Study of MHC polymorphism in Asiatic lions through Molecular tools

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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Sept. 2003
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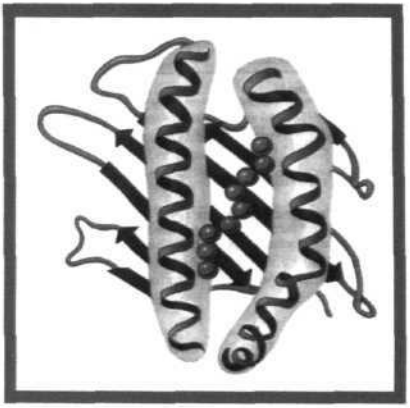

Dedicated to my Mentors

Dr. Lalji Singh

&

Dr. Reddanna

and

My Husband

Sundaram

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(A Central University Established in 1974 by an act of Parliament)
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DECLARATION

*I hereby declare that the work embodied in this thesis entitled '**Study of MHC polymorphism in Asiatic Sons through molecular tools**" has been carried out by me under the supervision of Prof. T. Reddanna & Prof. P.R.K.Reddy and this has not been submitted for any degree or diploma of any other University earlier.*

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This is to certify that ***Ms. Monika Sachdev*** *has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend her thesis* ***"Study of MHC polymorphism in Asiatic lions through molecular tools"*** *for submission for the degree of Doctor of Philosophy of this University.*

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ACKNOWLEDGEMENTS

I feel extremely privileged to expressed my veneration for my supervisor Prof. P.Reddanna, who has been a persistent source of encouragement and valuable guidance throughout the entire course of the present investigation. Working under him gave me a true sense of freedom with more confidence in addition to broaden my scientific outlook and positive attitude.

I express my heart-felt indebtedness to Prof. P.R.K.Reddy, for his extraordinary initiatives and suggestions time-to-time as well as constant encouragement throughout my research work.

Words fail to express my deepest sense of gratitude to Dr. Lalji Singh, Director CCMB Hyderabad. He guided and inspired me as a beacon light, during the difficult turns of my career and constantly rejuvenated my spirit to achieve the best. He magnanimously helped me, and allowed me to complete a major part of the dissertation work in CCMB providing me its marvelous research facilities. I never saw such a softhearted person with a strong and tough covering on his face like 'pistachio nut'. My experience of working under him made me a strong hearted person and increased my willpower tremendously.

I would like to express my profound thanks to Dr. Manjula Shridharan, member of my doctoral committee for her valuable suggestions, time to time and her constant encouragement.
I thank the head, Department of Animal Sciences and Dean, school of Life Sciences for providing necessary facilities.
My association with members of Dr. Reddanna's lab, Sreenath, Maya, Sridevi, Neerja, Shiv Kumar, G.V.Reddy, Mahipal, Mallikarjun, Aparna, Subhashini and Roy has been extreamly pleasant. I would like to specifically thank Sreenath and Maya for the nice times we had timely help.

I will pay my heartfelt gratitude to Dr. Sivaji and members of his research group Dr.Patil, Dr. Kholkute, Dr. Sontake & Dr.Umapathi for providing the blood samples of big cats. Without their help this project would have been impossible.
I extend my profuse sense of gratitude to Dr. Kshitij Majumdar, Dr. Ramesh Aggarwal, Dr. Thangraj & Dr.

Shankarnarayanan at CCMB for their valuable suggestions and technical help throughout the present investigation.

I would like to thank all my teachers till today for designing my career. Some of the names I can really recall at any moment of my life. Mrs. Kachroo, Mrs. Indu, Mrs. Pratibha, Mrs. Bhalla, Ms. Jyotsna, Mrs. Gupta and Mrs. Abha during my school days, gave me strong foundation. Dr. S.P.Singh, Dr. Raizada, Dr. Saxena, Dr. Bajpai and Dr. Awasthi during my graduation who linked my mind with a real competitive world. Dr. G.K.Garg, Dr. N.K.Singh, Dr. D.T.Singh and Dr.B.D.Lakhchaura during post-graduation, who could add molecular biology in depth of my inner core and made me curious about this fascinating world.

I pay my sincere thanks to Dr.Madhubala, Dr.Mohapatra and Dr. Bhattnagar who introduced me with the fascinating field of infectious diseases during my 2 years stay at JNU. Because of this experience I could hone up knowledge in the field of immunology.

I am obliged to Dr. Sarma from DRR for providing his laptop computer for the preparation of this manuscript and his fatherly personal concern about my career.

I owe my sincere thanks to instrumentation staff of CCMB, specially Giriji, Nagesh, Mehar, Asha, Prasad and Ramachari. They are always ready for their enthusiastic help to research scholars.
My sincere thanks are due to Suryanarayanji, Vishwanath, Haneef bhai and Ashok for their assistance in the labs of CCMB. I also appreciate the help provided by staff members of the Department of Animal Sciences Mr. Ankinaidu, Mr. Laxminarayana, Mr. Lallan and Mr. Jagan.

I express my thanks to my lab mates at CCMB Amritha, Jyotsna, Anjali, Geetanjali, Prashanth, Raman, Prasad, Rama, Raj, Anju, Ajay Gaur, Joshi, Avinash and Naidu.
It is always pleasant to think about the company of true friends like Ajay, Puneeta, Jagreet and Shubhendu who were like a breath of fresh air throughout the entire course.
I would like to recall and thank to all my doyens and predecessors during my stay at JNU, Preeti, Anirbhan, Bhagwan Das, Sammual Raj & Sidhu with whom I could learn something, which will be useful for me throughout my life.
It gives me a pleasant felling to remember my old friends Bipasha, Sagar, Sangeeta, Shamik, Monideepa, Anjani,

Tripti, Neeti who always inspired me through their e-mails and kept my spirits high.

I feel myself rich to have wonderful parents, who enlighten my life with full freedom and shaped me so far. My brothers Raman & Aman also proved to be a source of constant encouragement and stimulation. I wish to express my deepest gratitude to my in-laws who encouraged my ambitions; I thankfully acknowledge their support at home throughout the present study.

At last, but not least, I consider myself fortunate to have Sundaram as my life partner. Words would never be able to fathom the depth of my feeling for him, who displayed enormous strength, patience, courage and perseverance in helping me, throughout my research work. Without his love and support I wouldn't have made this task possible. I owe all my success to him.

[Monika]

List of Figures:

Fig. 15: Sequence polymorphism between clones of a single captive bred lion observed at a., locus

Fig.16: Dendrogram of sequence polymorphism at a_1 locus in lions

Fig. 17: Sequence polymorphism between clones of a single Asiatic lion observed at a_2 locus

Fig. 18: Sequence polymorphism between clones of a single hybrid lion observed at a_2 locus

Fig. 19: Sequence polymorphism between clones of a single captive bred lion observed at a_2 locus

Fig.20: Dendrogram of sequence polymorphism at a_2 locus in lions

Fig.21: Sequence polymorphism between clones of a single tiger observed at a_1 locus

Fig.22: Sequence polymorphism between clones of a single leopard observed at a., locus

Fig.23: Sequence polymorphism between clones of a single domestic cat observed at a_1 locus

Fig.24: Sequence polymorphism between clones of a single tiger observed at a_2 locus

Fig.25: Sequence polymorphism between clones of a single leopard observed at a_2 locus

Fig.26: Sequence polymorphism between clones of a single domestic cat observed at a_2 locus

Fig.27: Putative amino acid sequence polymorphism between clones of a single Asiatic lion observed at a_1 locus

Fig.28: Putative amino acid sequence polymorphism between clones of a single hybrid lion observed at a_1 locus

Fig.29: Putative amino acid sequence polymorphism between clones of a single captive bred lion observed at a., locus

Fig.30: Putative amino acid sequence polymorphism between clones of a single Asiatic lion observed at a_2 locus

Fig.31: Putative amino acid sequence polymorphism between clones of a single hybrid lion observed at a_2 locus

Fig.32: Putative amino acid sequence polymorphism between clones of a single captive bred lion observed at a_2 locus

Fig.33: Putative amino acid sequence polymorphism between clones of a single tiger observed at a_1 locus

Fig.34: Putative amino acid sequence polymorphism between clones of a single leopard observed at a_1 locus

Fig.35: Putative amino acid sequence polymorphism between clones of a single domestic cat observed at cc, locus

Fig.36: Putative amino acid sequence polymorphism between clones of a single dog observed at *a*: locus

Fig.37: Putative amino acid sequence polymorphism between clones of a single human sample observed at a_1 locus

Fig.38: Putative amino acid sequence polymorphism between clones of a single tiger observed at a_2 locus

Fig.39: Putative amino acid sequence polymorphism between clones of a single leopard observed at a_2 locus

Fig.40: Putative amino acid sequence polymorphism between clones of a single domestic cat observed at a_2 locus

Fig.41: Putative amino acid sequence polymorphism between clones of a single dog observed at a_2 locus

Fig.42: Putative amino acid sequence polymorphism between clones of a single human sample observed at α_2 locus

Fig.43: Polymorphism of putative translated product polymorphism at α_1, observed between clones of all Asiatic Lions

Fig.44: Polymorphism of putative translated product polymorphism at a_2 observed between clones of all Asiatic Lions

Fig.45: a_1 and a_2 domains of MHC, involved in inter-domain (p_2 & CD4a) binding

Fig.46: Amino acid residues in α_1 and α_2 domains of MHC class I involved in binding with foreign peptide

List of Tables:

List of Important Abbreviations

CBSG : Conservation Breeding Specialist Group

DNA : Deoxy Rebonucleic acid

FLA : Feline Leukocyte Antigen

HIV : Human Immunodeficiency Virus

HLA : Human Leukocyte Antigens

Ig : Immunoglobulin

IPTG : Iso Propyl Thio Galactoside

ILJCN : International Union for Conservation of Nature and Natural Resources

kDa : Kilo Dalton

kb : Kilo base pair

M : Molar

|i : Micro

MHC : Major Histocompatibility Complex

mM : Milli Molar

PAGE : Polyacrylamide gel electrophoresis

PBR : PCR Based RFLP

PCR : Polymerase Chain Reaction

RAPD : Random Amplification of Polymorphic DNA

RFLP : Restriction Fragment Length Polymorphism

SLA : Swine Leukocyte Antigen

SSCP : Single stranded conformational polymorphism

TCR : T-cell Receptor

X-gal : 5-Bromo-4-Chloro-3-Indolyl-(3-D-Glactoside

Contents

1. Introduction

The biological diversity of the planet is being rapidly depleted as a direct or indirect consequence of human actions. A large number of species are already extinct, while many other have reduced population sizes that put them at risk. Many species now require human intervention to optimize **their** management and ensure their survival. The scale of problem is enormous: 11.7% and 10.6% of existing species of mammals and birds, respectively, are categorized as threatened by the World Conservation Union (IUCN) (Mace, 1994), while an assessment by Conservation Breeding Specialist Group (CBSG) of IUCN classified 38% of 3559 vertebrate taxa as threatened, based on different criteria (Seal *et al,* 1993).

IUCN has recognized the need to conserve biodiversity at three levels: genetic diversity, species diversity and ecosystem diversity. The primary factors contributing to extinction are habitat loss, introduced species, overexploitation and pollution (Meffe and Carroll, 1994). In Indian perspective, there is a steady decline in biodiversity due to multiple reasons and many animals, which are the pride and natural heritage of not only of our country but also of the entire humanity are being reduced in terms of numbers every year. In wildlife parlance, India is unique country because it hosts five mega wild animals (lion, tiger, rhinoceros, elephant and leopard). All these are endangered to varying degrees due to altered habitat arising out of deforestation, agriculturalization, urbanization and poaching for pecuniary interests. Amongst the mega wild animals the most threatened ones are the great cats: lions and tigers (Khoshoo, 1997).

Asiatic lion *(Panthera leo persica),* a highly endangered species that once roamed in areas as far as Europe, Northern Africa, West Asia and the Indian subcontinent, now exists only as a single isolated relict population since 1880s in the Gir forests of Gujarat in India and as small groups in zoological parks. Their population is dwindling steadily and unless a careful strategy is drawn for their management in both wild and captive habitats, they may soon become extinct (O'Brien and Yukhi, 1999). Earlier studies by Yukhi and O'Brien (1988, 1990a, 1990b) reported total lack of any genetic variation in Asiatic lions. But studies done at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad by Lalji Singh and his group (Shankaranarayanan *et al.,* 1999; Shankaranarayan and Singh, 1998; Singh *et al.,* 2002) using RAPD and microsatellite markers indicated presence of moderate to abundant levels of genetic polymorphism in Asiatic lions. These observations, which were contrary to the earlier report of O'Brien and group, were also substantiated by semen analysis carried out in these wild cats (Shivaji *et al.,* 1998).

Major Histocompatibility Complexes (MHCs) are critical component of Cell mediated immune system. Their function is to bind antigenic peptides of foreign invader and activate the immune system. The MHC genes are the most polymorphic genes, presumably a means of enhancing immune responsiveness to a large array of antigens. The study of sequence polymorphism in the MHC system is important as it may provide information regarding the frequency and location of recombination events and thus may provide further information on the evolution of the Histocompatibility complex, and the rules that govern

recombination in general. Because of the critical role of MHC in immune recognition and defense, it would be useful to examine polymorphism of MHC in endangered species.

A study on immune loci polymorphism, particularly at MHC class I in Asiatic lions may provide clues regarding the vulnerability and survivability of these endangered wild cats to various diseases and other stresses and thus may pave the way for their scientific management in both wild and captivity. Hence, the present study was undertaken to have a detailed analysis of polymorphism at MHC loci in Asiatic lions through various molecular tools in order to verify the observations of O'Brien as well as Lalji Singh and group at CCMB, Hyderabad, India. The present study has been designed with the following objectives:

Objectives

1 To design consensus PCR primers for amplification of MHC loci in Asiatic lions, tigers, leopards, domestic cats, dogs and humans and to amplify the different MHC alleles using these primers.

2.To study the polymorphism at MHC loci in Asiatic lions through PCR based RFLP (PBR).

3.To study the polymorphism at MHC loci in Asiatic lions through Single stranded conformational polymorphism (SSCP).

4.To analyze the MHC loci polymorphism in wild Asiatic lions and compare the degree of polymorphism in Afro-Asian hybrid lions and captive lions through sequence based typing.

5. To analyze the MHC loci polymorphism at nudeotide level in a representative set of tigers, leopards, domestic cats, dogs and humans through sequence based typing.

6.To correlate the MHC polymorphism data of Asiatic lions with their management and breeding strategies.

