members of the particular lineage. Among the canines, red fox (*Vulpes vulpes*) that is categorized in separate vulpini tribe depicts discrete genetic dissimilarity and greater genetic distance from the members of canini tribe including Domestic dog (*Canis lupus familiaris*), Dhole or Wild dog

(*Cuon alpinus*) and Coyote (*Canis latrans*). Among the members of family Felidae sub-grouping was found to occur between Leopard (*Panthera pardus*) and Ocelot (*Leopardus pardalis*), Domestic cat *(Felis catus*) and Wild cat (*Felis silvestris*) and between Cougar (*Puma unicolor*) and Cheetah (*Acinomyx jubatus*). The findings could be a possible explanation to similar phenotypic characters, habitat ranges, feeding habits and prey preferences observed in the species belonging to same sub-group within a cluster. The nucleotide sequences of cytochrome b gene obtained in this study were found to be homologous with the reference sequences of leopard, domestic cat and dog obtained from NCBI Genbank, therefore no genetic dissimilarity was found to occur between the study sequences and their respective reference sequences.

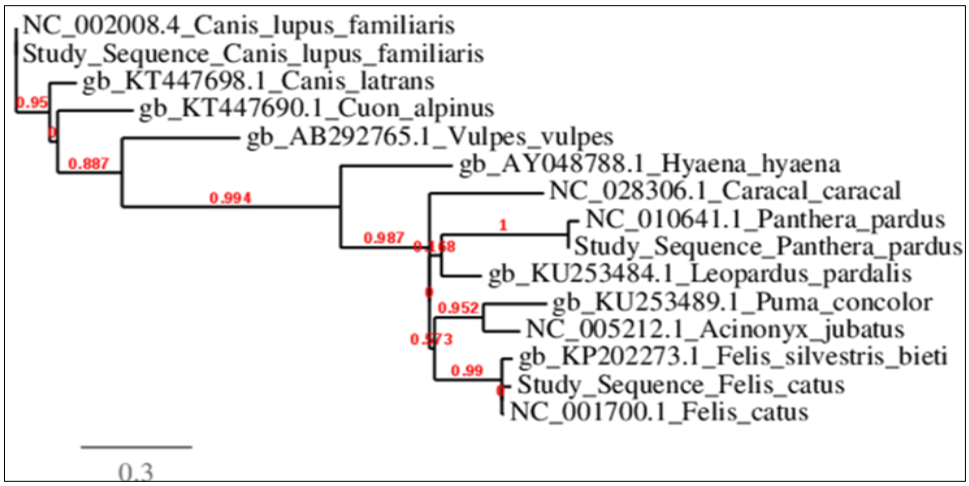

Fig 2: Phylogram showing Phylogenetic Analysis of Partial cytochrome b gene sequences in feline and canine species

4. Acknowledgement
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5. Conclusion
The findings of present study successfully highlighted the feasibility of non-invasive faecal DNA extraction as a source for providing sufficient and reliable genetic material that can be used for studying phylogenetic relationships among different species of animals and other downstream applications. The mitochondrial DNA marker cytochrome b gene having some highly conserved sequences with minimum intra-species and maximum inter-species variations can be effectively employed to differentiate animal species with varying degree of genetic relationships into distinct lineages and separate clades highlighting their evolutionary connections, diversification and the resultant genetic distance arising over a period of time. The nucleotide sequences of cytochrome b gene obtained in this study were found to be homologous with the reference sequences of leopard, domestic cat and dog obtained from NCBI Gen bank, therefore no genetic dissimilarity was found to occur between the gene sequences of animals of same species belonging to different geographical regions.

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