

Mitochondrial DNA sequence divergence among big cats and their hybrids

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Mitochondrial DNA sequence variation was used to distinguish between various big cat species and their hybrids. Mitochondrial D loop sequencing revealed the presence of only one haplotype among the Asiatic lions while the hybrids (Asiatic × African) exhibited a number of haplotypes, which showed homology with the African lion sequence. This strongly indicates the maternal contribution of African lions in the hybrid population. The sequence divergence reveals the two subspecies must have split about 80,000 to 100,000 years ago. Similar analysis also helped in distinguishing between Indian and Siberian tigers. Microsatellite studies had earlier shown the presence of Siberian tiger alleles in the Dhudhwa tiger reserve population. Mitochondrial cytochrome b gene sequence analysis of the hair samples from suspected Indian and Siberian tiger hybrids of the Dhudhwa tiger reserve revealed the presence of Indian tiger mitochondrial DNA haplotype.

THE precise definition of the taxonomy of a species has become essential to develop effective conservation strategies. The advent of molecular techniques in the field of conservation biology has provided an opportunity for the quantification of differences at the species and subspecies level and the precise characterization of taxonomic units¹. Multilocus DNA fingerprinting, microsatellites, RAPD and mitochondrial DNA analysis have proved to be invaluable tools in the reconstruction of phylogenetic relationships of populations within species²⁻⁴. The level of distinctiveness among populations is of extreme importance for identifying source populations for augmentation or reintroduction programmes. Mitochondrial DNA (mtDNA) is strictly maternally inherited and because of the constant mutation rate serves as a molecular clock, providing valuable clues about the genetic history of species^{5,6}. Mitochondrial DNA analysis of the humpback whale *Megaptera novaengliae* helped in the definition of its three subpopulations as separate conservation units⁷. Similar use of mtDNA sequence analysis has been done to detect hybridization in red wolf *Canis rufus* and coyotes⁸, the endangered Florida panther *Felis concolor coryi* and individuals from a local zoo stock of South American origin⁹. In this study, we have used mtDNA sequence analysis to assess

hybridization and introgression of African lion genes in Asiatic lions and the subspecies characterization of Indian and Siberian tigers.

The Species Survival Programme (SSP) for the critically endangered Asiatic lion was undertaken in 1981 to assist its conservation. However, it was discovered that some of the animals used in this programme were hybrids of Asiatic and African lions¹⁰. O'Brien and his group using allozyme analysis showed that the polymorphism observed in 3 of the 46 loci tested could be due to the African lineage. This raised serious doubts about the purity of the Asiatic lions housed in the various zoos in India. In our earlier study¹¹, using CA repeat microsatellites, it was shown that the hybrid lions can be differentiated from the pure Asiatic ones at loci Fca 77 and Fca 126. Pure Asiatic lions (lions of the Gir Forest Sanctuary, those housed in the Sakkarbaug zoo, Junagad and the lions whose parentage can be traced to the above are recognized as pure Asiatic lions according to the International Studbook for Asiatic lions) exhibited homozygosity and no variation while the hybrids showed variation and heterozygosity at these loci.

Twenty years ago, in an attempt to increase the genetic diversity of the tiger population, introduction of animals from different populations was planned in the Dhudhwa tiger reserve in India. The tiger cub chosen for reintroduction, a gift from Tycross zoo, UK, however, was discovered to be a hybrid between Indian tiger and Siberian tiger. This tiger managed to escape from a protected area into the wild and since then its existence has been a subject of controversy. Recently, a few tigers were spotted in the Dhudhwa tiger reserve, which had the typical Siberian tiger phenotype of white complexion, pale pelage, large head and wide stripes. Hair samples of tigers from the reserve were sent to our laboratory by Billy Arjan Singh, a conservationist, for microsatellite and mitochondrial sequence analyses. In this study we report the mtDNA analysis of the hair samples.

Materials and methods

Samples

Blood samples of Asiatic lion (*Panthera leo persica*), hybrids between Asiatic lion and African lion (*Panthera*

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leo leo), Indian tiger (*Panthera tigris tigris*) and Siberian tiger (*Panthera tigris altaica*) were collected from various zoos in India from the femoral vein by immobilizing the animals in squeeze cages or by anaesthetizing them. Genomic DNA was prepared from blood using standard procedures.