2. Review of Literature

2.1 Asiatic lion *(Panthera leo persica)*

The lions of India otherwise called as Asiatic lions (Fig. 1), are considered India's pride. The habitat of Asiatic lion ranged across Southwestern Asia, extending from Syria to Northern India, as recently as 200 years ago (Joslin 1973). 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 isolated population in the Gir Forest Sanctuary and the surrounding forests in the Gujarat state in western India. The Asiatic lions today number about 250-300 animals, but they had suffered a demographic contraction to less than 20 animals at the turn of the century due to over-hunting (O'Brien *et al.,* 1987). Asiatic lions are genetically and morphologically different from African lions *{Panthera leo leo),* which occur in the Sub-Saharan Africa. O'Brien *et al.,* (1987), based on biochemical and genetic studies estimated that the Asiatic lions have separated from the African population about 100,000 years ago, a period which is considered not long enough for reproductive incompatibilities to have evolved. Currently, the African lion population is mainly concentrated in two regions: Serengeti plains of Tanzania where a large group of about 3000 lions live and Ngorongoro Crater wherein a small population of about 100 lions live geographically isolated (Yukhi and O'Brien, 1990a). Like Gir forest lions, **the** Ngorongoro Crater lions have descended from a documented bottleneck in 1962, when the number of lions dropped to 10-15 as the result of an epizootic of biting flies (O'Brien ef *al.,* 1987). The Asiatic lions differ from African lions in many

Fig. 1. Asiatic lion *{Panthera leo persica)*

Asiatic lion, the pride of India is known as a highly endangered animal belonging to the wild cat family (Felidae)

morphological features. Asiatic lions are slightly smaller than African lions. The most striking morphological character, which is always seen in Asiatic lions and absent in African lions, is a longitudinal fold of skin running along its belly (O'Brien ef a/., 1987). In addition, male Asiatic lions have only moderate mane growth at the top of the head, so that their ears are always visible, while many African males develop full manes, which completely obscure the ears. Asiatic lions have bifurcated infraorbital foramina (small apertures which permit passage of blood vessels and nerves to the eyes), whereas in African lions, there is only one foramen on either side (Pocock, 1939). Mean pride size, measured by the number of adult females, tends to be smaller in Asiatic lions as compared to African lions; most Gir prides contain just two adult females, compared to averages of 4-6 for African lions (Walker, 1994). Coalitions of males defend home ranges containing one or more groups of females, but unlike African lions, Gir males generally associate with their pride females only when mating or on a large kill.

2.1.1 Biology of Asiatic lions

Reproductive season Year-round, but based on sightings of cubs there is a birth peak from late winter to early summer (Ravi Chellam and JohnSingh, 1993)

Litter size

In wild: mean, 2.5, range 1-5 (observed only after young cubs are fully mobile) (Walker, 1994)

In captivity: 2-6 (Chavan, 1993)

Age at first reproduction

In wild: females 4 years, males 5-8 years;

In captivity: 3 years (males and females) (Walker, 1994)

Age at last reproduction

In wild: females 15-16 years (Chavan, 1993)

In captivity: both sexes 15 years (Walker, 1994)

Adult sex ratio:

1 male: 2.2 females (Ravi Chellam and Johnsingh, 1993)

Juvenile mortality (< 12 Months)

In wild: 33%

In captivity: 36%

Adult mortality

In wild: 8-10% (Walker, 1994)

Longevity

Females: 17-18 years

Males: 16-17 years (Chavan, 1993)

2.1.2 Habitat and distribution of Asiatic lions

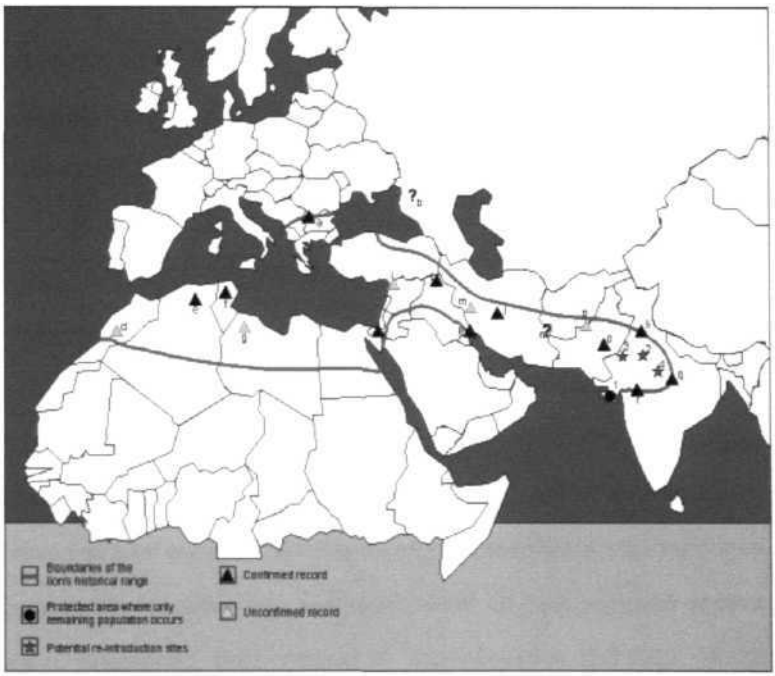
In ancient times, the habitat of Asiatic lions ranged from North Africa, Europe, and South-western Asia to India. It became extinct in Eastern Europe around AD 100 and in Palestine around the time of the Crusades (Guggisberg, 1961). It remained widespread elsewhere until the mid-1800s, when the advent of firearms led to its extinction over large areas. By the late 1800s, the lion had disappeared from Turkey (Ustay, 1990); the last reports from Iran and Iraq date

to 1942 (Joslin, 1973) and 1918 (Hatt, 1952) respectively. In India, lions ranged east to the state of Bihar, but declined under heavy hunting pressure. By the turn of the century, the Asiatic lions were confined to the Gir forest, Junagadh, Gujarat State (Kinnear, 1920). The Gir forest is a dry deciduous forest dominated by teak. The drier eastern part of the Gir is vegetated with acacia thorn savanna and receives about 650-mm annual rainfall; rainfall in the west is higher at about 1000 mm a year (Ravi Chellam and Johnsingh, 1993). The forest, which covered about 2,600 km^2 at the turn of the century (Oza, 1983), has since shrunk to less than half this size. Most of the remaining forest is included in the Gir National Park and Wildlife Sanctuary (259 + 1,153 = 1412 km^2). The Gir forest is the last representative block of the natural vegetation of the semi-arid Saurashtra peninsula, and is surrounded by cultivation. There is a progression in human settlement and activity around the Gir forest, which threatens the survival of the Asiatic lions (Walker 1994). Fig. 2 shows the area of distribution of Asiatic lions during ancient times and today.

2.1.3 Population status of Asiatic lions

The Gir lion population had been reduced to a very low number by the early years of the 20^{th} century; fewer than 20 according to the Chief Forester of Junagadh (Winter-Blyth and Dharmakumarsinhji, 1949). The first census of the Gir forest lions was conducted in 1936, which yielded an estimate of 234 adults (Winter-Blyth and Dharmakumarsinhji, 1951). Subsequent studies estimated the population at around 100 adults (1968-1979), 191 adults (1985), 221 adults (1990). In addition, about 30-40 lions are believed to live in the agricultural

Fig. 2. Past and present distribution of Asiatic lion



Asiatic lion, which once roamed in areas as far as Northern Africa, Southern Europe and West Asia is now restricted to a small area in the Gir Forest Sanctuary, Gujarat State, India

mosaic surrounding the reserve boundaries (Chavan, 1993 and Walker 1994). Based on Radio-telemetry studies, Ravi Chellam (1993) estimated the home range of male lions to be 110 km^2 and that of females to be 50 km^2. Density is estimated at one lion per 7 km^2. This density is comparable to the upper range of estimates of lion density in Sub-Saharan Africa.

2.1.4 Principal threats to Asiatic lions of Gir forest

The Asiatic lion currently exists as a single relict population, and is thus vulnerable to extinction from unpredictable events, such as epidemic or large forest fire. Even though, Walker (1994) has estimated them to be a healthy population and predicted a zero percent chance of extinction over the next 100 years, the growing human activity is threatening their habitat and is one of the reasons for their inbreeding. Inbreeding among the Gir forest lions reduces their genetic variability and renders them susceptible to diseases and other stresses. Genetic studies (O'Brien *et ai,* 1987) of 28 lions from India's Sakkarbaug Zoo at Gir forest revealed total genetic uniformity among the animals. A high incidence of spermatozoal abnormalities has also been found for both wild and captive Asiatic lions (O'Brien *et ai,* 1987; Wildt *et ai,* 1987; Fouraker and Wildt, 1992). Walker (1990, 1994) has noted high rates of infant mortality among the Sakkarbaug Zoo lions and has reasoned inbreeding to be the principal cause for this.

Considering the threats faced by the Gir forest lions from an ever-increasing human encroachment into their habitat (which forces them to inbreed and also reduces their food and water resources), it is imperative to find

alternative habitat for these lions in addition to developing a scientific strategy for their management. All these depend on an objective and scientific assessment of the level of their genetic diversity through conventional and molecular tools, which can provide vital clues to manage their population scientifically and also help in effective captive breeding of these big cats.

2.2 Conservation biology

2.2.1 Genetics in conservation biology

Considering the importance of maintenance of genetic diversity for conservation of endangered wild animals like Asiatic lions, it is imperative to understand the role of genetics in conservation biology. Factors like habitat loss, overexploitation, pollution etc., which contribute towards extinction (of endangered species), reduce population sizes of species where they are susceptible to stochastic effects, whether environmental, catastrophic, demographic, or genetic (inbreeding depression, loss of genetic variation, and accumulation of deleterious mutation) (Frankham, 1995).

There are five major genetic issues in conservation biology: (a) inbreeding depression, (b) accumulation of deleterious mutations, (c) loss of genetic variation in small populations, (d) genetic adaptation to captivity and its effect on reintroduction success, (e) outbreeding depression (Frankham, 1995).

2.2.2 Inbreeding depression in Wildlife

Inbreeding depresses reproductive fitness in out bred animals (Wright ,1977). Rails and Ballou (1983) have provided compelling evidence that captive

populations of vertebrate wildlife suffer from inbreeding depression. Inbred individuals showed reduced juvenile survival compared to outbreds in 42 mammalian populations (Rails and Ballou, 1986). Miller (1994) has reported that inbreeding depression should be more severe in the wild than in captivity since it is typically more severe in harsher environments. Thornhill (1993) has reported about inbreeding depressions in lions. Vrijenhock (1994) demonstrated that a genetically variable sexual species of fish numerically dominated a related parthenogenetic species until a drought eliminated their habitat. He also showed that when the populations were subsequently reestablished, the sexual species possessed reduced genetic variation from a founding event and was consistently less abundant than the parthenogenetic species. The sexual species reestablished its numerical dominance following the deliberate addition of genetic variation via replacement of 30 sexual individuals by fish from elsewhere. Overall there is clear and unequivocal evidence of inbreeding depression in the wild. Inbreeding is presumed to increase the risk of extinction, since it depresses components of reproductive fitness in naturally outbreeding species (Soule, 1986). A substantial number of wildlife populations have very low levels of Allozyme heterozygosity (Frankham, 1995, Nevo *et ai,* 1984;Yukhi and O'Brien, 1990a). Birth and death rates are susceptible to inbreeding depression (Charlesworth and Charlesworth, 1987), and sex-ratio distortions are sometimes found in inbred populations (Soule and Wilcox, 1980). Heschel and Paige (1995) have reported that loss of genetic variation decreases the ability of wild populations to survive climatic extremes, pollutants, diseases, pests and

parasites. Mills and Smoose (1994) showed that inbreeding is likely to contribute to population decline, especially for species with low reproductive rates. Suggestive evidence for the role of inbreeding and loss of genetic variation in decline and extinction of wild populations exists for Florida panthers, Puerto Rican parrots, Isle Royale wolves, inbreeding colonial spiders, native mice *(Peromyscus polionotus),* heath hens, bighorn sheep, and middle spotted wood peckers (Brock and While, 1992; Frankham, 1995).

Since inbreeding depression reduces genetic variability of endangered species and ultimately results in their extinction, it is necessary to design conservation strategies, which can overcome the ill effects of inbreeding depression. One obvious way to overcome inbreeding depression in small populations is to introduce immigrants from elsewhere. This has been demonstrated by the studies of Heschel and Paige (1995); Spielman and Frankham (1992) and Vrinjenhock (1994)

2.2.3 Accumulation of deleterious mutations

Deleterious mutations are added to populations by mutation and removed by natural selection. However, some become fixed in small populations and reduce reproductive fitness (Lande 1995). Lande (1995) has predicted that the accumulation of new mildly deleterious mutations in populations up to effective sizes of 1000 or more may be a more important cause of extinction than demographic stochasticity, and of similar importance to environmental stochasticity. Captive populations of endangered species are often founded from small numbers such that inbreeding becomes unavoidable (Hedrick and Miller,

1992). Templeton and Read (1983, 1984) devised a scheme to eliminate inbreeding depression in such populations. This involved increasing population size, equalizing founder representation, choosing healthy inbred individuals as parents, and dissortative mating with respect to pedigree. When the scheme was implemented in Speke's gazelle, they reported a significant reduction in inbreeding depression after only two to three generations.

2.2.4 Genetic deterioration in captivity

Captive breeding is being used as a means of saving endangered species from extinction, with reintroduction into the wild usually being the desired end point (Rails and Ballou, 1986a; 1986b). Three adverse genetic changes occur in captivity: inbreeding depression, loss of genetic variation, and genetic adaptation to the captive environment. Selection for tameness and other adaptations to the captive environment are likely to jeopardize reintroduction success. Genetic adaptation to captivity has been documented in fish and several *Drosophila* species (Frankham, 1995; Frankham, 1992; Lachance and Mangan, 1990). It is generally disadvantageous on return to the natural environment (Lachance and Mangan, 1990). Considerable difficulty has been encountered in the reintroduction of endangered vertebrate species into the wild (Serena, 1995). Genetic adaptation to captivity is one of many possible reasons for this (Frankam, 1995). It is important to minimize genetic adaptation to captivity for populations likely to be used for reintroduction into the wild. This should occur when generations in captivity, selection in captivity and heritability of reproductive fitness in the captive environment are minimized, and then generation length and

the proportion of immigrants are maximized (Frankham, 1995; Allendorf, 1993; Frankham, 1992; Lande, 1992).

