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Genetic variation in Asiatic lions and Indian tigers

Previous reports have suggested that Asiatic lions and tigers are highly inbred and exhibit very low levels of genetic variation. Our analyses on these species have shown much higher degrees of polymorphism than reported. Randomly amplified polymorphic DNA (RAPD) analysis of 38 Asiatic lions, which exist as a single population in the Gir Forest Sanctuary in India, shows an average heterozygosity of 25.82% with four primers. Sperm motility studies by our colleagues corroborate this data. In Indian tigers, microsatellite analysis of five CA repeat loci and multilocus fingerprinting using Bkm 2(8) probe on a population of 22 individuals revealed a heterozygosity of 22.65%. Microsatellite analysis at loci Fca 77 and Fca 126 revealed polymorphism amongst the Asiatic × African lion hybrids, which has enabled us to use these as markers to discriminate the pure Asiatic lions from the hybrids. A similar analysis was used to identify hybrids of Indian and Siberian tigers through polymerase chain reaction (PCR) amplification of hair samples. To ascertain the variation which existed before the population bottleneck at the turn of the present century, microsatellite analysis was performed on 50- to 125-year-old skin samples from museum specimens. Our results show similar levels of genetic variability as in the present population (21.01%). This suggests that low genetic variability may be the characteristic feature of these species and not the result of intensive inbreeding. DNA fingerprinting studies of Asiatic lions and tigers have helped in identifying individuals with high genetic variability which can be used for conservation breeding programs.

1 Introduction

The habitat of the Asiatic lion (*Panthera leo persica*) ranged across southwestern Asia, extending from Syria to Northern India, as recently as 200 years ago [1]. The subspecies became extinct in Syria, Iraq, Iran, Afghanistan and Pakistan in the latter part of the 19th century. At present, Asiatic lions exist as a single relict population isolated since the 1880s [1] in the Gir Forest Sanctuary and the surrounding forest in the Gujarat state in western India. Large-scale hunting had reduced the population to less than twenty at the turn of the present century. Conservation efforts have brought this number up to 350. O'Brien *et al.* [2–4] reported a total absence of variation at each of 46 allozyme loci in 28 individuals of Asiatic lions of Gir Forest in contrast to several African lion (*Panthera leo leo*) populations which showed moderate levels of allozyme variation at the same loci. Lack of genetic variation in the Asiatic lion was correlated with a high incidence of morphologically abnormal spermatozoa and low levels of circulating testosterone, a critical hormone for spermatogenesis [4]. Based on these observations, the prediction was made [2–4] that the Asiatic lion is a severely endangered species that has suffered a population bottleneck or a series of bottlenecks followed by inbreeding in their recent history. The Indian tiger (*Panthera tigris tigris*), is another critically endangered felid. The population of this animal has dwindled from 40 000 individuals at the beginning of this century to the present figure of approximately 3500. Poaching for bones and hair, which have purported medicinal properties, and habitat shrinkage have been the major causes for the drastic reduction in number. Furthermore, the lions and tigers presently kept in various Indian zoos are suspected to be hybrids between Asiatic and African lions, and Indian and Siberian tigers.

The present study was, therefore, undertaken (i) to examine the extent of inbreeding in the Asiatic lion and Indian tiger population, (ii) to identify pure Asiatic lion from the hybrid between Asiatic and African lion, and pure Indian tiger from the hybrid between Indian tiger and Siberian tiger, and (iii) to identify the lions and tigers showing extensive genetic variation for selective breeding for their proper genetic management. To answer the above questions we have used random amplified polymorphic DNA (RAPD) and microsatellite analyses. For RAPD, short arbitrary oligonucleotides were used as primers in a PCR reaction to generate a DNA pattern. The advantages of this technique are that no prior sequence information of the genomes under investigations is required, it covers the genome more extensively than other techniques, and minute amounts of DNA are sufficient to carry out the analysis. RAPD, therefore, has been used to study many animal and plant genomes [5–7]. Microsatellites, due to their abundance in the genome and high mutation rates, prove useful in addressing questions in dynamics of populations. They have been used in conservation biology to assess the levels of genetic diversity and plan future strategies as in the case of the bottlenecked wombat and endangered Ethiopian wolf [8, 9]. We have also used banded krait minor satellite DNA (Bkm)-derived 2(8) multilocus DNA finger-

