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 units[1]. Multilocus DNA fingerprinting, microsatellites, RAPD and mitochondrial DNA analysis have proved to be invaluable tools in the reconstruction of phylogenetic relationships of populations within species[2–4]. 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 units[7]. Similar use of mtDNA sequence analysis has been done to detect hybridization in red wolf *Canis rufus* and coyotes[8], the endangered Florida panther *Felis concolor coryi* and individuals from a local zoo stock of South American origin[9]. 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 lions[10]. 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 study[11], 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 primers[12],

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 primers[13]. 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 units[15]. 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 heteroplasmic[12]. 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 studies[16].

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 age[17]. 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 forest[18].

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 ATÇAAAAAGT
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 SIBTIGDLOO 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 SIBTIGDLOO 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 SIBTIGDLOO  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 SIBTIGDLOO  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  ---,---  SIBTIGDLOO   <==

                                  260       270       280       290       300
1  251...300 LIONDLOOP   GTGTACGTGT ATACGTGTAC GTGTATACGT GTACGTGTAT ACGTGTACGT
4  ---,---               <==
2  ---,---  TIGERDLOOP   <==
5  ---,---               <==
3  ---,---  SIBTIGDLOO   <==

                                  310       320       330       340       350
1  301...340 LIONDLOOP   GTGTACGTGT ACGTGTGTAC GTGTACGTGT GTACGTGTGT
4  ---,---               <==
2  ---,---  TIGERDLOOP   <==
5  ---,---               <==
3  ---,---  SIBTIGDLOO   <==
```

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.

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                                    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.

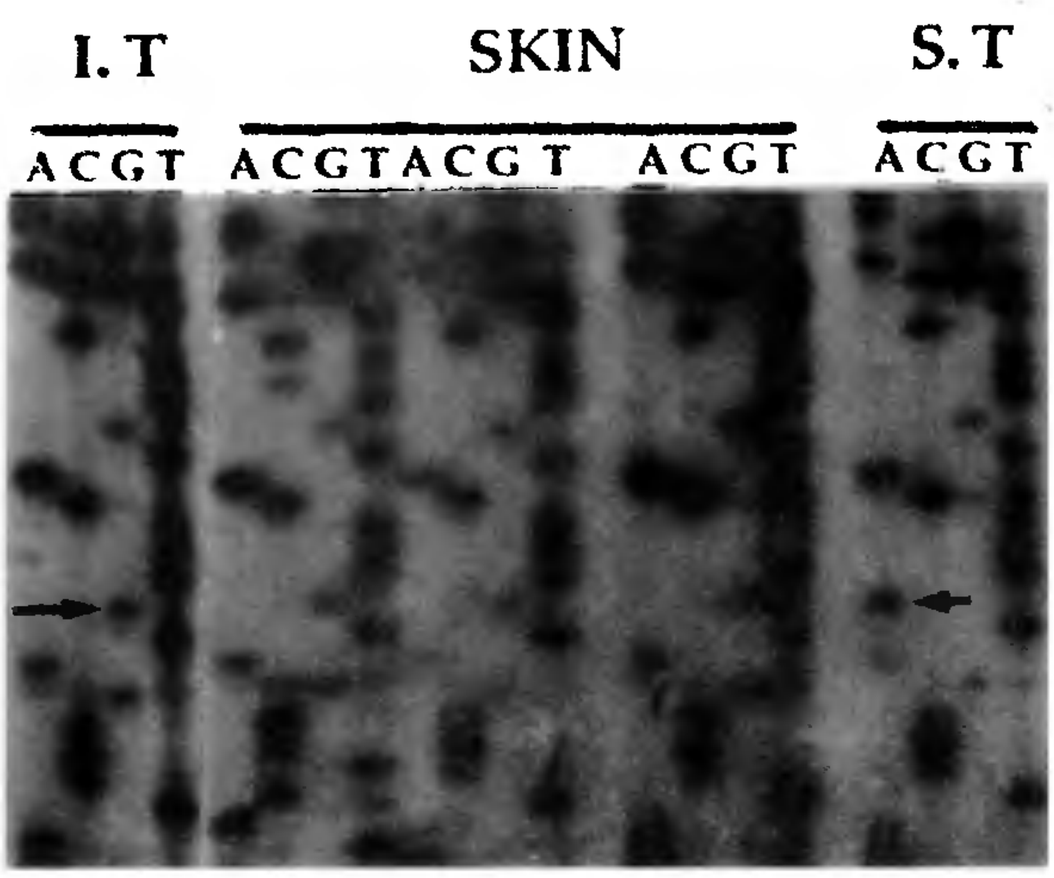

Figure 3. Autoradiogram of the cytochrome b gene sequence showing the presence of 'A' in Siberian tiger, while showing 'G' in Indian tiger and the skin and hair samples.

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, reintroduction 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 scats[19]. 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.

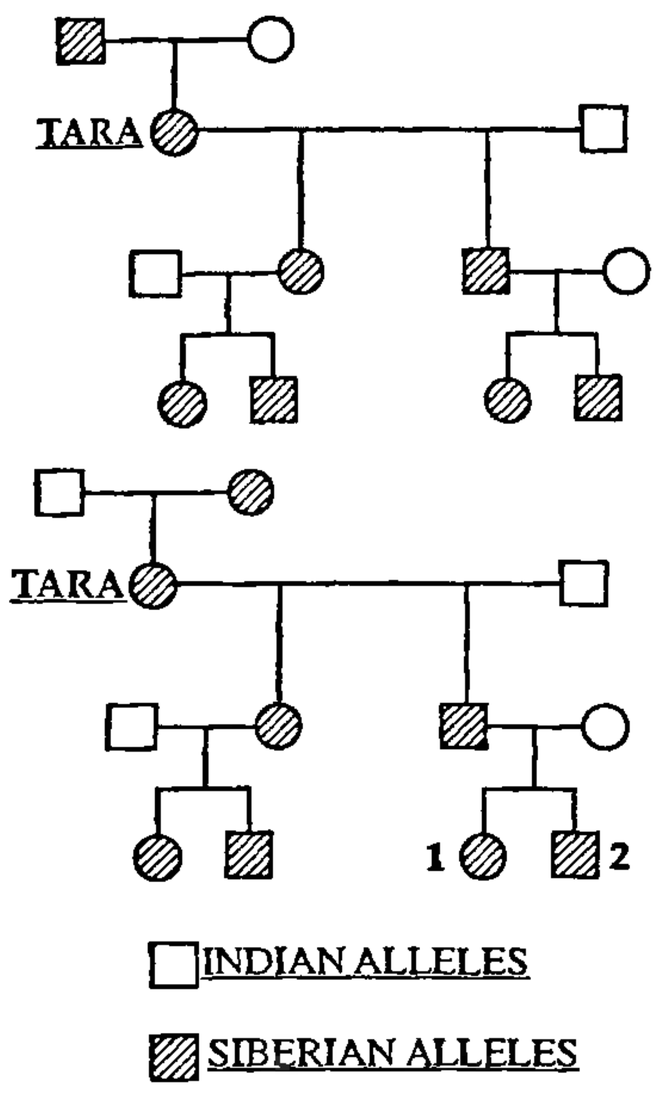

Figure 4. Hypothetical pedigrees showing the possibility of the presence of Indian tiger mtDNA haplotype in hybrid tigers. Animals numbered 1, 2 represent those which are hybrid possess an Indian tiger mtDNA haplotype.

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Assessment of inbreeding depression in big cats: Testosterone levels and semen analysis