2.2.5 Genetic management of threatened populations

It is clearly desirable to minimize inbreeding and loss of genetic variations in the genetic management of threatened species. Most researchers assume that heterozygosity represents evolutionary potential (Falconer, 1989), though others have stressed the need to retain allelic diversity (Allendorf, 1986; Fuerest and Maruyama, 1986). Although allelic diversity and heterozygosity are frequently related (Borlase ef a/., 1993; Briton ef a/., 1994; Nevo *et* a/., 1984), heterozygosity is less affected by severe population size bottlenecks than allelic diversity. Rails and Ballou (1986) proposed that for captive populations, initiating populations with at least 20-30 unrelated founders could maximize initial heterozygosity and by ensuring that the sampling of the gene pool is representative. Many captive populations of wildlife have been founded when only small numbers are left (Hedrick and Miller, 1992). It was observed that the captive population of Speke's gazelle in the U.S. was founded from one male and three females. These founders often contribute unequally, such that the rate of inbreeding and the loss of genetic variation are increased. Consequently, it has been recommended that such populations be managed to equalize founder representation (Frankham, 1995). This procedure reduced inbreeding and improved retention of genetic variation, but produced no benefits in reproductive fitness when evaluated in *Drosophila* (Loebel *etal.,* 1992).

Generation interval can be maximized by cryopreservation (Moore *et al.,* 1992) or by breeding from older animals. Embryo freezing technology is used in conservation of domestic mammals (Smith *et al.,* 1994) and for stock maintenance in mice, but is not available for most wildlife. Semen freezing is used in conservation of domestic mammals and birds, but is not available for most wildlife. For the majority of non-domesticated species like Asiatic lion, breeding from older animals is currently the only means for extending the generation interval, which may be difficult to achieve. Ballou and Lacy (1995) have predicted that minimizing kinship is the optimum means for managing small pedigreed populations with unequal founder contributions to maximize retention of heterozygosity and allelic diversity as this procedure combines the benefits of equalizing family sizes and adjusting founder representation and thus has good scope in management of lions in captivity.

2.2.6 Molecular studies in conservation biology

Avise (1994) first proposed the use of molecular markers in conservation biology. Genetic and phylogenetic analysis of endangered species using molecular markers has been advocated by O'Brien (1994). Genetic markers have been used to resolve taxonomic uncertainties and to define effective conservation units (Wayne ef *al.,* 1994). Polymerase chain reaction has been proposed as a strategy for non-invasive genotyping in vertebrate conservation. Animals can be monitored from hairs, feathers, museum specimens, or excreta, birds can be sexed and diet can even be determined (Morin and Woodruff, 1995). Bruford and Wayne (1993) have used highly variable microsatellite DNA

markers in conservation related studies. They found these markers to be highly informative. Genetic monitoring has been used to identify genetically distinct populations for augmentation of small, inbred populations to alleviate inbreeding and increase genetic variation (Butler *et al.,* 1994; *Olney et al.,* 1994). Vrijenhoek (1994) has proposed the utilization of genetic monitoring of wild populations as a tool to identify the best populations for reintroduction. Geyer *et al.,* (1993) have used DNA fingerprints to analyze the genetic relatedness among founders in the populations of California condors, so that their genetic management can be optimized. DNA profiles have been used for identification of kin structure among Guam rail founders (Haig ef *al.,* 1994). Gottelli *et al.* (1994) used genetic markers to detect introgression from wild into captive Ethiopian wolf, *Canis simensis.* Morin and Woodruff (1995) have used genetic markers to determine paternity in studies of the basic biology of endangered species. Molecular genetic markers have been used to detect illegal hunting of whales (Baker and Palumbi, 1994). Crazier and Ksmierski (1994) have used genetic distances as calculated using molecular markers for setting conservation priorities according to taxonomic distinctness.

Khoshoo (1997) has advocated a study on the extent and nature of genetic variation in the important tiger reserves located in different parts of India and in Gir sanctuary in the case of lion, since such an estimate of genetic variability is a prerequisite for drawing a meaningful conservation strategy. He also advocated the deployment of a whole range of DNA fingerprinting techniques, which are not only quick but also highly reliable for such estimation

studies. Molecular approaches as applied to tiger and lion would help to clear taxonomic uncertainties regarding species, subspecies, hybrids, and inbred population in the wild stock. Such studies would be helpful in captive breeding of wild populations and proper management of these animals.

Species survival is critically dependent on reproductive performance, which in turn is directly related to the level of genetic heterozygosity (Rails *et al.,* 1979). Wildt *et al.* (1987) based on a comparative study of the Asiatic lions of Gir forest and several African lions *{Panthera leo* leo) have observed that in the lions of Sakkarbaug zoo (in Gir forest) and Ngorongoro (which are inbred) exhibited more abnormal spermatozoa and lower serum testosterone levels compared to the outbred lions of Serengeti. These studies have established a direct correlation between the lack of genetic variability in the Asiatic lion and the high incidence of morphologically abnormal spermatozoa and low levels of the male steroid hormone testosterone and predicted that the Asiatic lion has suffered a population bottleneck followed by inbreeding. However, detailed studies undertaken at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad by Shivaji *et al.,* (1998) on assessment of inbreeding depression in big cats through analysis of testosterone and semen analysis have shown that majority of lions and tigers analyzed in the study exhibited good spermatozoal number, high percentage of motile spermatozoa and low incidence of abnormal spermatozoa similar to that observed for the outbred lions of Serengeti reserve in Africa, 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

also support the view that the Asiatic lions and Indian tigers are not completely inbred. They have also advocated the identification of animals with the best semen profiles for use in controlled breeding programmes to ensure propagation and genetic variability.

In a study on Allozyme polymorphism in Asiatic lions of Gir forest and African lions, O'Brien *et al.* (1987b) reported a total absence of variation at each of the 46 Allozyme loci analyzed in the 28 Asiatic lions. Their study also showed moderate levels of Allozyme variation in African lions. Based on this observation, they predicted 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. Later O'Brien *et al.* (1990) did a quantitative analysis of restriction fragment length polymorphism of MHC class I genes in Asiatic and African lions. In this study, they were unable to detect any RFLP in the Asiatic lions and a moderate level of heterozygosity (21.8%) was observed in African lions. On the contrary, studies at CCMB by Shankaranarayanan *et al.* (1997) on tigers and lions based on randomly amplified polymorphic DNA (RAPD), microsatellite analysis of five repeat loci and multilocus fingerprinting indicated a high degree of heterozygosity. In their study, RAPD analysis (using four primers) of 38 Asiatic lions showed an average heterozygosity of 26% (ranging from 15% to 35% for individual primers). Shankaranarayanan and Singh (1998) did a detailed analysis of mitochondrial DNA sequence divergence among big cats and their hybrids. 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. Average nucleotide diversity of 9% was observed between Asiatic and hybrid lion sequences. Singh ef *al.* (2002) reported isolation of highly polymorphic microsatellite loci from a partial genomic library of the Asiatic lion. These loci were characterized and have shown high levels of variation ranging from six to 11 alleles per locus in the population studied. They have reported a high heterozygosity in both African and Asiatic lions at these microsatellite loci.

Considering the conflicting nature of the scientific reports and in order to obtain a clear picture about the level of genetic polymorphism among the Asiatic lions, which can aid in designing a scientific strategy for their conservation and management, it is imperative to look for more specific molecular techniques. Immune loci of vertebrates play a vital role in their survival and adaptation to various stresses and such studies have indicated their utility in conservation biology (Ellegren ef *al.,* 1993; Mikko and Andersson, 1995; Takahata, 1995; Richman *et al.,* 2003).

2.3 Immune system

2.3.1 Basics of vertebrate immune system (Delves and Roitt, 2000)

The immune system is an organization of cells and molecules with specialized roles in defending against infection. There are two fundamentally different types of responses to invading microbes called innate response and acquired response.

(i) Innate (natural) responses occur to the same extent, every time the infectious agent is encountered, whereas acquired (adaptive) responses improve on

repeated exposure to a given infection. The innate responses use phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer cells. The molecular components of innate responses include complement, acute-phase proteins, and cytokines such as the interferons.

(ii) Acquired responses, which involve the proliferation of antigen-specific B and T cells, occur when the surface receptors of these cells bind to antigen. Specialized cells called antigen-presenting cells, display the antigen to lymphocytes and collaborate with them in the response to the antigen. B cells secrete immunoglobulins, the antigen-specific antibodies responsible for eliminating extracellular microorganisms. T cells help B cells to make antibody and can also eradicate intracellularpathogens by activating macrophages and by killing virally infected cells. Innate and acquired responses usually work together to eliminate pathogens. All these cells develop from pluripotent stem cells in the fetal liver and in bone marrow and then circulate throughout the extracellular fluid. B cells reach maturity within the bone marrow, but T cells must travel to the thymus to complete their development. Adaptive immune responses are generated in the lymph nodes, spleen, and mucosa-associated lymphoid tissue. These are referred to as the secondary lymphoid tissues. In the spleen and lymph nodes, the activation of lymphocytes by antigen occurs in distinctive B- and T-cell compartments of lymphoid tissue. A striking morphologic feature of the B-cell area is the secondary follicle containing the germinal center, where B-cell responses occur within a meshworkof follicular

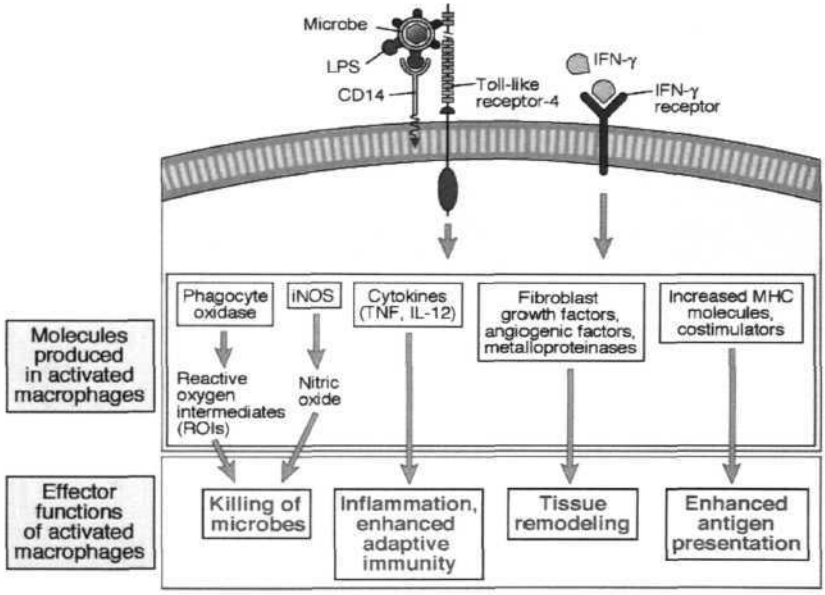
dendritic cells. The mucosa-associated lymphoid tissues, including the tonsils, adenoids, and Peyer's patches, defend mucosal surfaces. Diffuse collections of lymphoid cells are present throughout the lung and the lamina propria of the intestinal wall.

To establish an infection, the pathogen must breach through three levels of resistance of the host (i) the pathogen must first overcome numerous surface barriers, such as enzymes and mucus that either are directly anti-microbial or inhibit attachment of the microbe. Because neither the keratinized surface of skin nor the mucus-lined body cavities are ideal habitats for most organisms, microbes must breach the ectoderm. Any organism that breaks through this first barrier encounters the two further levels of defense, (ii) the innate and (iii) acquired immune responses.

2.3.2 Immune Recognition

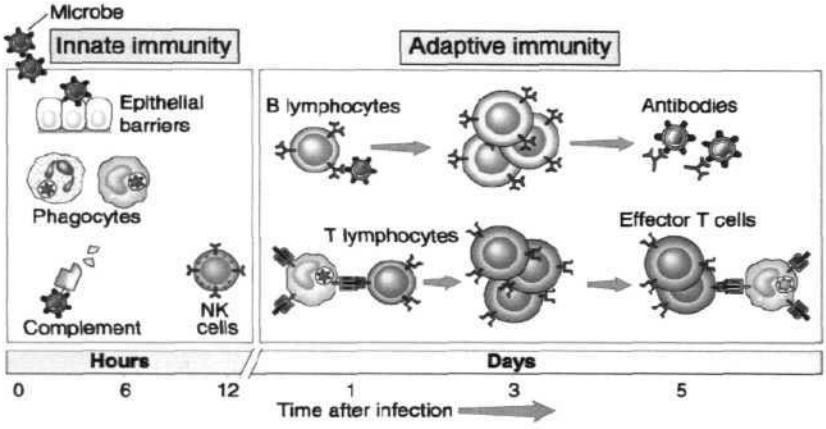
The body can potentially respond to almost anything that can be bound by the receptors of either the innate or the acquired immune system (Fig.3 & 4). Molecules recognized by receptors on lymphocytes are generically referred to as antigens and can range from small chemical structures to highly complex molecules. Both the T-cell receptor and the antibody that is embedded in the B-cell membrane, the B-cell receptor, have binding sites (Novatny *et a*/., 1983 and Garcia *et at.,* 1999) that are only 600 to 1700 A^0. Therefore, these receptors recognize only a small part of a complex antigen, referred to as the antigenic

Fig. 3. Triggering of immune response



The vertebrate immune system responds to pathogen (microbe) invasion by triggering the release of a number of molecules from activated macrophages, which aid in elimination of the pathogen

Fig. 4. Components of immune system



Innate immunity and Adaptive immunity are the two principal components of vertebrate immune system. Innate immune responses are mediated by phagocytes and also by the epithelial tissues which act as barriers. Adaptive immune responses are mediated by T and B lymphocytes

epitope. For these reasons, complex antigens consist of a mosaic of individual epitopes.

Antigens that elicit immune responses are termed immunogens. Not all antigens are naturally immunogenic. Small, non-immunogenicantigens are called haptens and must be coupled to larger immunogenic molecules, termed carriers, to stimulate a response (Mitchinson, 1971). Large protein antigens usually contain epitopes equivalent to carriers and haptens and are therefore inherently immunogenic. Carbohydrates, by contrast, must often be coupled to proteins in order to be immunogenic, as is the case for the polysaccharide antigens used in the *Haemophilus influenzae* type b vaccine. Even large protein antigens with adequate numbers of carrier epitopes can be made more immunogenic by combining them with an adjuvant— a substance that nonspecifically enhances antigen-specific immunity (Stewart, 1995). Many microorganisms inherently possess adjuvant activity in the form of immunostimulatory molecules such as lipopolysaccharideand muramyl dipeptide.