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Nonstandard abbreviations: Bkm, banded krait minor satellite DNA; **OTU,** operational taxonomic unit; **RAPD,** randomly amplified polymorphic DNA

Keywords: Genetic variation / Randomly amplified polymorphic DNA / Microsatellites / Lions / Tigers / Conservation

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Table 1. Collection of blood samples of lions, tigers and tiger skin and hair samples from various zoos in India

Zoo	Animals	Number of animals (males + females)	International Stud Book No.
Chandigarh	Hybrid lion (Asiatic × African)	20 (12 + 8)	
	Indian tiger	2 (1 + 1)	
Calcutta	Hybrid lion (Asiatic × African)	10 (6 + 4)	
	Indian tiger	10 (7 + 3)	
	Tigon (male Indian tiger and female hybrid lion)	1 (0 + 1)	
	Litigon (male hybrid lion and female tigon)	2 (1 + 1)	
Bhubaneshwar	Hybrid lion (Asiatic × African)	18 (13 + 5)	
	Asiatic lion	2 (0 + 2)	304, 1017
	Indian tiger	43 (26 + 17)	
Guwahati	Hybrid lion (Asiatic × African)	2 (1 + 1)	
	Indian tiger	6 (4 + 2)	
Sakkarbaug Gir Forest	Asiatic lion	41 (18 + 23)	299, 312, 321, 323, 1201, 1224, 1225, 1236, 1237, 1243, 1256, 1258, 1272, 1274
	Indian tiger	5 (3 + 2)	
Hyderabad	Hybrid lion (Asiatic × African)	13 (7+ 6)	
	Indian tiger	5 (2 + 3)	
Darjeeling	Siberian tiger	5 (2+3)	

Total No. of hybrid lions (Asiatic × African) = 63
Total No. of Asiatic lions = 33
Total No. of Indian tigers = 71
Total No. of Siberian tigers = 5
Total No. of tigon = 1
Total No. of litigons = 2

Skin samples from National Museum, Calcutta (50–125 years old) of Indian tiger = 15.
Hair samples of hybrid between Siberian and Indian tiger = 2

printing to detect minisatellite variations. In order to probe into the genetic history of lions and tigers, microsatellite analysis on five CA repeat loci was carried out using skin samples from museum specimens (some of them 125 years old).

2 Materials and methods

2.1 Samples

Blood samples of the Asiatic lion (*Panthera leo persica*), Indian tigers (*Panthera tigris tigris*), hybrids between Asiatic lion and African lion (*Panthera leo leo*) and Siberian tigers (*Panthera tigris altaica*) from various zoos in India were collected from the femoral vein by immobilizing the animals in squeeze cages or by anesthetizing them (Table 1).

2.2 DNA isolation and multilocus fingerprinting

Genomic DNA prepared from blood [10] was digested with *Hinf*I and electrophoresed in a 0.9% agarose gel. Southern blotting and filter hybridization with Bkm 2(8) probe were done as described [11, 12].

2.3 Bkm 2(8) probe

The 2(8) probe is a Bkm-positive subclone of the *Drosophila* clone CS314 [13], subcloned in M13mp9, containing a 545 base pair insert consisting of 66 copies of GATA repeat, a highly conserved component of Bkm interspersed with a variable number of dinucleotide repeats of CA in several locations.

2.4 ^{32}P-labeling of probes

The ^{32}P-labeled single-stranded probe (specific activity of 0.7–3.0 × 10^8 cpm/μg) was prepared by using M13 universal primer [14], and [α-^{32}P]dATP (specific activity 3000 Ci/mmol; Jonaki, BARC, India).

2.5 RAPD analysis

The RAPD analysis was performed with 30 primers obtained from Operon Technologies (Alameda, CA, USA). The primers used for the detailed analysis were OPJ 13, OPAV 16, OPC 04, and OPC 02. PCR was performed with 50 ng of genomic DNA, 5 pmoles of the primer, 200 μM each of dATP, dGTP, dCTP, dTTP, 2 mM $MgCl_2$, and 0.5 U *Taq* polymerase. The reaction conditions were 94°C for 1 min, 37°C for 1 min, 72°C for 2 min, for 45 cycles, followed by a 7 min extension at 72°C. The PCR products were analyzed on a 1.4% agarose gel and the bands visualized by ethidium bromide staining under UV. Band sharing index and heterozygosity were calculated according to Yuhuki and O'Brien [15]. The heterozygosity was calculated as the average of the number of fragments differing in each pairwise combination divided by the total number of fragments.