0.1 g of skin with hair was incubated overnight at 55°C in a 5% Chelex (BioRad, USA) solution. The sample was then kept at 100°C for 8 min and subsequently spun at 12,000 rpm for 10 min. The supernatant was treated with GeneClean Bio 101 kit and DNA was eluted from glass milk at 55°C. The DNA was then used for subsequent PCR reactions.

Mitochondrial D loop analysis

Mitochondrial D loop was amplified from whole cell DNA using the following primers¹²,

5' GCATCTGGTTCTTACTTCAGG 3' (forward)
5' ATTTTCAGTGTCTTGCTTTT 3' (reverse).

The reactions were performed with 50 ng of DNA, 5 pmoles of each primer, 200 µM of each dNTP and 1 unit Taq polymerase in a Perkin Elmer 9600 thermal cycler. The reaction conditions were 94°C 15 s, 48°C 15 s, 72°C 1 min for 30 cycles followed by 7 min extension at 72°C. Single product of around 1 kb was amplified. 400 ng of the product was treated with exonuclease I and alkaline phosphatase to remove the primers and dNTPs using Sequenase PCR product sequencing kit (United States Biochemical Inc.). The above reaction was directly sequenced with Perkin Elmer Ready Reaction sequencing kit with 5 pmoles of either of the primer. The sequencing reaction was electrophoresed and analysed on a ABI Prism 377 automated sequencer. Sequence comparisons were performed using the Autoassembler software. Cyclist (Stratagene) PCR sequencing kit was used to perform the manual sequencing. The products were resolved on a 8% polyacrylamide gel containing 8 M urea. The gel was dried, exposed to X-ray film and the sequences read manually.

Cytochrome b analysis

A 325 bp region of the mitochondrial cytochrome b gene was amplified using L14843 and H15149 primers¹³. The PCR conditions were initial denaturation of 2 min at 94°C followed by 35 cycles of 94°C 15 s, 50°C 15 s and 72°C 30 s. The product was sequenced manually as mentioned above.

Results and discussions

The mitochondrial D loop is composed of AT-rich stretches which flank the central conserved region (CCR).

The 3' end of the CCR contains several conserved sequence blocks (CSB), which have been implicated in the initiation of heavy strand replication. These CSBs are flanked by stretches of repetitive sequences (RS)¹⁴. DNA slippage followed by polymerase and endonuclease-facilitated repair is thought to be the primary mechanism for the evolution of these repetitive regions. This involves the mispairing of a slipped strand in an array of repeated motifs followed by polymerase repair which results in the gain or loss of repeat units¹⁵. Earlier studies have shown that, in carnivores, RS 3, which is present between CSB 1 and CSB 2, is highly variable in the arrangement of specific motifs and is heteroplasmic¹². The motifs are composed of simple sequence stretches which are variants of the basic motif 'ACGT'. The arrangement of these motifs is species-specific with the basic arrangement of motifs being conserved, like a signature. However, the number of repeats in each motif varies. The sequence arrangement is shown in Figure 1 (Genbank accession numbers AF088864, AF088865, AF088866). The sequence of RS 3 as motifs is shown in Figure 2.

While no variation was observed among the mtDNA of Asiatic lions, the hybrid lions showed extensive variation, indicating that many haplotypes exist in the population. High degree of homology observed between the hybrid and African lion sequences suggests the contribution of African lion founder females in the SSP. Average nucleotide diversity of 9% was observed between Asiatic and hybrid lion sequences. Applying the molecular clock rate of 1% substitutions every 8,000 to 10,000 years for D loop in humans, a divergence time of 80,000 to 100,000 years was obtained. This result is consistent with the findings of other molecular and palaeontological studies¹⁶.

It was hypothesized earlier that the Asiatic lions and many other species like the African cheetah and large number of mammals underwent a population bottleneck about 10,000 years ago at the end of the Pleistocene ice age¹⁷. The lack of variation in the mitochondrial sequence among the Asiatic lions and the tigers suggests that only a few mitochondrial haplotypes survived this bottleneck which were subsequently lost due to inbreeding. The African lions in the Serengeti National Park, because of their widespread distribution compared to the single relict population of Asiatic lions in the Gir forest, show markedly lesser effects of the bottleneck. The lions in the nearby Ngorongoro crater, which were derived from only a few lions, however, show the effect of the bottleneck similar to that of the lions of Gir forest¹⁸.