2.3.3 Innate Immune Responses

2.3.3.1 Cellular Components of Innate Responses

The innate immune system consists of all the immune defenses that lack immunologic memory. Thus, a characteristic of innate responses is that they remain unchanged however often the antigen is encountered. These types of responses developed earlier in evolution than acquired responses. Macrophages

(derived from blood-borne monocytes) possess receptors for carbohydrates that are not normally exposed on the cells of vertebrates (Fraser *et* a/., 1998), such as mannose, and therefore can discriminate between "foreign" and "self molecules. In addition, both macrophages and neutrophils have receptors for antibodies and complement, so that the coating of microorganisms with antibodies, complement, or both enhances phagocytosis (Aderem *et at.,* 1999). The engulfed microorganisms are subjected to a wide range of toxic intracellular molecules, including superoxide anion, hydroxyl radicals, hypochlorous acid, nitric oxide, antimicrobial cationic proteins and peptides, and lysozyme. Phagocytes also remove the body's own dead or dying cells. Dying cells in necrotic tissue release substances that trigger an inflammatory response, whereas cells that are dying as a result of apoptosis (programmed cell death resulting in the digestion of DNA by endonucleases) express molecules on their cell surface, such as phosphatidyl serine, that identify them as candidates for phagocytosis (Savill, 1997).

A key cellular component of innate immunity — and one of the most intensely studied components during the past decade — is the interdigitating dendritic cell (Bell, 1999). Cells of this type, which include Langerhans' cells in skin, constantly but quietly endocytose extracellular antigens. However, they become activated and behave as antigen presenting cells when pattern-recognition receptors on their surface recognize distinctive pathogen-associated molecular patterns on the surface of microorganisms (Medzitov, 1997). Endogenous danger signals, such as the release of interferon from virally infected cells or an increase in heat-shock proteins as a result of necrotic cell

death also activate dendritic cells. Molecules that act as pattern-recognition receptors on dendritic cells include the lipopolysaccharide receptor, the mannose receptor, and members of a family of molecules called toll. Pathogen-associated molecular patterns include yeast-cell-wall mannans, lipopolysaccharides on the surface of gram-negative bacteria, and teichoic acids, which are present on gram-positive bacteria (Medzitov, 1997). Activation causes dendritic cells to up-regulate the expression of B7 costimulatory molecules (also known as CD80 and CD86)on their surface. Co-stimulatory molecules are molecules that provide the signals necessary for lymphocyte activation in addition to those provided through the antigen receptor. These activated dendritic cells migrate to the local draining lymph node, where they present antigen to T cells. The antigen is processed intracellularly into short peptides by means of proteolytic cleavage before it is presented by major-histocompatibility-complex (MHC) molecules on the surface of dendritic cells (Delves, 2000).

Unlike macrophages and neutrophils, eosinophils are only weakly phagocytic and, on activation, probably kill parasites mainly by releasing cationic proteins and reactive oxygen metabolites into the extracellular fluid. They also secrete leukotrienes, prostaglandins, and various cytokines (Wardlaw *et al.,* 1995).

Basophils and mast cells have similar functional characteristics, but there is little evidence that blood basophils develop into tissue mast cells. Both types of cells possess high-affinity receptors for IgE (FceR) (Kinet *et al.,* 1999) and

thereby become coated with IgE antibodies. These cells are important in atopic allergies such as eczema, hay fever, and asthma, in which allergen binding to the IgE cross-links the FCER. This event triggers the cell to secrete inflammatory mediators such as histamine, prostaglandins, and leukotrienes.

Natural killer cells destroy infected and malignant cells (Biron *et al.,* 1999). They recognize their targets in one of two ways. Like many other cells, they possess Fc receptors that bind IgG (FcyR). These receptors link natural killer cells to IgG-coated target cells, which they kill by a process called antibody-dependent cellular cytotoxicity. The second system of recognition that is characteristic of natural killer cells relies on the killer-activating receptors and killer-inhibitory receptors of these cells. The killer-activating receptors recognize a number of different molecules present on the surface of all nucleated cells, whereas the killer-inhibitory receptors recognize MHC class I molecules, which are also usually present on all nucleated cells (Moretta *etal.,* 1997). If the killer-activating receptors are engaged, a "kill" instruction is issued to the natural killer cell, but this signal is normally overridden by an inhibitory signal sent by the killer-inhibitory receptor on recognition of MHC class I molecules. Although all nucleated cells normally express MHC class I molecules on their surface, they can sometimes lose this ability. This loss may occur as a result of either microbial interference with the expression mechanism — for example, after herpes virus infection — or malignant transformation. Therefore, cells that lack MHC class I surface molecules are in some way abnormal. This lack of MHC class I molecules means that there is no inhibitory signal from the killer-inhibitory

receptor, and the natural killer cell kills the abnormal target cell by inserting the pore-forming molecule perforin into the membrane of the target cell and then injecting it with cytotoxic granzymes (Peter and Roitt, 2000).

2.3.3.2 Role of inflammation in innate immune response (Peter and Roitt, 2000)

Infection with a pathogen triggers an acute inflammatory response in which cells and molecules of the immune system move into the affected site. The activation of complement generates C3b, which coats the surface of the pathogen. The neutrophil chemoattractant and activator C5a is also produced, and together with C3a and C4a triggers the release of histamine by degranulating mast cells. This in turn causes the contraction of smooth muscles and a rapid increase in local vascular permeability. Substances released from the pathogen and from damaged tissues up-regulatethe expression of adhesion molecules on vascular endothelium, alerting passing cells to the presence of infection. The cell-surface molecule L-selectin on neutrophils recognizes carbohydrate structures such as sialyl-Lewis on the vascular adhesion molecules (Lasky, 1995). The neutrophil rolling along the vessel wall is arrested in its course by these interactions. As the neutrophil becomes activated, it rapidly sheds L-selectin from its surface and replaces it with other cell-surface adhesion molecules, such as the integrins. These integrins bind the molecule E-selectin, which appears on the blood-vessel wall under the influence of inflammatory mediators such as bacterial lipopolysaccharide and the cytokines interleukin-1 and tumor necrosis factor a.

Complement components, prostaglandins, leukotrienes, and other inflammatory mediators, all contribute to the recruitment of inflammatory cells, as does an important group of chemoattractant cytokines called chemokines. The activated neutrophils pass through the vessel walls, moving up the chemotactic gradient to accumulate at the site of infection, where they are well placed to phagocytose any C3b-coated microbes.

2.3.4 Acquired immune response

This response is mediated by B-lymphocytes and T lymphocytes (Peter and Roitt, 2000). The development of lymphocytes and the myeloid lineage from primordial stem cells in the fetal liver and in bone marrow is guided by interactions with stromal cells (such as fibroblasts) and by cytokines (including stem-cell factor and various colony-stimulating factors) (Metcalf and Nicola, 1995). The initial stages of lymphocyte development do not require the presence of an antigen, but once these cells express a mature antigen receptor, their survival and further differentiation become antigen-dependent.

2.3.4.1 The B-Cell Receptor and Soluble Antibodies

Antibodies consist of two identical heavy chains and two identical light chains that are held together by disulfide bonds (Edelman, 1973). The N terminal of each chain possesses a variable domain that binds antigen through three hypervariable complementarity-determining regions. The C terminal domains of the heavy and light chains form the constant regions, which define the class and

subclass of the antibody and govern whether the light chain is of the «or Atype. The amino acid sequence of the constant region of the heavy chains specifies five classes of immunoglobulins (IgG, IgA, IgM, IgD, and IgE), four subclasses of IgG, and two subclasses of IgA. These classes and subclasses have different functions. Each type of antibody can be produced as a circulating molecule or as a stationary molecule. The latter type has a hydrophobic transmembrane sequence that anchors the molecule in the B-cell membrane, where it functions as the B-cell receptor.

All immunoglobulins are glycoproteins and contain 3-13% carbohydrates, depending on the class of the antibody. The carbohydrate is essential in maintaining the structure of the antibody. The basic antibody "monomeric unit" (which is biochemically a tetramer) is bivalent, with two antigen-binding arms of identical specificity. Each of these arms can be cleaved proteolytically in the laboratory to yield individual monovalent antigen-binding fragments (Fab) (Davis *et a*/., 1998). Another part of the immunoglobulin molecule, the Fc region, contains most of the constant region of the heavy chains. The secretory IgA at mucosal surfaces is a tetravalenf'dimer," whereas circulating IgM is a decavalent "pentamer." These IgA and IgM polymers are stabilized by a polypeptide, the J (joining) chain. Secretory IgA also contains a molecule called secretory component, which may protect the IgA against proteolytic cleavage within the gastrointestinal tract.

The B cells that develop earliest during ontogeny are referred to as B1 cells. Most B1 cells express CD5, an adhesion and signaling cell-surface molecule. They are the source of the so-called natural antibodies, which are IgM antibodies and are frequently polyreactive (i.e., they recognize several different antigens, often including common pathogens and autoantigens). In most cases, natural antibodies have a relatively low affinity (Hayakawa *etal.,* 1999).

Most B cells lack the CD5 molecule, and because they develop slightly later in ontogeny, they are referred to as B2 cells. Before they encounter antigen, mature B2 cells coexpress IgM and IgD antibodies on their cell surface, but by the time they become memory cells, they have usually switched to the use of IgG, IgA, or IgE as their antigen receptors. Complexes of antibodies with a newly encountered antigen and complement are localized in the follicular dendritic cells (a different type of cell from the interdigitating dendritic cell) within secondary lymphoid tissues. This event initiates the formation of the germinal centers, which are discrete areas within the spleen and lymph nodes where B-cell responses occur. Within these germinal centers, B2 cells that encounter the antigen undergo immunoglobulin class switching and begin to produce IgG, IgA, or IgE, and somatic hypermutation of their antigen-receptor genes occurs. Memory cells and plasma cell precursors are also generated in the germinal centers. The final stages of differentiation of B2 cells into antibody-secreting plasma cells occur within the secondary lymphoid tissues but outside the germinal centers. Although generally short-lived, with a half-life of only a few days, some plasma cells survive for weeks, especially within the bone marrow (Slifka and Ahmed, 1998).

2.3.4.2 T-cells **of the Thymus and** T-cell **receptors**

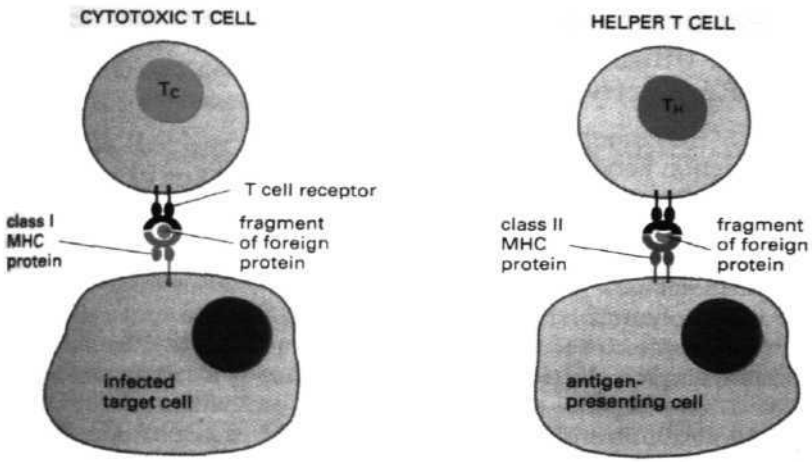
Stem cells continuously migrate from the bone marrow to the thymus, where they develop into T cells (Kriusbeek, 1999). Recent evidence suggests that, despite the partial degeneration of the thymus that occurs at puberty, T cells continue to develop in the thymus throughout life (Jamieson *et al.,* 1999). T cells with a/fi T-cell receptors initially remain in the thymus, where they are subjected to a series of selection procedures. Unlike the antibody molecule, which acts as the antigen receptor on B cells and recognizes antigen in its native (natural) state, the a/(i T-cell receptor recognizes short peptides that result from the intracellular processing of protein antigens, which are presented to the T-cell receptor by MHC molecules on the cell surface. The amino acids recognized by the T-cell receptor derive from both the MHC molecule and the antigenic peptide. Thus, the T-cell receptor recognizes an individual's own MHC molecules (self) together with peptides derived from foreign antigens. Since MHC molecules are highly polymorphic, the desirable immature T cells in each person in an outbred population are those that can recognize self-MHC molecules but those are not autoreactive. This objective is achieved by thymic education, a process that involves both positive and negative selection (Fink and Bevan, 1995; Kruisbeek and Amsen, 1996; Rathmell and Thompson, 1999). Cells are positively selected if they express a T-cell receptor capable of interacting with the MHC complexes on the person's own epithelial cells in the thymic cortex. Positive selection switches off the signal for spontaneous apoptosis that is otherwise triggered naturally in developing T cells. More than 95 percent of T cells are not selected at this stage

and therefore die in the thymus. In contrast, negative selection involves the induction of apoptosis in any lymphocyte that expresses a T-cell receptor with a high affinity for the complex of a self-peptide plus a self-MHC molecule on dendritic cells and macrophages in the thymic medulla.

During thymic education, the expression of a large number of T-cell-surface molecules is switched on and off in a highly regulated manner. Some of these, and many other cell-surface molecules with a role in immune responses, were originally characterized on the basis of their reactivity to panels of monoclonal antibodies (Peter and Roitt, 2000). The antibodies produced by various laboratories were said to form a cluster when they could be grouped together because they recognized the same cell-surface molecule. This led to a nomenclature in which a given molecule was assigned a "cluster of differentiation," or CD, number — for example CD1, CD2, and CD3. This CD nomenclature has become the standard way of referring to these cell-surface molecules.

The CD4 and CD8 molecules are of particular note with regard to T-cell development; together with the CD3 group of molecules, they form an essential part of the T-cell-receptor complex. CD4 binds to an invariant part of the MHC class II molecule, whereas CD8 binds to an invariant part of the MHC class I molecule (Fig.5). CD4 T cells usually act as helper T cells and recognize antigens presented by MHC class II molecules, whereas CD8 T cells are usually cytotoxic and recognize antigen presented by MHC class I molecules. Early in T-cell

Fig. 5. MHC class I and class II molecules



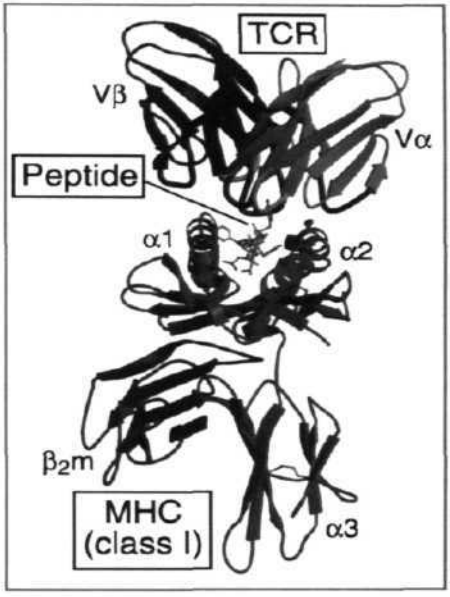
The Cytotoxic and Helper T cells recognize two different types of MHC molecules. Cytotoxic T cells recognize class I molecule of MHC, while Helper T cells recognizes MHC class II molecule

development in the thymus, immature T cells express both CD4 and CD8 (Ellmeier, 1999). If they have an appropriate T-cell receptor, these double-positive immature T cells have the potential to recognize an antigen-derived peptide presented by either MHC class II molecules or I. As T cells mature in the thymus, the expression of one of these molecules is lost, resulting in single-positive CD4 or CD8 T cells that recognize a peptide presented only by MHC class II or MHC class I molecules, respectively.