2.6 Microsatellite analysis

The following CA repeat loci were analyzed [16].

Fca 35: 5′ CTTGCCTCTGAAAAATGTAAAATG 3′
5′ AAACGTAGGTGGGGTTAGTGG 3′
Fca 43: 5′ GAGCCACCCCTAGCACATATACC 3′
5′ AGACGGGATTGCATGAAAAG 3′
Fca 77: 5′ GGCACCTATAACTACCAGTGTGA 3′
5′ ATCTCTGGGGAAATAAATTTTGG 3′
Fca 90: 5′ ATCAAAAGTCTTGAAGAGCATGG 3′
5′ TGTTAGCTCATGTTCATGTGTCC 3′
Fca 126: 5′ GCCCCTGATACCCTGAATG 3′
5′ CTATCCTTGCTGGCTGAAGG 3′

One of the primers for each locus was 5′ end-labeled with gamma [γ-^{32}P]ATP (specific activity 3000 Ci/mmol; Jonaki). Reactions were performed with 50 ng of genomic DNA, 5 pmoles of each primer (one of them radioactively labeled), 200 μM each of dATP, dCTP, dGTP, dTTP, and 0.5 U *Taq* polymerase. The reaction conditions were 94°C for 30 s, 55°C for 15 s, 72°C for 15 s, 30 cycles, followed by a 7 min extension at 72°C. The PCR products were electrophoresed in an 8% denaturing polyacrylamide gel and exposed overnight to X-ray film (Kodak X-omat film) or visualized with Phosphor Imager (Molecular Dynamics, CA, USA) using ImageQuant software.

2.7 Skin and hair samples

Skin with hair (0.1 g) was incubated overnight at 55°C in a 5% Chelex (Bio-Rad) solution. The sample was then kept at 100°C for 8 min and subsequently spun at 12000 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 for microsatellite analysis.

2.8 Multivariate map-like graphical representation

The raw data obtained from fingerprinting using Bkm 2(8) and RAPD analyses were transformed into distance matrices according to Nei [17] which were then used to derive the relatedness among individuals (operational taxonomic units or OTUs). A new multivariate approach was used to obtain graphical relative positions of OTUs with respect to the computed genetic distances (Chandrika B.-Rao and K. C. Majumdar, submitted for publication).

3 Results

3.1 DNA fingerprinting

Multilocus fingerprinting with Bkm 2(8) probe was performed on 31 Asiatic lions using several restriction enzymes (*Hinf*1, *Taq*1, *Bst*N1, *Hae*III, *Mbo*1). No polymorphism was detected in the populations studied. However, in 22 tigers, Bkm 2(8) probe in combination with *Hinf*1 restriction enzyme revealed polymorphism (Fig. 1). Per individual, 25–27 bands were scored in the 1–8 kbp molecular weight range. The majority of polymorphic bands were found in the 2–6 kbp region. An average heterozygosity of 28% was observed (Table 2).

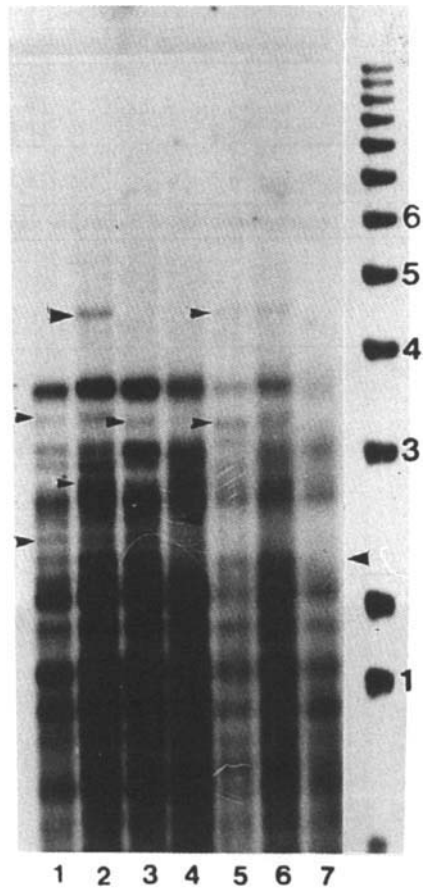

*Figure 1. Hinf*1 DNA profiles of 7 Indian tigers (lanes 1–7) from Bhubaneshwar zoo developed after hybridization with Bkm 2(8) probe showing polymorphism. Molecular weight marker lane represents 1 kbp ladder.