An average nucleotide diversity of 8% was observed among the Indian and Siberian tiger sequences, which translates to a divergence time of 80,000 to 100,000 years (Figure 1). Sequence of the cytochrome b region

		10	20	30	40	50	
1	1... 50	LIONDLOOP	GGGGGGTAAG	GGGGGTTTGT	TTAAGCTAAT	TGTTTACTAA	ATCAAAAAGT
4	1... 50		1111111111	1111111111	1111111111	1111111111	1111111111
2	1... 50	TIGERDLOOP	GGGGGGTAAG	GGGGGTTTGT	TTAAGCTAAT	TGTTTACTAA	ATCAAAAAGT
5	1... 50		1111111111	1111111111	1111111111	1111111111	1111111111
3	1... 50	SIBTIGDLOC	GGGGGGTAAG	GGGGGTTTGT	TTAAGCTAAT	TGTTTACTAA	ATCAAAAAGT
			60	70	80	90	100
1	51...100	LIONDLOOP	TTGCATGTGT	ATACGTGTAT	ACGTGTACGT	GTGTACGTGT	GTACGTGTGT
4	51...100		1111111111	1000110001	0011011111	1100001111	0000111101
2	51...100	TIGERDLOOP	TTGCATGTGT	ACGTGTACGT	GTGTATACGT	GTACGTGTGT	ACGTGTGTAT
5	51...100		1111111111	1000110000	1111111111	1111111111	1111111110
3	51...100	SIBTIGDLOC	TTGCATGTGT	ATACGTGTAC	GTGTATACGT	GTACGTGTGT	ACGTGTGTAC
			110	120	130	140	150
1	101...150	LIONDLOOP	ACGTGTGTAC	GTGTACGTGT	ACGTGTACGT	GTACGTGTAC	GTGTACGTGT
4	101...150		1111110111	1111111111	1111110000	1100110000	1111111111
2	101...150	TIGERDLOOP	ACGTGTATAC	GTGTACGTGT	ACGTGTGTAC	GTGTGTACGT	GTGTACGTGT
5	101...150		0011111000	1111111111	0001001100	1100110011	0100001111
3	101...150	SIBTIGDLOC	GTGTGTACGT	GTGTACGTGT	GTATACGTGT	GTACGTGTGT	ATACGTGTGT
			160	170	180	190	200
1	151...200	LIONDLOOP	ACGTGTACGT	GTGTACGCGT	ATACGTGTAC	GTGTACGTGT	GTACGTGTAC
4	151...200		0000110011	0011001000	0100110111	1111111111	0000110000
2	151...200	TIGERDLOOP	GTACGTGTGT	ACGTGTGTAC	GTGTGTATAC	GTGTACGTGT	ACGTGTACGT
5	151...198		0111111101	1111110111	1111011000	1101111111	10001100
3	151...198	SIBTIGDLOC	ATACGTGTAT	ACGTGTATAC	GTGTATACGT	GTATACGTGT	ATACGTGT
			210	220	230	240	250
1	201...250	LIONDLOOP	GTGTGTACGT	GTACGTGTGT	ACGTGTACGT	GTGTACGTGT	ACGTGTATAC
4	201...218		1100110011	00001111			
2	201...218	TIGERDLOOP	GTACGTGTGT	ACGTGTGT			
5	---		<==				
3	---	SIBTIGDLOC	<==				
			260	270	280	290	300
1	251...300	LIONDLOOP	GTGTACGTGT	ATACGTGTAC	GTGTATACGT	GTACGTGTAT	ACGTGTACGT
4	---		<==				
2	---	TIGERDLOOP	<==				
5	---		<==				
3	---	SIBTIGDLOC	<==				
			310	320	330	340	350
1	301...340	LIONDLOOP	GTGTACGTGT	ACGTGTGTAC	GTGTACGTGT	GTACGTGTGT	
4	---		<==				
2	---	TIGERDLOOP	<==				
5	---		<==				
3	---	SIBTIGDLOC	<==				

Figure 1. Comparison of the mt D loop sequences of Asiatic lions, Indian tigers and Siberian tigers. '1' represents a matching base, while '0' represents a mismatch.

		10	20	30	40	50
1	1... 39		3232322221	2322223232	2231232232	232232231
2	1... 50		3232322221	2322212322	2123212212	1212121212 1212123222
3	1... 48		3232322212	3222123222	2121212121	2123221232 21211222
4	1... 24		3232111111	1232322212	2221	
5	1... 37		3231112221	2322212322	2212111212	1212322
6	1... 47		3232111122	2222223221	2121212232	2322322321 2121131

Figure 2. Comparison of the mt D loop sequence motifs from hybrid lions (sequences 1 to 5) and Asiatic lion sequence. 1 = ACGTGTGT, 2 = ACGTGT, 3 = AT.