MHC class I molecules are expressed on all nucleated cells. This allows infected cells to signal their plight to cytotoxicCD8 T cells and establish intimate intercellular contacts by presenting the complex of foreign peptide and MHC molecule to the T-cell receptor of the effector cell (Fig. 6). Since MHC class II molecules signal CD4 helper T cells to secrete cytokines, the effector function of the helper T cell does not always depend on the establishment of intimate contact with the cell that will respond to the cytokine. This explains why the immune system needs only a few kinds of specialized "professional antigen-presenting cells" (dendritic cells, B cells, and activated macrophages) to express MHC class II molecules.

Unlike antibodies, T-cell receptors are produced only as transmembrane molecules. They consist of a/B or y/5 heterodimers; each a, ft, y, and 8 chain contains a variable domain and a constant domain. As in the antibody molecule, the variable domains contain three complementarity-determining, which in the case of the a/fi T-cell receptor recognize a complex formed by a peptide seated

Fig. 6. Recognition of peptide-MHC complex by T-cell receptor



The a, and a_2 domains of MHC class I are involved in presentation of antigenic peptide to T-cell receptor

within the groove of an MHC molecule regions (Garcia *et al.,* 1999, Davis *et al:* 1998). Most y/6 T cells do not recognize antigen in the form of peptide-MHC complexes, although MHC-like ("nonclassicMHC") molecules such as CD1 may present certain antigens (particularly lipids and glycolipids) to some y/5 T cells. Other y/8 T cells do recognize antigen directly, just as antibody molecules do *{Bom et al.,* 1999).

2.3.5 Major Histocompatibility complex (MHC)

The major histocompatibility complex (MHC) is a set of genes with immunological and non-immunological functions and present in all vertebrates studied so far (Trowsdale 1995; Gruen and Weissman, 1997). It was discovered during transplantation studies in mice (as the H-2 complex) by Peter Gorer in the Lister Institute in London in 1937, who later collaborated with George Snell of the Jackson Laboratories in Ben Harbor (Gorer, 1937). Jean Dausset described the first human MHC antigen Mac (HLA-A2) (Dausset, 1959) followed by the discovery of 4a and 4b (HLA-Bw4 and -Bw6) by the Leiden group led by Jon van Rood (Van Rood, 1963). It plays a vital role in histocompatibility (Snell, 1981) and in immune regulation (Benaceraff, 1972; Zinkernagel, 1974; Doherty **and** Zinkemagel, 1975). The main function of the main MHC molecules is peptide binding and presentation of them to T lymphocytes. Among the non-immune functions, the noteworthy ones are interactions with other receptors on the cell surface (Svejgard and Ryder, 1976), in particular with transferrin receptor (TfR),

epidermal growth factor (Schreiber *et al.,* 1984) and various hormone receptors (Philips *et al.,* 1986), and signal transduction (Schafer *et al.,* 1995).

2.3.5.1 MHC structure in humans

The MHC in humans is called Human Leukocyte Antigens (HLA). It is located on chromosome 6p21.31 and covers a region of about 3.6 Mbp depending on the haplotypes (Trowsdale, 1995; Anonymous, 1999). The HLA complex is divided into three regions: class I, II, and III regions (Klein, 1976). The telomeric region to the classical HLA complex is now called the class Ib region; and there has also been a suggestion for a class IV region located at the telomeric end of the class III region (Gruen and Weissmann, 1997). The classical HLA antigens encoded in each region are HLA-A, -B, and -C in the class I region, and HLA-DR, -DQ and -DP in the class II region. All class I genes are between 3 and 6 kb, whereas, class II genes are between 4-11 kb (Browning and McMichael, 1996). The 1998 Nomenclature Committee recognized more HLA genes, all of which are in the class I, and Ib regions: HLA-E, -F, -G, -H, -J, -K and -L (Bodmer ef *al.,* 1999). Among those, only HLA-E, -F and -G are expressed. The massive sequencing project of a human MHC haplotype has just been completed and the map positions of all of these genes are known (Anonymous, 1999). The class III region has the highest gene density but some of the genes are not involved in the immune system (Gruen and Weissmann 1997; Aguado ef *al.,* 1996). Among the genes, which are located in the MHC region, HSP70, TNF, C4A, C4B, C2, BF and CYP21 are important. The HSP70

genes encode cytosolic molecular chaperons and might have donated to the peptide-binding region (PBR) to the ancestor MHC gene (Flajnik *et al.,* 1991). It has also been proposed that HSP70 may be the functional forerunners of MHC molecules because of their peptide binding and presenting abilities (Srivastava and Heike, 1991). TNF (A) and TNFB (LTA) genes encode cachectin and lymphotoxin-a molecules, respectively (Gruen and Weissmann, 1997; Webb and Chaplin, 1990). C2, C4A and C4B are the genes for some of the complement proteins, whereas, BF codes for factor B which is also involved in immune response (Campbell *et al.,* 1986). CYP21 is the gene for 21-hydroxylase, which is an important enzyme in corticosteroid metabolism. Its complete deficiency causes congenital adrenal hyperplasia, which was the first disease identified to be the result of a structural change in an HLA-linked gene (Levine and Zachmann, 1978). Other genes of interest in the class III region are the human homologue of the mouse mammary tumor integration site Int-3, NOTCH4, and the homologue of a homeobox gene similar to PBX1 involved in t(1;19) translocation in pre-B cell ALL encoded on chromosome 1q23, PBX2 (or HOX12) (Gruen and Weissmann, 1997).

A highly relevant feature of the MHC antigens is their co-dominant expression. Since both alleles contribute to the phenotype equally, it is important to investigate the genotypes in disease association studies rather than the alleles on their own. If susceptibility to a disease is a recessive trait, allelic association studies may not yield a positive result. Also important is the fact that the MHC is inherited *en bloc* as a haplotype with the exception of the rare recombinational

events. Recombination occurs at 1-3% frequency mostly at the HLA-A or HLA-DP ends, i.e., in 100 meiosis the haplotype will be broken and reconstituted in one to three of them. The large segment from HLA-B to HLA-DQB is almost always inherited as a whole. This also has important implications in disease associations. A haplotypical association is usually stronger and more meaningful than an allelic association.

Despite the enormous number of alleles at each expressed loci, the number of haplotypes observed in populations is much smaller than theoretical expectations. This is to say that certain alleles tend to occur together on the same haplotype rather than randomly segregating together. This is called linkage disequilibrium (LD) (Begowich *et ai,* 1992).

2.3.5.2 Polymorphism at MHC

Genes in the major histocompatibility complex, many of which are fundamental aspects of the immune system, are the most polymorphic loci known in mammals and have become widely studied in man and vertebrates. Among the expressed loci, the MHC has the greatest degree of polymorphism in the human genome (Beck and Trowsdale, 2000). The numbers of alleles recognized at the classical loci by December 1998 are presented in Table 1 (Bodmer *et ai,* 1991; Schreuder ef a/., 1999; Marsh *etal.,* 2000).

MHC polymorphism is at such an extent that it is theoretically possible for each animal to possess a different set of MHC alleles. It is important to recognize

Table-1: **Number of alleles at the classical HLA loci**

Locus	Genotypical Alleles	Serological Equivalents
HLA-A	119	40
HLA-B	245	88
HLA-C	**74**	9
HLA-DRB1	201	80
HLA-DQB1	**39**	7
HLA-DPB1	**84**	(-)

Table shows the content of polymorphism at classical HLA loci till 1998. (Bodmer ***etal*, 1991).**

that within the allelic polymorphism at the DNA level, which seems endless, there are ancient lineages, which predate speciation and maintain themselves in closely related species. This is the basis of the trans-species polymorphism theory proposed by Jan Klein and has found widespread support (Klein *et al.,* 1993). Allelic lineages may be shared by related species, such as human and apes (Kupferman *et al.,* 1992) or even human and mice (Lundberg and McDevitt, 1992), having been present in their common ancestor. The high polymorphism at MHC genes also extends to individual amino acids that are putatively involved with recognition of antigens from pathogens (Hedrick ef a/., 1991; Salomon *et al.,* 1999).

Examining factors that can influence genetic variation, Hedrick and Thomson (1983) and Markow *et al.* (1983) concluded that some form of balancing selection has been important in maintaining the genetic variation for HLA loci. The most cited balancing selection explanation is that different MHC alleles determine the level of resistance to various pathogens; substantial evidence supports this hypothesis (Hedrick and Kim, 2000).

A major function of MHC molecules is to present foreign antigens to stimulate an immune response to invading pathogens (Zinkernagel, 1979). HLA alleles have been shown to differ in their ability to create an immune response to a variety of infectious agents (Hill, 2001), suggesting that the epidemic diseases of the past, and even more modern infectious diseases, may have played a central role in determining the frequencies of HLA alleles observed. Based on

these and other data, several different genetic and population genetic models have been proposed to explain how MHC variation is maintained: heterozygote advantage, frequency-dependent selection, and variable selection in time and/or space (Hill, 1991; Apanius *et al.,* 1997; Hedrick and Kim, 2000; Meyer and Thomson, 2001). Even though it is not clear which of these selection models is most important, Hedrick (2002) proposed that all the three types of balancing selection might be overlapping. For example, frequency-dependent selection models in which alleles when rare have a high fitness and may also have a constant advantage for heterozygotes. It is also likely that the extent of selection on an allele under a heterozygote advantage or a frequency-dependent model varies over both time and space because of temporal and spatial variation in the presence or density of pathogens.

2.3.5.3 MHC and disease

For an efficient immune response to a pathogen to occur, MHC molecules must bind peptides derived from microbial proteins and the T-cell repertoire must include clones that can be activated by such MHC-bound peptides (Andrews, 1999). Non-fulfillment of either of these requirements may render a person carrying a particular combination of HLA alleles more susceptible to given infectious diseases than one who has a different combination of alleles.

The best example of this resistance is the association of specific class I and class II alleles with protection against severe malaria in sub-Saharan Africa (Hill *et al.,* 1991). In the Gambia, infection with *Plasmodium fal.ciparum,* which

causes malaria, is extremely common, although the mortality rate among children with malarial anemia or cerebral malaria is low. Both complications are believed to be the consequence of a failure to clear the parasites from the blood, leading to increased hemolysis and blockage of cerebral blood vessels by parasitized erythrocytes. HLA typing of the relevant population revealed the presence of the HLA-B*53 allele at a frequency of approximately 25 percent among healthy persons or children with mild malaria (the allele is rare in non-African populations). By contrast, the frequency of HLA-B*53 among patients with severe malaria was approximately 15 percent. The comparison suggests that possession of the HLA-B*53 allele reduces the risk of death from severe malaria by approximately 40 percent. Presumably, the HLA-B53 molecules bind very efficiently certain peptides produced by processing the malarial circumsporozoite protein and present them to CD8+ T cells, whose progeny attack the liver-stage parasites. Such cytotoxic T cells have indeed been found in patients with malaria, and circumsporozoite peptideshave been eluted from the HLA-B*53 molecules of these patients (Hill *et at.,* 1992). Protection against severe malarial anemia is also afforded by possession of the class II HLA-DRB1*1302/DQB1*0501 haplotype. In other sub-Saharan populations, different class I and class II alleles are involved in the resistance to severe malaria.

Other examples of associations between infectious diseases and specific MHC alleles have been reported (Hill, 1998), and resistance-conferring alleles are prevalent in areas in which the disease is endemic. Stronger resistance of persons who are heterozygous for specific MHC alleles associated with an

infectious disease has also been noted and has been explained in terms of the fact that a heterozygote can present a wider range of peptides to T cells than a homozygote can (Carington *et a*/., 1999). In general, however, the associations are difficult to demonstrate, because they are often obscured by numerous complicating factors, which may overcome the resistance an MHC allele would otherwise confer. A striking example of this effect is the production by the parasite or infectious agent of variant peptides, or altered-peptide ligands, that differ from T-cell-stimulating peptides by one or more amino acids (Klenerman *et a*/., 1994; Bertoletti, 1994; Gilbert, 1998). The variant peptides bind to MHC molecules, but the assemblage does not stimulate a T-cell response: they act as antagonists of the response-stimulating peptides.

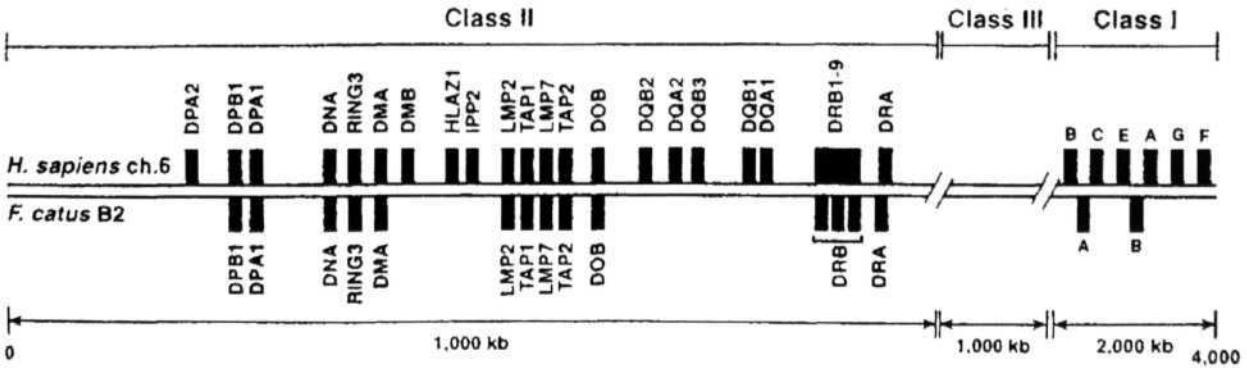
2.3.5.4 Feline MHC

The feline MHC was once perceived as inefficient or weak, since neither blood transfusion nor pregnancy regularly induced allogenic antibodies, and because organ transplants, particularly bone marrow, are not immunologically rejected as vigorously as in other species (Pollack *et ai,* 1982). Nonetheless, a controlled skin graft analysis of 59 domestic cats done by Winkler *et al.* (1989) demonstrated consistent acute graft rejection, although only a quarter of these resulted in allogenic antibody production. The antibody specificities define overlapping serospecificities in outbred cats allowing the recognition of 24 haplotypes. O' Brien and Yukhi (1999) cloned feline MHC class I and II gene homologs and genetically mapped them to feline chromosome B2q11, syntenic

with some nine non-MHC genes whose homologs reside on human chromosome six adjacent to HLA (Fig. 7). The Major Histocompatibility complex (MHC) encodes two classes of antigen-presenting molecules from T-cell receptors (Class I and Class II molecules) (Klein, 1986). Both molecules appear to have similar peptide binding grooves in their extracellular portions (Bjorkman *et al.,* 1987). Highly polymorphic features found in these molecules contribute to the formation of various shapes of these grooves (Bjorkman *et al.,* 1987) and also to the capacity of each molecule for binding various spectra of immunological peptides (Van Bleek and Nathenson, 1990; Rotzsehke *et al.* 1990; Falk ef *al.,* 1991).