3.2 RAPD analysis

RAPD patterns were assessed in Asiatic lions and Indian tigers using 30 random primers. Four of these produced polymorphic pattern in Asiatic lions and were used for population studies (Figs. 2, 3). A total of 38 individuals were analyzed and an average heterozygosity of 25.82% was observed. The heterozygosities ranged from 16.71% to 34.39% for individual primers (Table 3). The primers which showed polymorphism in lions did not reveal any polymorphism in tigers.

3.3 Microsatellite analysis

Microsatellite analysis was carried out on five CA repeat loci which are polymorphic in the felids. Asiatic lions, however, did not show any variation at all at the five microsatellite loci analyzed. Analysis of locus Fca 77 (Fig. 4) and locus Fca 126 (Fig. 5) showed differences between the Asiatic lions and hybrid lions. While Asiatic lions were monomorphic and homozygous at these loci, the hybrid lions were polymorphic and not all alleles matched those found in Asiatic lions (Fig. 4, 5). These alleles are probably due to the contribution of African lion to their genome. Analysis at these loci was also able to confirm that two Asiatic lions in the Bhubaneshwar zoo were indeed pure Asiatic lions.

Table 2. Analysis of DNA profiles of Indian tigers developed with Bkm 2(8) probe

Population	Number of animals	Fragment size	% Heterozygosity
White skin color	7	2.0–8.8 kbp	20.0
Normal skin color	7	2.0–8.3 kbp	28.8
Heterozygous for skin color	8	2.0–5.5 kbp	23.0

Table 3. RAPD analysis of Asiatic lions

Primer	Number of animals	Average number of fragments scored/individual	% Heterozygosity
OPAV 16	38	8	30.54
OPD 05	36	7	16.71
OPC 04	39	8	34.39
OPJ 13	35	6	21.67

Average % heterozygosity = 25.82%

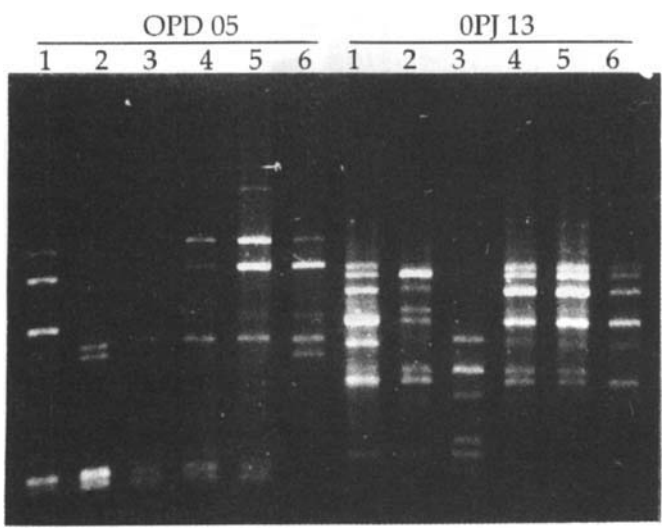

Figure 2. RAPD pattern using primers OPD 05 and OPJ 13 showing polymorphism in Asiatic lions from Sakkarbaug zoo.

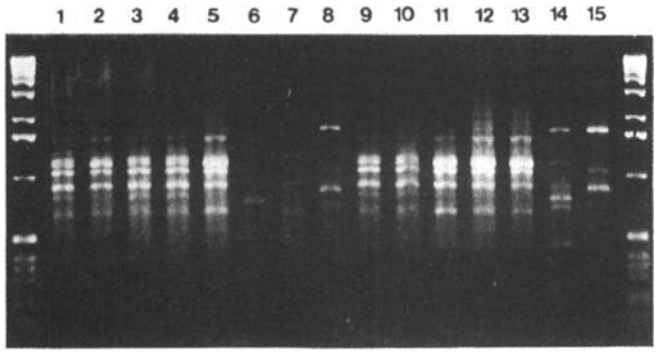

Figure 3. RAPD pattern using primer OPAV 16 showing moderate levels of genetic variability in the Asiatic lion population of Sakkarbaug zoo.