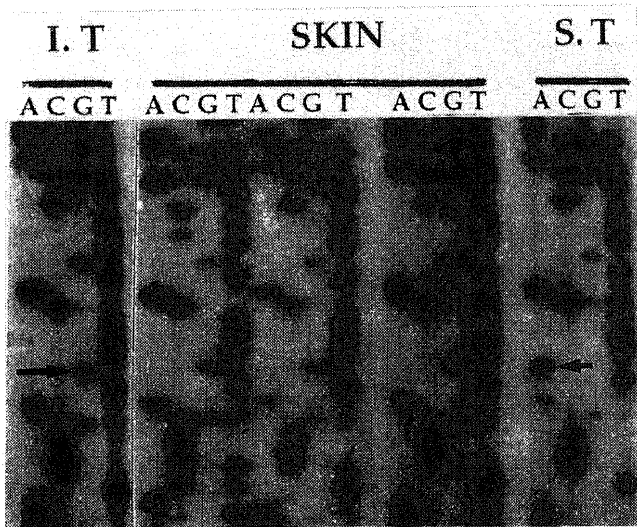


Figure 3. Autoradiogram of the cytochrome b gene sequence showing the presence of 'A' in Siberian tiger, while showing 'G' in Indian tiger and the skin and hair samples.

showed a single transition from 'G' to 'A' in all the Siberian tigers at position 342. Cytochrome b sequence from the hair samples showed 'G' in that particular position, indicating an Indian tiger haplotype (Figure 3). Since, mitochondria are strictly maternally inherited, the results indicate that these animals had an Indian tiger mother. Assuming that Tara had a Siberian father and an Indian mother, then all the progeny including Tara would exhibit only an Indian mitochondrial haplotype. While, if the mother had been Siberian and the father Indian, the F1 generation, i.e. Tara, would have a Siberian haplotype and the F2 generation would have Indian or Siberian haplotypes depending upon the female they mate with (Figure 4). The microsatellite data coupled with the present study suggest that the tigers of the Dhudhwa tiger reserve could be hybrids of Indian tiger and Siberian tiger. The D loop amplification was, however, unsuccessful.

Our present report raises several serious issues. First, if the tigers are indeed hybrids, what is the conservation status of such animals and the population? An example of this problem is the case of the American red wolf, which had hybridized with the other species. The Indian and Siberian tigers are considered different subspecies because of their geographical separation, otherwise, according to the cytochrome b, mitochondrial D loop and microsatellite data the split between the two could have occurred only 70,000 to 100,000 years ago, as in the case of lions where interbreeding is very much viable. Secondly, if the escaped tiger did breed in the reserve areas, as appears to be the case, then it would be the first instance of a successful reintroduction of zoo-bred tiger into the wild. As populations of tigers are reducing in size and getting fragmented, reintroduc-

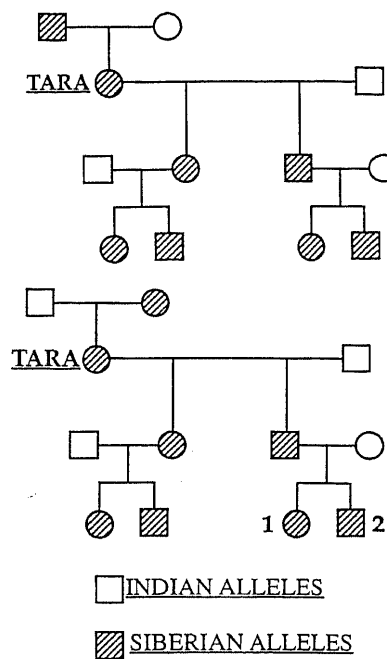


Figure 4. Hypothetical pedigrees showing the possibility of the presence of Indian tiger mtDNA haplotype in hybrid tigers. Animals numbered 1, 2 represent those which are hybrid possess an Indian tiger mtDNA haplotype.

tion programmes gain importance as genetically variant individuals from the zoo population can be reintroduced to invigorate the wild population. We have also developed a rapid method for isolating DNA from dried scats¹⁹. The protocol reduces the processing time drastically, thereby permitting the analysis of large number of samples. This non-invasive procedure opens up the possibility of large scale sampling of the wildlife. These results call for a detailed study of the tiger population of the Dhudhwa reserve in India and a reappraisal of our conservation policy, especially of tigers.

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