In a study to characterize MHC cDNA clones in the domestic cat, Yukhi ef *al.* (1989), isolated eight molecular clones of feline MHC class I genes from a cDNA library of a cat T cell lymphoma line. DNA sequence analysis of eight clones revealed that they all fell into one of two internally identical allelic groups, which differed, by 9% of their nucleotide sequences. Comparison of the feline leukocyte antigen (FLA) class I coding sequence with other class I genes from other species revealed that the domestic cat genes display 81-82% sequence identity with human, and 73-79% sequence identity with mouse class I genes. Feline and human class I genes have similar sequences and protein structures, with three extracellular domains (a), one transmembrane domain and one cytoplasmic domain. Variable codons detected in FLA class I alleles, were, in most cases, in positions which were also variable in humans and mice, whereas

Fig. 7. An abridged map of Human Leukocyte Antigen (HLA)



An abridged genetic linkage map of FLA on human chromosome No. 6 shows the position of Feline Leukocyte Antigen (FLA) homologs with HLA

invariant positions with defined functional constraints were generally conserved and invariant between the three species as well.

Yukhi and O'Brien, (1990a) have analyzed the DNA variation in class I MHC loci of three feline species (domestic cat, African cheetah and lion) through RFLP and Southern analysis. In an attempt to measure MHC-associated DNA variation within single species, they examined three free-ranging populations of lions (Ngorongoro Crater population, Serengeti population, consisting of a larger group of lions and the third group consisting of Asiatic lions of Gir forest in India). The Asiatic lions were observed to reveal no RFLP whatsoever when 73 MHC fragments were monitored using 5 restriction enzymes with 6-nucleotide recognition sites. The Serengeti lions exhibited a high Heterozygosity value of 21.8% and the Ngorongoro crater lions had a low Heterozygosity value of 8%.

In another study, Yukhi and O' Brein (1990b) performed detailed analysis of the Feline MHC class I genes of domestic cat compared to the human and mouse MHC. Their study revealed that MHC class I genes consist of eight coding exons which encode the following protein domains: leader sequence, ai and a_2 polymorphic extracellular domains which are involved in antigen and T-cell recognition, a_3 extracellular domain, transmembrane domain and three cytoplasmic domains. The analysis by Yukhi and O'Brien revealed that certain residues of the first two extracellular domains are invariant and others are highly polymorphic. They observed presence of polymorphic region in the a! domain of domestic cat that encodes the first a-helix (amino acid residues 58-86) had

patchwork or mosaic pattern between FLA transcripts with highly polymorphic regions in the 5' and 3' ends of the first a-helix region separated by a strongly conserved 23-nucleotide stretch. Further variant and invariant amino acids are generally conserved between cats, humans and mouse. The majority of highly polymorphic residues of feline class I molecules (9 out of 10 highly polymorphic sites which show over three different residues) are located on the site facing the antigen binding groove.

Yukhi and O'Brien (1994) studied the pattern of sequence divergence at MHC I in three feline species (domestic cat, ocelot and cheetah). He observed highly mosaic MHC class I molecules in these animals consisting of short polymorphic sequence motifs in their entire coding region. A majority of similar motifs were recognized in class I alleles of at least two feline species. However, none of these class I alleles had an identical combination of these motifs in their mosaic structures. Yukhi and O'Brien also have enumerated factors which contribute to the origin and sustenance of abundant MHC allelic diversity in Felidae. These factors are: (1) gradual accumulation of spontaneous nucleotide substitution; (2) negative selection in regions involved in T-cell receptor interaction and recognizing common features of peptides; (3) positive selection pressure to produce functional sequence variation in residues that bind to variable antigens; and (4) periodic intragenic (interallelic) and intergenic DNA recombinations within class I genes based on the nature of highly mosaic structure among domestic cat MHC class I sequences.

O'Brien and Yukhi (1999) analyzed the pattern of nucleotide variation in feline MHC genes (class I and II) in the context of their role in presenting peptides to T-cell receptors and this revealed strong parallels to observations in human and murine systems. They also identified that the variant and invariant amino acid residues are generally in homologous amino acid sites in cats, humans and mouse.

Smith and Hoffman (2001) sequenced the antigen recognition site (exon 2 and 3) of a single MHC class I locus from representatives of the three felid lineages in order to provide a starting point for identifying MHC variability that is potentially relevant to embryo transfer success. Seven unrelated domestic cats and single individuals from eight wildcat species, representing all three felid lineages, were included in their study. BLAST comparisons of the alleles from the study against human MHCs indicated that they are all most similar to subtypes of HLA-B. A greater diversity was observed by Smith and Hoffman at the FLA-Z locus than among all previous reported FLA sequences; cloning and sequencing of only 15 animals yielded 12 alleles with distinct amino acid sequences. This level of diversity suggests a high amount of divergence between species.

3. Materials and Methods

3.1 Materials

Blood samples were collected from 10 Asiatic lions maintained at Sakkarbaug Zoo in Gujarat, but originally derived from the Gir forests and from 3 hybrid lions (hybrids between Asiatic and African lions), 3 captive bred lions, 3 tigers, 3 leopards kept at the Nehru Zoological park, Hyderabad and Nandakanan Zoological park, Bhubaneswar. In addition, blood samples were also collected from 3 domestic cats and 3 dogs maintained at the Veterinary College, A.N.G.R Agricultural University, Hyderabad and 3 human volunteers.

Proteinase -K, Ribonuclease A (DNAse free), Agarose, Ethidium bromide, Tris HCI, EDTA, Sodium dodecyl sulphate (SDS), Phenol, Bromophenol blue, Xylene cyanol, Acrylamide, Methylene bis acrylamide, TEMED, and Ammonium per sulphate and other molecular biology grade reagents were purchased from M/s Sigma Chemical Company (St. Louis, USA).

100 bp DNA ladder, 50 bp DNA ladder, Bovine Serum Albumin (BSA) DpnII, AluI, HinfI, DdeI, RsaI, HhaI, HaeIII were purchased from M/s New England Biolabs, USA.

DNA silver staining kit, Gel extraction kit and pMOS cloning kit were purchased from M/s Amersham Biosciences, USA.

Taq DNA polymerase, dNTPs, AmpliTaq DNA polymerase, Exonuclease, Alkaline phosphatase and Dye terminator dideoxy sequencing kit were purchased from M/s Perkin Elmer, USA.

All other chemicals, which have not been mentioned here, were procured from the local companies and were of high quality.

3.2 Methodology

3.2.1 Blood sample collection

Blood samples were collected in heparinized blood collection vials from the lions, tigers, leopards, dogs and cats from the femoral vein by immobilizing them in squeeze cages or by anesthetizing them (Shankaranaryanan et. al. 1997). From human volunteers, blood was collected from the strained forearm vein in heparinized blood collection vials.

3.2.2 DNA isolation

DNA isolation from blood samples was carried out as per the procedure described by Lang ef *al.* (1993). Immediately after collection, blood samples were frozen at -70°C with equal volume of lysis buffer I (30 mM Tris-HCI, pH 8.0, 5 mM EDTA and 50 mM NaCI) When required for DNA isolation the frozen blood samples were thawed in ice, transferred to 50 ml round bottom centrifuge tubes (M/s Sorvall, USA), kept at 65°C for 10 minutes and centrifuged at 12,000 g at room temperature. The pellet was transferred to a 10ml glass homogenizer (M/s Borosil, India) and homogenized in 5 ml of lysis buffer II (50 mM NaCI and 2 mM EDTA). The homogenate was then transferred to 50 ml round bottom centrifuge tubes and 5 ml of lysis buffer II was added to it. To this, Proteinase-K and SDS were added to a final concentration of 150 ^g/ml and 2% respectively and incubated at 37°C for 4 hours Then, Tris saturated Phenol (pH 8.0) was added, mixed gently and centrifuged at 1500 g for 10 minutes at room temperature. **The**

aqueous supernatant was transferred to another 50 ml round bottom centrifuge tube, added with equal volume of Tris saturated Phenol: Choloroform:Iso-amyl alcohol (25:24:1), mixed gently and centrifuged at 1500 g for 10 minutes at room temperature. The supernatant was then transferred to a fresh 50 ml centrifuge tube, added with 1/30 volume of 3 M Sodium acetate (pH 5.2) and equal volume of iso-propyl alcohol and centrifuged at 12000 g at 4°C for 10 minutes. The precipitate was washed with 70 % Ethanol twice, vacuum dried and dissolved in 400-500 til of TE buffer (10 mM Tris-HCI, pH 8.0 and 1 mM EDTA). The isolated DNA was checked for its quality and quantity by reading its absorbance at 260 and 280 nm in a Beckman DU650 spectrophotometer.

3.2.3 Designing of PCR primers

PCR primers for ai and a_2 domains of MHC class I genes of cat family were designed based on the Feline MHC class I cDNA sequences published by Yukhi & O'Brien (1990) using the Genetool® software (Wishart *et ai,* 2000). Care was taken to ensure a minimum of 60% G and C bases and to avoid formation of secondary structures within the primer sequence. The sequences of the primers are given below:

ai **Primer sequence**

F Primer: CCA CTC CCT GAG GTA TTT CTA CAC C

R Primer: GGA CTC GCT CTG GTT GTA GTA GCG

a_2 **Primer sequence**

F Primer: CGC ACA ACA TCC AGA GAA TGT ACG

R Primer: TGT CCA GGT ATT TGG CGA GC

For humans and dogs, the ai PCR primers given above were used. For amplification of a_2 region of MHC class I genes in humans and dogs, consensus PCR primers given below were designed based on the sequences available at the website http://www.ihwg.org.

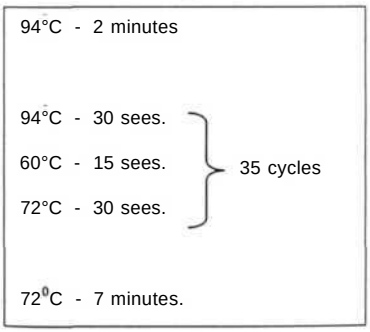
F Primer: CCA GAG AAT GTA CGG CTG TGA C

R Primer: ACG CAC GCG CCC TCC AGG TAG

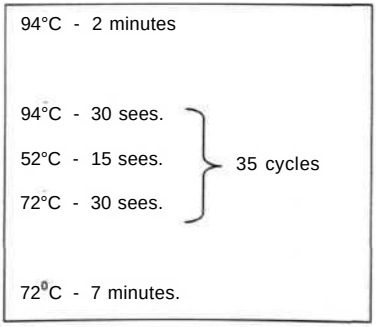
3.2.4 Polymerase chain reaction

Polymerase chain reaction was performed using the genomic DNA isolated from blood of lions, tigers, leopards, domestic cats, dogs and humans in a MJ Research Thermal Cycler (M/s MJ Research, USA) in 20 µl reaction volumes. The PCR mixture included 200 µM dNTPs, 5 pico moles each of forward and reverse primers, 20 µg of BSA, 10 X PCR buffer of M/s Perkin Elmer, USA (20 mM Tris-HCl (pH 8.8), 10 mM potassium chloride, 10 mM ammonium sulphate, 2 mM magnesium sulphate, 0.1% Triton X-100), 50 ng genomic DNA and 0.5 Units of Amplitaq DNA polymerase (M/s Perkin Elmer, USA). The thermal cycler (M/s MJ. Research, USA) was programmed to maintain the following cycling condition for amplification of ai and a_2 loci.

ai



a_2



The PCR products were loaded on a 2% Ethidium bromide (50 ng/ml) stained Agarose-TAE gels, resolved at 50 volts for 1 hour, visualized under UV light and photographed.

3.2.5 PCR based RFLP (PBR)

The PCR amplicons generated by both ai and *a.2* specific primers were digested with the restriction enzymes - DpnII, AluI, HinfI, DdeI, RsaI, HhaI and HaeIII (from M/s New England Biolabs, USA) as follows: The digestion reaction mixture consisted of 10 p.l of amplicon, 15 |al of appropriate 10 X reaction buffer, 1 (il of restriction enzyme and 25JJ.I of sterile distilled water. The mixture was incubated at 37°C overnight for complete digestion and the digested samples were loaded in a 15% mini native polyacrylamide gel (prepared using Protean II gel assembly of M/s Biorad, USA) The gel was prepared as per the protocol described in Sambrook and Russell (2001).

Preparation of 15% native polyacrylamide gel

Volume - 10 ml

1. Acrylamide:bis-acrylamide (20:1) solution (30%) - 5 ml
2. 10XTBE-1 ml
3. 10% ammonium per sulphate - 60^1
4. TEMED-5^1
5. Distilled water to make up volume to 10 ml

The gel was allowed for polymerization at room temperature for about 2 hours.

The restricted samples were mixed with 6X gel loading dye consisting of 40% sucrose and 0.025% Xylene cyanol (final concentration of 1X) were electrophoresed at 90 volts for 4 hours, stained with ethidium bromide solution (50 ng/ml) for about 10 minutes, destained in distilled water for about 5 minutes and visualized & documented under UV light in a gel documentation system (M/s Amersham Pharmacia, Sweden).