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Studies on Asiatic lions earlier suggested that they were highly inbred and have very low levels of genetic variations. However, subsequent studies indicated higher degree of DNA polymorphism in Asiatic lions and Indian tigers and suggested that the low genetic variability was a characteristic feature of these species and was not the consequence of intensive inbreeding. Therefore, the present study was undertaken to ascertain whether inbreeding depression has set in these species. A total of 16 tigers and 7 lions from three Indian zoos were evaluated for inbreeding depression effects by analysis of semen samples with respect to spermatozoal number, percentage motile spermatozoa, percentage morphologically abnormal spermatozoa, ejaculate volume and fertilizing ability of spermatozoa. Majority of the animals exhibited good spermatozoal number, high percentage of motile spermatozoa and low incidence of abnormal spermatozoa unlike inbred animals, thus implying that inbreeding depression has not yet affected these animals. The high fertilizing ability of the semen samples and the high levels of serum testosterone further support the view that the Asiatic lions and Indian tigers are not completely inbred.

THE Indian tiger (*Panthera tigris tigris*) and the Asiatic lion (*Panthera leo persica*) are today the most critically endangered animals. The decimation of these mega cats to a few thousand tigers and a few hundred lions could be attributed to habitat shrinkage, large-scale indiscriminate hunting for sport and pleasure and due to poaching for their body parts which are in great demand in China, South East Asia, Middle East, Western Europe and USA[1]. Thus there is a need to conserve these mega cats and increase their numbers.

Species survival is critically dependent on reproductive performance which in turn seems to correlate with genetic heterozygosity[2–7]. Wildt *et al.*[7] based on a comparative study of the Asiatic lions of Gir forest and several African lions (*Panthera leo leo*) established a direct correlation between the lack of genetic variability in the Asiatic lion and the high incidence of morphological abnormal spermatozoa and low levels of the male steroid hormone testosterone[8,9] and predicted[7–9] that the Asiatic lion has suffered a population bottleneck followed by inbreeding. However, a recent study[10] on the tigers and lions of India based on randomly amplified polymorphic DNA (RAPD), microsatellite analysis of five repeat loci and multilocus fingerprinting indicated a higher degree of genetic heterozygosity than reported and they concluded that the low genetic variability may be a characteristic feature of these species and not the result of intensive inbreeding. The present study was, therefore, undertaken to evaluate the extent of inbreeding depression in the Indian tigers and the Asiatic lions based on semen characteristics such as spermatozoal number, percentage motile spermatozoa, incidence of abnormal spermatozoa, levels of serum testosterone and fertilizing ability of spermatozoa. Our analyses corrobo-

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