Microsatellite analyses of 30 Indian tigers revealed an average heterozygosity of 22.65% at three of the five loci (Fig. 6). Two of the loci were monomorphic, as in the case of Asiatic lions (Table 4). Microsatellite analysis of 15 skin samples of tigers, ranging in age from 50 to 125 years, was performed (Fig. 7). The average heterozygosity observed was 21.01% and the difference between the present and the past populations was not statistically significant (Table 5). Only those loci which were polymorphic in the present population showed polymorphism in individuals 50–125 years ago. The loci found to be monomorphic in the present population were found to be monomorphic in the ancient population as well. The levels of genetic variability revealed are much higher than reported. The Siberian tigers showed an average heterozygosity of 29.66% in the loci tested. Hair samples of two suspected Indian and Siberian tiger hybrids were tested to confirm hybridization. The analysis of locus Fca 126 and Fca 35 revealed that both animals had alleles contributed by each of the species (Fig. 8).

3.4 Graphical representation

The multivariate graphical representation of the fingerprinting and RAPD data revealed the clustering pattern of the genetic variability in the population. In the representation of the Asiatic lion population (Fig. 9), points D, E, I, and J, which lie outside the cluster, represent the animals having high amounts of genetic variation. The cluster represents the animals having low genetic variability. Three distinct clusters are observed in the representation of the tiger population (Fig. 10). Each cluster represents animals having a particular skin color. The representation thus enables us to identify heterozygotes for skin color by their position on the graph.

4 Discussion

As natural habitats disappear, many species are reduced to small populations as a direct or indirect consequence of human actions. Large outcrossing populations that suddenly decline to a few individuals show reduced viability and fecundity, known as inbreeding depression [18, 19]. Although harmful effects of inbreeding on individuals may diminish as deleterious recessive genes are removed from the population by selection [20, 21], the population as a whole loses the evolutionary flexibility conferred by genetic diversity [22]. Inbreeding and loss of genetic variation decreases the ability of wild populations to adapt to extreme climatic changes and make them vulnerable to new diseases, parasites, pollutants, competitors, and food supplies [23–25]. The importance of disease outbreaks on selecting genetically resistant survivors was first emphasized by Haldane [26]. Infec-

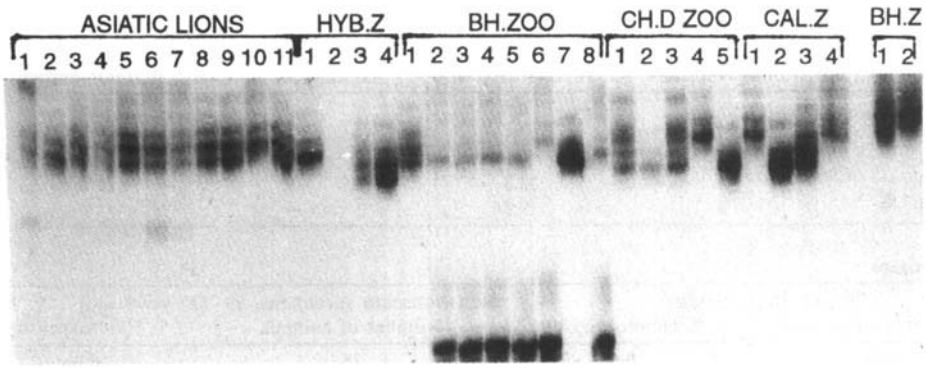

Figure 4. Fca 77 microsatellite analysis of pure Asiatic lions and hybrid lions from various Indian zoos. Last two lanes representing lions from Bhubaneshwar zoo show similar pattern as that of pure Asiatic lions, which are different from the hybrids, thus confirming their purity. Lane numbers indicate particular animals from each zoo. Similar numbered animals are identical in both Figs. 4 and 5. HYB. Z, Hyderabad Zoo; BH. Z, Bhubaneshwar Zoo; CAL. Z, Calcutta Zoo; CHD. Z, Chandigarh Zoo. Pure Asiatic lions were from Sakkarbaug Zoo.