3.2.6 Single stranded conformational polymorphism (SSCP)

The amplicons generated using ai and a2 locus-specific primers were mixed with equal volumes of 2X gel loading dye (consisting of 95% formamide, 10 mM NaOH, 20 mM EDTA, 005% bromophenol blue and 005% xylene cyanol) The samples were denatured at 94°C for 10 minutes and immediately kept on ice till loading on gel. A composite gel consisting of 1% Agarose and 6% Polyacrylamide was prepared by mixing the ingredients as follows (Orita *et* a/., 1989), :

Total volume - 50 ml

1. 29:1 Acrylamide:bis acrylamide solution - 10 ml
2. Glycerol - 8 ml
3. 10XTBE-5ml
4. 10% ammonium per sulphate - 175 |il
5. 2% agarose solution (maintained at -60-65°C) -25 ml
6. TEMED-12nl
7. Distilled water to make up volume to 50 ml

After preparation of the solution, it was immediately poured in Protean II gel assembly (M/s Biorad, USA) and allowed for polymerization for about an hour The samples were loaded on to the gel and electrophoresed at 200 volts for 4-5 hours at 4°C. The gel was then subjected to silver staining as per the procedure described by Peng *et al.* (1995).

Silver staining

The gel was fixed in a solution containing 12% acetic acid, 50% methanol and 0.02% formaldehyde for about 2 hours under gentle agitation. Then the gel was washed in 50% ethanol for 20 minutes twice. Subsequently the gel was pre-treated in freshly prepared 0.02% sodium thiosulphate for 1 minute, rinsed in distilled water thrice and impregnated with 0.2% Silver nitrate and 0.03% formaldehyde for 20-30 minutes and rinsed in distilled water thrice. The amplicons were then visualized in a solution containing 6% sodium carbonate, 0.02% formaldehde and 0.0005% sodium thiosulphate for 3-5 minutes and transferring the gel into a solution containing 50% methanol and 16% acetic acid stopped the reaction. The gel was then dried on filter paper and documented.

3.2.7 Gel extraction of PCR products

The PCR products amplified using CM and 012 specific primers were resolved in 1% agarose-TBE gel and the amplified bands were extracted using GFX™ PCR DNA and Gel band purification Kit (Amersham Biosciences, USA). Using a clean razor blade the slice of agarose containing the DNA band to be purified was excised and the slice was cut into several smaller pieces and transferred into a pre-weighed 15 ml microcentrifuge tube. The agarose piece

was weighed (-100 mg) and for each 100 mg of gel pieces, 10 (il of capture buffer was added. The microcentrifuge tube was closed and mixed by vortexing vigorously and incubated at 60°C until all the agarose is completely dissolved. The sample was then transferred to *GFX*™ column and incubated at room temperature for 1 minute and centrifuged at 12,000 g for 30 seconds. The flow through collected in the *collection tube* fixed at the bottom of *GFX*™ column was discarded. 500 \i\ of *wash buffer* was added to the column and centrifuged at 12,000 g for 30 seconds and the flow collected in the *collection tube* was discarded. A fresh 1.5 ml microcentrifuge tube was fixed to the *GFX*™ column and 50 nl of elution buffer (10 mM Tris-HCI pH 8.0, TE pH 8.0 or autoclaved double-distilled water) was added to the top of the glass fiber matrix in the GFX™ column and incubated at room temperature for 1 minute. The column was then centrifuged at 12,000 g for 1 minute to recover the purified DNA collected in the 1.5 ml microcentrifuge tube. After checking the concentration of DNA the gel extracted PCR amplicons are used for cloning.

3.2.8 Cloning

For cloning of PCR amplicons, *pMOSBlue* Blunt ended cloning Kit (Amersham Life Sciences, USA) was used. The *pMOSBlue* Blunt ended cloning kit consists of *pMOSBlue* vector of size 2887 bp, MOSfi/ue competent cells, polynucleotide kinase enzyme mix and T4 DNA ligase for carrying out the cloning. The amount of insert (gel extracted PCR amplicons) required for the ligation reaction was calculated as follows. For achieving a vectorinsert ratio of 1:25, wherein the size of vector was 2887 bp and sizes of inserts were 246 bp

and 270 bp respectively for ai and a_2 locus specific amplicons, and 50 ng of vector used (as per manufacturer's instructions). The following formula was used for calculating the amount of insert to be used in the ligation reaction:

$$\frac{Z \times 50 \times 25}{2887 \times 1} = \text{ng of insert}$$

where Z= size of insert in bp

Based on the above formula, the amount of a-i and o$_2$ locus specific amplicons to be used in the ligation reaction was calculated (11.4 ng and 10.7 ng respectively for on and a_2 locus specific amplicons). After calculation of amount of insert DNA, the required quantity of insert DNA was set up for polynucleotide kinase (pk) reaction for generating blunt ends suitable for cloning in the pMOSS/ue vector.

3.2.8.1 Setting up of polynucleotide kinase reaction

For each product to be cloned, the pk reaction was set up as follows:

VI of 10X pk buffer

05 nl of 100 mM dithiothreitol

1 ^l of pk enzyme mix

X jal (11-12 ng) of gel extracted PCR product

Y |il of sterile distilled water to make up volume to 10 nl

The pk reaction mixture was incubated at 22°C for 40 minutes and centrifuged briefly to collect the contents at the bottom of the tube. The reaction mixture was then heat inactivated by incubation at 75°C for 10 minutes, cooled on ice for 2 minutes and centrifuged briefly to collect the condensate and proceeded for ligation.

3.2.8.2 Setting up of Ligation reaction

For ligation, 50 ng of vector was used for ligation with the pk reaction mixture. The ligation reaction was prepared as follows:

10(il of pk reaction mixture

1 \x\ of pMOSB/ue vector (50 ng)

1 (il of T4 DNA ligase (4 Weiss units)

The ligation mixture was incubated at 22°C overnight and used for transformation

3.2.8.3 Setting up of transformation

Before setting up the transformation, adequate numbers of Luria Bertani (LB) agar antibiotic plates were prepared as follows (Sambrook and Russell, 2001):

Tryptone- 10 g

Yeast extract - 5 g

NaCI-10g

Agar- 15 g

The mixture was sterilized by autoclaving and ampicillin was added to a final concentration of 80 ng/ml after filter sterilization and after the medium has cooled to <50°C. The plates were stored at 2-8°C and used as per the

requirement. For preparation of LB broth all the above said ingredients except agar were used.

The *pMOSBlue* competent cells were first thawed on ice and the 15 ml microcentrifuge tubes on which transformation reaction is to be set up were also pre-chilled. In addition to pMOSB/ue competent cells, competent cells of *Ecoli* strains DH5a and DH10B were also prepared and used as per the procedure described in Sambrook and Russell (2001). Briefly, 2ml of LB liquid medium was inoculated with single isolated colony of either DH5a or DH1 OB. About 500^1 of the overnight grown culture was used for inoculation of 50 ml of LB liquid medium and grown until the culture reached an ODeoo of 0.6. The culture solution was centrifuged at 3000 g for 10 min at 4°C, the supernatant was discarded and the pellet was dissolved in 8 ml of 100 mM CaCb. The solution was once again centrifuged at 3000 g for 10 minutes at 4°C, the supernatant was discarded and the pellet was dissolved in 1 ml of 100 mM CaCb- The solution was left on ice for 2 hours and 100^1 of it was used for single transformation reaction.

For carrying out transformation, 100^.1 of competent cells were pipetted to the pre-chilled microcentrifuge tube to which 1 (j1 of ligation mixture was added and stirred gently to mix. The tubes were left on ice for 30 minutes and subjected to heat shock by placing at 42°C for 90 seconds. Then immediately the tubes were placed on ice for 2 minutes and 200 nl SOC medium (2% bactotryptone, 0.5% bacto-yeast, 0.5% sodium chloride, 10mM magnesium chloride, 20mM glucose) kept at room temperature was added to the tubes. The tubes were subjected to shaking at 200-250 rpm at 37°C exactly for 45 minutes

and 100 (il of transformation mixture from each tube was plated on to the Luria Bertani agar plates containing X-gal and IPTG (prepared by spreading 35 |al of 50 mg/ml X-gal and 20 ^l of 100 mM IPTG per agar plate 30 minutes prior to plating the transformation mixture). The plates were then incubated overnight at 37°C. The pMOSB/ue vector allowed for blue-white screening after overnight incubation, with recombinants colony appearing white and non-recombinant colonies appearing blue in color. The white colonies were subjected to screening through direct colony PCR.

3.2.8.4 Screening through direct colony PCR

White colonies were picked up using sterile toothpick and inoculated on Luria Bertani agar plates (with 80 (ig/ml of ampicillin). The same toothpick was then used in colony PCR for adding template (bacterial cells) to the PCR mixture kept in 96 well PCR plates (M/s Axygen, USA). The PCR mixture consisted of 200 |iM dNTPs, 5 pico moles each of forward (T7) and reverse primer (U19) primers of the vector, 20 |ag of BSA, 10 X PCR buffer of M/s Perkin Elmer, USA and 1unit of Taq Polymerase (M/s Bangalore Genei, India). Polymerase chain reaction was carried out in a M.J. Research Thermal Cycler (M.J. Research Inc, USA) by adopting the following cycling profile.

94°C - 2 minutes

94°C - 30 sees
52°C - 15 sees — 35 cycles
72°C - 30 sees

72°C - 7 minutes

The amplified products were loaded on a ethidium bromide stained 2% Agarose-TAE gels and electrophoresed for 2 hours, visualized under UV light and documented. The positive colonies produced amplicons of size -400 bp and false positive colonies gave -130 bp amplicons. The positive colonies selected on the basis of colony PCR were used for plasmid preparation.

3.2.8.5 Plasmid preparation (Sambrook and Russell 2001)

The positive colonies were inoculated in culture tube containing 3 ml LB broth and were incubated at 37°C for overnight. After incubation, the culture was centrifuged in 1.5 ml microcentrifuge tubes at 15,000 g for 2 minutes. The supernatant was discarded and the pellet was resuspended in 100 JJl. of sterile distilled water. 100 ^l of lysis buffer (1% sodium lauryl sulphate, 10 mM EDTA, 100 mM sodium hydroxide) was then added to the resuspended solution and kept in boiling water bath for 2 minutes. After boiling, 50 |il of 1M magnesium chloride was added to the tubes, kept on ice for 2 minutes and centrifuged at 15,000 g for 2 minutes at 4°C. After this, 50 ^l of 5 M potassium acetate was added to the tubes, kept on ice for 2 minutes and centrifuged at 15,000 g at 4°C for 2 minutes. The supernatant was collected in another fresh 1.5 ml microcentrifuge tube, after which 600 *yi* of isopropanol was added and incubated at 4°C for 10 minutes. This was then centrifuged at 15,000 g for 15 minutes at 4°C, supernatant was discarded and the pellet was washed with 70% ethanol twice and left for air-drying. The completely dried plasmid DNA pellet was dissolved in 30 |il sterile distilled water and used directly for sequencing.

3.2.9 DNA sequencing

For direct sequencing of gel extracted PCR amplicons generated using a^ and α_2 locus specific primers and also the insert in the plasmid DNA isolated from the clones (about 15-20 clones from each amplicons sequenced), Thermal cycler GeneAmp PCR system 9700, ABI PRISM DNA sequencer 3700 and BigDye Terminator Cycle Sequencing FS Ready Reaction kit available from M/s Applied Biosystem, USA were used. The plasmid clones from the same individual and also from different individuals were used for sequencing. As per the manufacturer's instructions sequencing reaction mixtures were prepared in a 96-well MicroAMP tray as follows:

Terminator ready Reaction Mix	-	2 \x\
Plasmid DNA	-	2 jil (30 ng)
Sequencing primer (T7 or U19)*	-	1 jal (Ipicomole)
Total reaction volume	-	5 µl

* For sequencing of the insert in the forward direction the vector primer T7 and for sequencing in the reverse direction, the vector primer U19 were used.

PCR was carried out by adopting the following cycle sequencing reaction condition (as per manufacturer's instructions)

94°C - 10 sees
50°C - 5 sees } 30 cycles
60°C - 4min

4°C - kept till the time of precipitation

The 96-well MicroAMP tray was removed from the thermal cycler after completion of the PCR and the cover flap over the plate was removed. 25 nl of precipitation solution (25:1 Absolute alcohol:3 M Sodium acetate, pH 5.2) was then added to each well of the 96-well MicroAMP tray, incubated at room temperature for 10 minutes and centrifuged at 3000 g for 30 minutes at 16°C. The supernatant was then discarded by inverting the tray onto paper towel folded to the size of the tray. 100 \i\ of 70% ethanol was added to each and centrifuged at 3000 g for 15 minutes at 16°C after which the supernatant was discarded as explained above. The tray was inverted along with the paper towel and centrifuged for 3 seconds at 300 g and the pellet was dried for about 10 minutes at room temperature. The tray was stored at 4°C till sequencing run was carried out. Before starting the sequencing run, 10 nl of loading dye (M/s Applied Biosystems, USA) was added to each well of the tray and subjected to capillary sequencing in the ABI PRISM sequencer 3700.

3.2.10 Sequence analysis

The raw sequence data obtained from the capillary sequencing machine ABI PRISM sequencer 3700 was first edited by using 'Sequence analysis' software of the manufacturer (M/s Applied Biosystems, USA). This software also ensured that the vector backbone sequences and artifacts were removed and the direction of sequencing was identified. Next, using the software 'Autoassembler 2.0' (supplied by the manufacturer, Applied Biosystems, USA), the sequences were assembled and aligned facilitating the reading of all the sequences in the forward direction. Setting a minimum overlap value of '50bp' and minimum error

value of '0%', the exactly identical sequences were identified and removed from the raw data. Then, setting an overlap value of '50bp' and error values between '10-25%', the percent variation in sequence between clones from the same individual and also between different individuals was analyzed. The clones, which were inserted in the reverse direction in the vector, were read in the forward direction with the help of 'Sequence editor' utility of 'Genetool' software (Wishart *et al.,* 2000). This utility also ensured the identification of ambiguous nucleotides. The clones were then aligned and pairwise identify between clones from the same individual and between different individuals were checked using 'multiple alignment' utility of Genetool software. The software was also used to calculate percentage variation in sequence between the different clones. Finally the finished sequence data was compared with previously reported or obtained ones using 'BLASTn' software available at the website http://www.ncbi. nlm.nih.gov/BLAST (Altschul ef a/.,1989).

The sequences, which were compared with already available sequences in the web using BLASTn software utility, were checked for alignment of translational frame using 'BLASTx' software utility. This ensured the identification and removal of alleles, which had stop codons in the middle of reading frame (pseudoalleles) and also homology comparison of the sequence of different clones with that of published amino acid sequence of MHC.

The heterozygosity was calculated based on the procedure described by Shankaranarayanan ef *al.* (1997) and Yukhi and O'Brien (1990). The heterozygosity was calculated as the average of the number of nucleotides

differing in each pairwise combination of lions divided by the total number of nucleotides. Heterozygosity was calculated separately for both ai and 012 loci. The consensus sequence of 10 clones with respect to each lion was considered for this analysis.