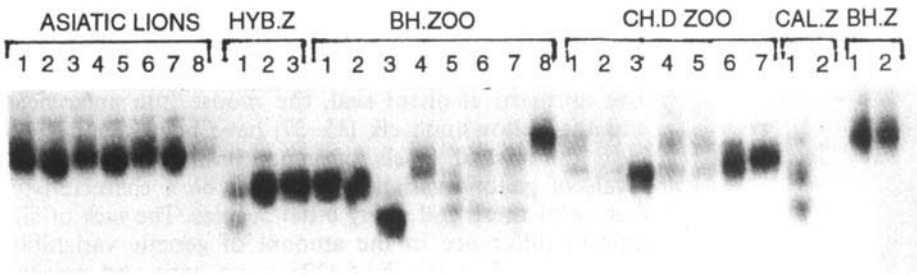

Figure 5. Fca 126 microsatellite analyasis of pure Asiatic and hybrid lions from various Indian zoos. Two lions from Hyderabad zoo (HYB. Z, lanes 1, 2) which were considered pure Asiatic lions, showed alleles different from those of Asiatic lions but similar to those of the hybrids from other zoos. Similar numbered animals are identical in both Figs. 4 and 5. Key to the zoos is the same as in Fig. 4.

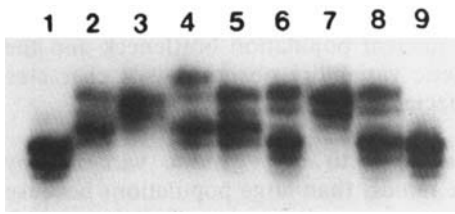

Figure 6. Allelic variability in the Indian tigers at CA repeat locus Fca 126. The presence of stutter bands is usual in dinucleotide microsatellites.

tious diseases, like any ecological factor, are now considered to be one of the important factors in determining the selective pressure on the genomes of surviving species [27, 28].

Molecular studies on the African cheetah showed that it possessed extremely low levels of genetic variation [15, 16, 29–31]. O'Brien *et al.* [31] suggested that in a highly inbred population there is a higher rate of juvenile mortality and species susceptibility to pathogens leading to extinction. The above evidence indicating that lack of genetic variation is the cause of the cheetah's plight was seriously contradicted [32] and the necessity of carrying out detailed ecological studies to identify the factors responsible was suggested.

O'Brien *et al.* [2, 33] conducted a genetic survey of 46 electrophoretic allozyme systems resolved from blood of 28 Asiatic lions wild-caught or captive-bred, maintained at the Sakkarbaug Zoo in India but originally derived from the Gir Forest. They found no variation at any of the 46 loci tested. In contrast, they observed moderate levels of allozyme variation at the same loci in several African lions (*Panthera leo leo*). They observed that, like the cheetah (71%), the Asiatic lions show a high incidence of morphologically abnormal spermatozoa (79%) compared to free-ranging African lions (25–61%) and other species such as bull or dog (20–30%), which is nearly always associated with infertility in these species [2]. They speculated that the lack of genetic variation in genes affecting sperm development could be the cause for such high levels of spermatozoal abnormalities [2]. O'Brien *et al.* [2] have stated: "The Gir lion population may thus represent another example of a severely endangered species that has suffered a population bottleneck or series of bottlenecks followed by inbreeding in their recent history".

In contrast to the bove observations, our present study of 38 Asiatic lions using RAPD analysis with four primers showed an average heterozygosity of 26% (ranging from 15% to 35% for individual primers). Only two of the lions analyzed by O'Brien's group were analyzed in our study. DNA fingerprinting using Bkm-derived 2(8) multilocus probe, which gives extensive variation in most of the eukaryotes, including human and crocodile [10, 11, 34] and microsatellite analysis of five CA repeat loci, however, failed to reveal any variation at all. This suggests that perhaps the sequences which are hypervariable are different in different species and therefore, based on a limited study, one needs to be cautious before deriving conclusions of far reaching importance.

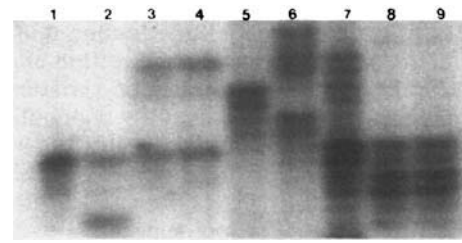

Figure 7. Allelic variability at CA repeat locus Fca 43 in the present population of Indian tigers (lanes 1–4) and 50- to 125-year-old skin samples from museum specimens (lanes 5–9).

Table 4. Microsatellite analysis of Asiatic lions, Indian tigers and Siberian tigers

Locus	Asiatic lions		Indian tigers		Siberian tigers	
	Number of alleles	% Heterozygosity	Number of alleles	% Heterozygosity	Number of alleles	% Heterozygosity
Fca 35	1	0.0	1	0.0	1	0.0
Fca 43	1	0.0	5	22.41	5	31.45
Fca 77	1	0.0	1	0.0	1	0.0
Fca 90	1	0.0	3	23.3	3	27.87
Fca 126	1	0.0	3	29.24	–	–

Table 5. Microsatellite analysis of Indian tigers

Locus	Number of alleles	Blood (live animals)		Skin (museum specimens, 50–125 years old)	
		Number of animals	% Heterozygosity	Number of animals	% Heterozygosity
Fca 35	1	23	0.0	15	0.0
Fca 43	5	23	22.41	15	24.68
Fca 77	1	23	0.0	15	0.0
Fca 90	3	23	23.3	15	18.57
Fca 126	3	23	29.24	15	19.79

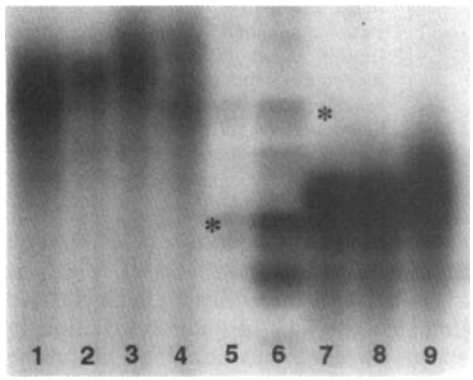

Figure 8. Identification of hybrids between Indian tigers and Siberian tigers using microsatellites. Hair samples (lanes 5, 6) from two suspected hybrids and blood samples from pure tigers were analyzed at locus Fca 126. The upper band (shown by asterix) matches with that of Indian tigers (lanes 1–4) while the lower band (shown by asterix) matches with that of Siberian tigers (lanes 7–9).

Microsatellite analysis of the hybrids between Asiatic lion and African lion showed the presence of alleles which are absent in the Asiatic lions studied and may, therefore, serve as a marker for distinguishing hybrids from pure Asiatic lions. Sperm morphology and motility studies of a large number of Asiatic lions from Gir forest carried out by our colleague in CCMB (Dr. S. Shivaji, personal communication) using computer-aided motility analysis did not reveal a high level of sperm abnormalities, similar to that reported by O'Brien *et al.* [2]. This discrepancy may be attributed to many factors such as small sample size, seasonality, age, sexual activity of the animals and the procedure of serial electroejaculation itself [2].

The mutation rates of various genetic loci, coding, minisatellite, microsatellite, *etc.*, vary considerably within and amongst themselves. Reconstitution of genetic variation in a population reduced to homozygosity by a population bottleneck depends upon the mutation rates of the loci examined and the generation time of the species. The wide range in heterozygosity levels observed in the various loci and within the RAPD loci suggests a wide range in their rates of mutations. Toward the end of the last ice age, some 10000 years ago, nearly 75% of all the mammals in North America and Europe became extinct. The northern elephant seal, the moose, the polar bear and the Yellowstone elk [35–37] have been reported to have diminished levels of genetic variation. The low levels of genetic variation seems to be a characteristic feature of these and many other species. The lack of significant difference in the amount of genetic variability was observed in the past (125 years ago) and present populations as ascertained by analysis of skin and blood samples. These observations suggest that the species went through an ancient population bottleneck and the low level of genetic variability observed is a characteristic of these species.

Small populations tend to lose genetic variation by genetic drift more rapidly than large populations because of random sampling of a small number of alleles at each generation. An immediate effect of depletion of genetic variability is increased homozygosity. Though the impact of increased homozygosity is still widely debated, it often leads to lower viability, that is, increased susceptibility to diseases and fecundity (inbreeding depression). It becomes imperative that conservation programs for small populations must devise methods to minimize,

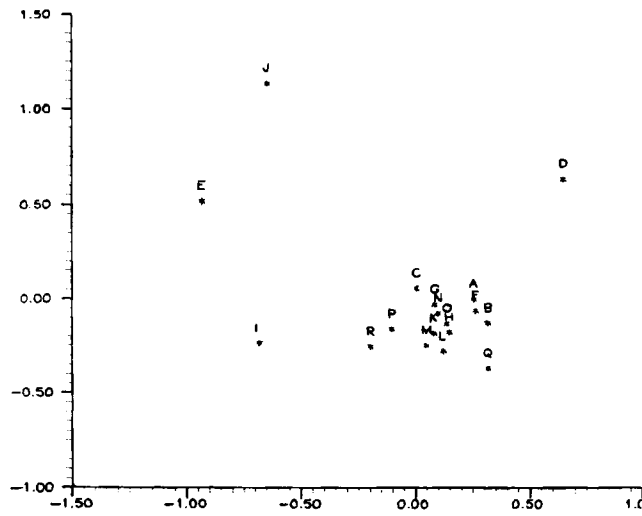

Figure 9. Multivariate map-like representation of the Asiatic lion population in the Sakkarbaug zoo based on RAPD analysis. Outlying points D (wild caught), E (Stud No. 1237), I (Stud No. 1256), J (wild caught) represent the animals showing greater genetic variation. *X* and *Y* axes are arbitrarily scaled.

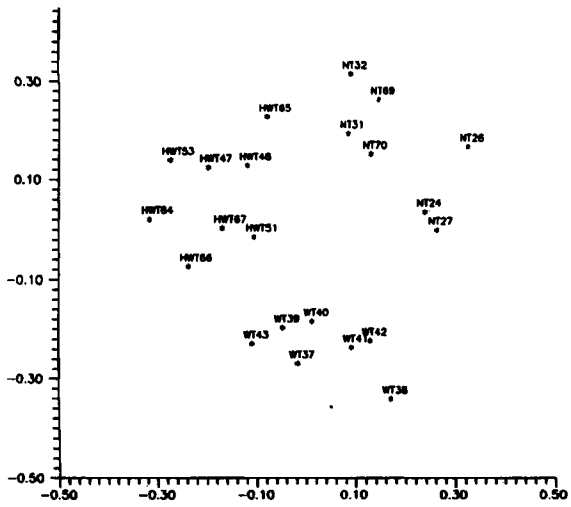

Figure 10. Multivariate map-like representation of the Indian tigers in Bhubaneshwar zoo based on Bkm 2(8) fingerprinting. The representation depicts the clustering of white tigers (WT), heterozygote for white skin (HWT), and normal skin color (wild) tigers (NT). The *X* and *Y* axes are arbitrarily scaled.

halt, or even reverse the decline in genetic variability that occurs. The powerful technique of molecular genetics has the ability to reveal a population's past history, present status and future prognosis for survival. Cluster analysis of the RAPD data showed that while most of the Asiatic lions cluster together, some individuals exist as outgroups. These individuals exhibit variation from the population. These can be used for conservation breeding program to increase the genetic variability of the population.

Our finding that the hair samples from two tigers from the wild population in Uttar Pradesh, India are probably those of hybrids between Indian and Siberian tigers, is very disturbing. DNA profiling of all the tigers in that population must be carried out at the earliest opportunity to identify and segregate the hybrids in order to preserve the genetic identity of the Indian tiger. Genetic markers are the indices of genomic variation and not the object genes for natural selection and adaptation. Human, mammalian, and model species genome projects are beginning to identify genes of would-be candidates for selective adaptation. When DNA probes for population analysis of such genes become available, the effect of lack of genetic variation on the viability of the species will become clearer. Until then, we have to be content with the indirect correlation. In this tudy, the use of RAPD and microsatellite analysis have revealed a much higher level of genetic variability in Asiatic lions and tigers than previous studies using conventional methods. A cautious approach should be taken when discussing implications of studies on population genetic variation as the low levels of variation detected could be due to the limitations of the experimental technique used.

We express our sincere thanks to the directors and other staff of Delhi, Sakkarbaug, Hyderabad, Bhubaneshwar, Calcutta, Guwahati, Chandigarh, Lucknow, Mysore and Darjeeling Zoos for their whole-hearted support in immobilizing the animals and for collecting the blood samples. Without their active cooperation, the present study would not have been possible. Financial support by a grant from the Central Zoo Authority to Dr. Lalji Singh is gratefully acknowledged. Our thanks go to Mr. S. C. Sharma, Member Secretary, Central Zoo Authority, Ministry of Environment, Govt. of India for providing all possible support for the present work. We are also thankful to Dr. K. Thangaraj, Scientist, CCMB for help during the study.

Received February 26, 1997

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