Genetic distances were calculated based on the similarity and differences between consensus sequences of each lion with respect to ai and 012 sequences using the software "Clustal" (Thompson *et al.,* 1997).

The software 'Swissprot' (M/s Swiss Institute of Bioinformatics, Switzerland, Boeckmann *et al.,* 2003) was used for translation of the DNA sequence data in the right reading frame. The software 'Peptool Lite' (Wishart ef *al.,* 2000) was used for multiple alignment of protein sequences of different clones, checking for pairwise identities of the translated sequences and their comparison with known/published MHC sequences. Computed alignments were carried out by an alignment algorithm that aligns regions of sequences exhibiting the highest degree of similarity. The 'hot-spots' for mutation in the amino acid sequences of the different clones were also identified using this software utility.

The nucleotide and amino acid sequences of the clones of the same animal and between different animals were subjected to multiple sequence and profile alignments with the help of 'Clustalx' software and the output postscript file generated by the software was analyzed using 'Phylip' software. In this analysis, the utilities 'DNAdist' and 'Prodist' were used for calculation of genetic distance between the different individuals/animals.

Results & Discussion

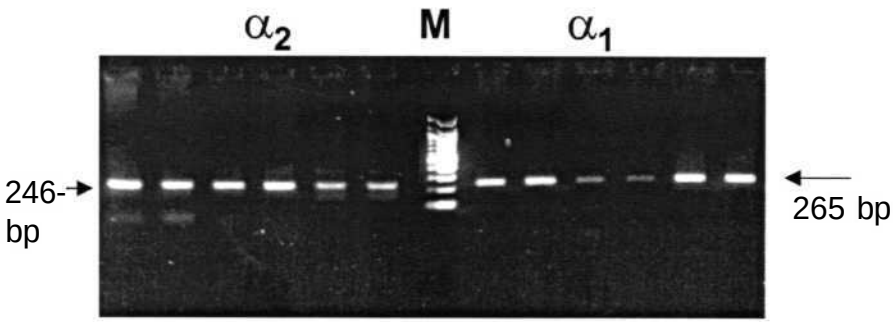
4. Results and Discussion

4.1 PCR amplification using MHC ai and *0.2* locus specific primers

Blood samples of Asiatic, hybrid and captive/circus lions were collected under sterile conditions from Sakkarbaug zoo, Gujarat, Nehru zoological park, Hyderabad and Nandankanan Zoological Park, Bhubaneshwar. Genomic DNA was isolated separately from each blood sample. PCR amplification was done with genomic DNA using the designed primers (as described in materials and methods), which gave amplified products (amplicons) of 265 bp in case of on (exon-2), and 246 bp in case of a_2 (exon-3) domain (Fig. 8). The Feline MHC primers for a_2 failed to amplify human and dog DNA samples. When the human and Feline MHC sequences for a_2 region was analyzed online through BLAST (http://ncbi.nlm.gov), it was observed that the region from which Feline MHC a_2 primers were designed was not conserved across species. This may be the reason for failure of Feline MHC a_2 primers to amplify human and dog samples. Therefore, a separate set of consensus primers was designed from published sequences of a_2 (exon-3) region of humans for amplification of human and dog DNA. These primers gave amplicons of the size of 210 bp in both human and dog samples (Fig. 9).

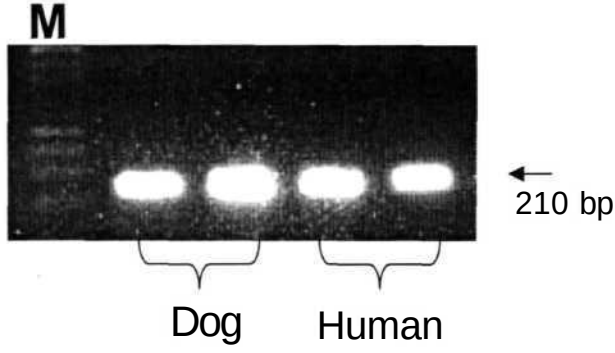
The published information regarding MHC sequence of lions is very scarce in the literature and this necessitated designing of consensus primers from the conserved region of published sequences of MHC of domestic cat. Even though large amounts of data regarding human MHC is available in published literature (Takahata *etal.,* 1992; Satta era/., 1994), several studies have shown

Fig. 8. Amplification pattern of a_2 and GL, specific primers in lions



PCR primers, designed from published sequences of MHC class I of domestic cat were used for amplification of six lion samples. α_2 primer gave amplicons of size 246 bp, while a, primer amplified a 265 bp band. M-Molecular weight marker (100 bp ladder)

Fig. 9. Amplification pattern of u_2 specific primers in dogs and humans



Consensus primer were designed from published sequences of ct_2 (exon-3) region of humans & dogs for amplification of a_2 region from genomic DNA of human & dog samples. This primer gave amplicons of the size of 210 bp in both human and dog samples. M - Molecular weight marker (100 bp ladder)

that human MHC data may not be relevant for studying MHC in other genus. Richman *et al.,* (2001), based on phylogenetic analyses, inferred that evolution of MHC polymorphism has proceeded independently across genus and hence studies using human data may not be relevant to studies involving non-primate species. They have studied MHC polymorphism in *Peromyscus maniculates* (American deer mouse) using conserved sequences from several other *Peromyscus* species. Even though O'Brien ef *al.* (1994) designed consensus primers from human DRB class II MHC sequence for amplification in domestic cat, these primers failed to give clear amplicons in case of lions, tigers and leopards in the present study. Hence, the present study was restricted to analysis with respect to ai and 012 domains of MHC class I loci using the primers designed from sequences reported by Yukhi and O'Brien (1990).

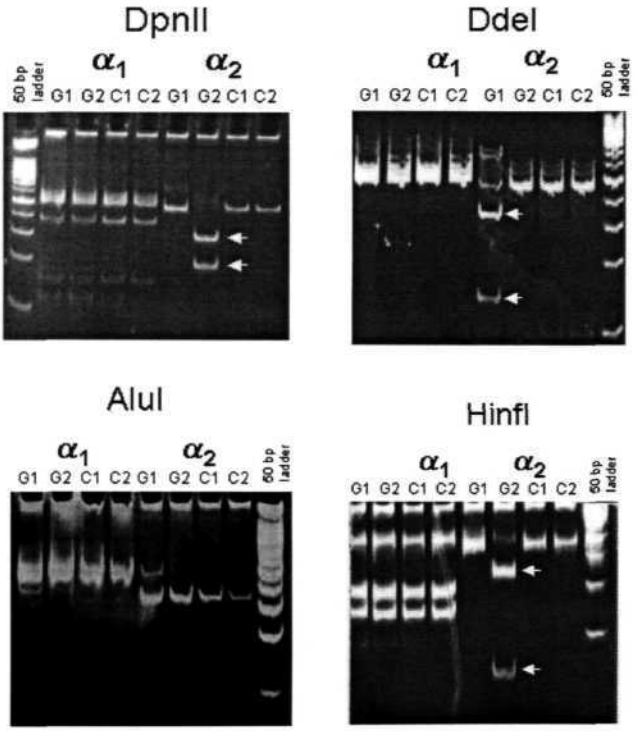
4.2 PCR Based Restriction Fragment lengths Polymorphism (PBR) analysis of MHC amplicons

The PCR amplicons generated using genomic DNA isolated from 10 wild Gir forest lion, 3 Afro-Asian hybrid lions and 3 captive lions, were subjected to PBR (PCR-based RFLP) with seven different 4-base pair cutter restriction enzymes as mentioned in materials and methods. In the present study, only tetra cutter restriction enzymes were considered since they are expected to have more restriction sites when compared to hexa or octa cutters. Sawhney *et al.* (2001) used PBR technique to study polymorphism of bovine MHC class I genes using the tetra cutter restriction enzymes Taql and Ddel.

It was observed that five (DpnII, DdeI, HinfI, HaeIII, HhaI) out of the seven restriction enzymes tested revealed PBR in Gir forest and hybrid lions but not in captive lions with respect to amplicons generated using a_2 specific primers (Fig. 10A & 10B). The PCR amplicons generated using <xi specific primer pair did not exhibit any PBR. Hviid *et al.,* (1997) in their study on HLA class I polymorphism in a_2 locus of humans reported a very high degree of sequence polymorphism. The restriction enzyme DdeI exhibited the maximum PBR in both wild and hybrid lions. 6 out of 10 wild (Gir forest) lions exhibited PBR whereas only 1 out of the 3 hybrid lions tested exhibited PBR. The captive lions did not exhibit any PBR. In the present study, the absence of PBR amongst captive bred lions might be due to the limited polymorphism at MHC class I loci in these lions, when compared to wild and hybrid lions. A summary of observations with respect to PBR is given in Table 2.

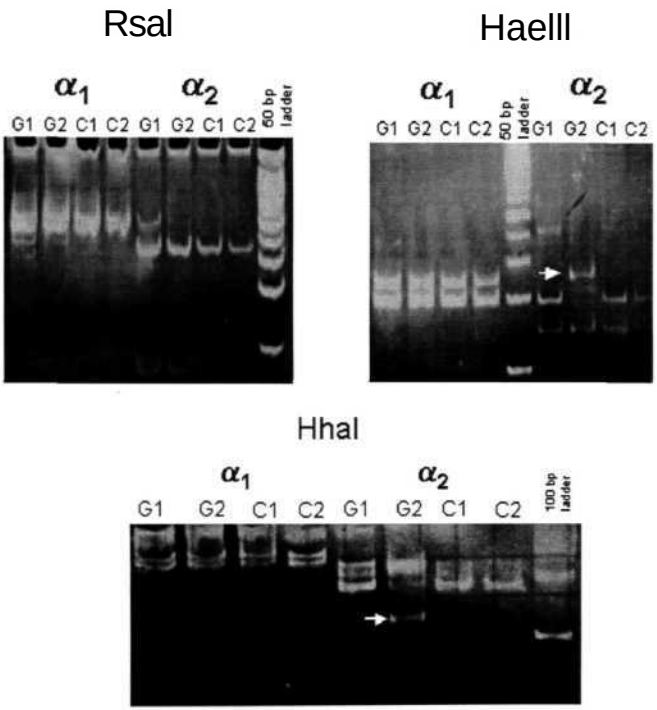
The use of PBR methodology for studying genetic polymorphism is a promising approach. Klebs ef *al.* (2003) used PBR as an alternative to flow cytometry for detection of H-2 in the screening of transgenic mice. Similarly Ando ef *al.* (2001) have also used the PBR strategy to study genetic polymorphism of the swine major histocompatibility complex (SLA) class II DMA gene. The methodology of PBR can be used to study MHC polymorphism in areas in which no serological reagents are available, wherein each polymorphic band observed after PBR analysis in the wild lions can be used as a genetic marker, which would allow the use of a binary scoring system to define class I haplotypes independently of serological or other typing data. Another possible application of

Fig. 10A. PCR Based RFLP (PBR) analysis of a, and a_2 locus specific amplicons



The amplicons generated using a_1 and a_2 specific primers were restricted using 7 four base pair restriction enzymes. G1 & G2 - Gir forest lions. C1 & C2 - Captive bred lions. The Gir forest lions exhibited PBR at a_2 with DpnII, Ddel and Hinfl, whereas captive bred lions did not show any PBR at both *a,* and a_2. Arrow indicates Polymorphic band

Fig. 10B. PCR Based RFLP (PBR) analysis of a, and a_2 locus specific amplicons (Cont'd)



PBR was observed in Gir forest lions at $a_2$when analyzed using HaeII and HhaI. Arrow indicates Polymorphic band

Table-2: Summary of observation of PBR analysis in wild and captive lions

Restriction enzyme	a i		<X2	
	wild	captive	wild	captive
DpnII	**X**	**X**	**'(D**	X
DdeI	**X**	**X**	**^(2)**	X
AluI	**X**	**X**	**X**	X
HinfI	**X**	**X**	**•-(1)**	X
RsaI	**X**	**X**	**X**	X
HaeIII	**X**	**X**	**•d)**	X
HhaI	**X**	**X**	**'(1)**	X

Table shows a comparison of PBR (PCR based RFLP) in wild and captive lions, at en and 012 loci. X-represents absence of PBR, ^-represents presence of PBR and (n) represents the number of polymorphic bands.

PBR especially in relation to disease susceptibility/resistance would be in determining linkage between phenotype and restriction fragment polymorphism.

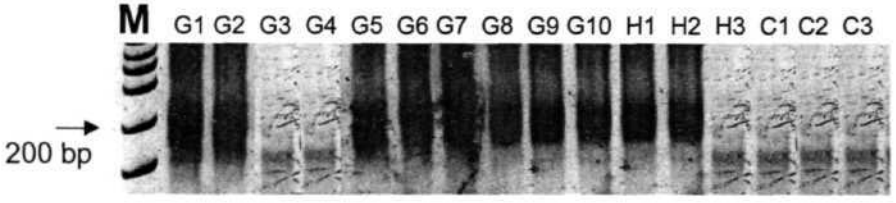
However, in the present study, the level of polymorphism at MHC loci as revealed by PBR analysis among the different lions appears to be limited and additional restriction enzymes or other strategies may be required for analysis of polymorphism.

4.3 Single stranded conformational polymorphism (SSCP) analysis

The PCR amplified products of 10 wild (Gir forest) lions, 3 hybrid lions and 3 captive lions generated using both CM and a_2 specific primers were subjected to SSCP analysis through a silver stained composite gel electrophoretic analysis as described in materials and methods. In this analysis, with respect to amplicons generated using ai-specific primers, it was observed that eight out of 10 wild lions (from Gir forest) and two out of 3 hybrid lions exhibited a smear in the SSCP and rest of the lions including the 3 captive lions produced a non-polymorphic band (Fig. 11). Similarly, with respect to a2-specific primers, all the 10 wild lions, 2 out of 3 hybrid lions and one out of 3 captive lions exhibited smear like pattern in the SSCP and rest of lions exhibited a clear band in the gel.

The methodology of SSCP is a powerful technique for analyzing sequence polymorphism in PCR amplicons. Andersen *et al.* (2003) developed a high throughput SSCP strategy for mutation detection at MHC loci by automated capillary array electrophoresis. A SSCP based assay for major histocompatibility complex B genotyping in chickens was developed by Goto *et al.* (2002). In this study, allelic differences at chicken B-F (class I) and B-L (class II) loci were

Fig. 11. Single stranded conformational polymorphism (SSCP) analysis of a., and a_2 locus specific amplicons



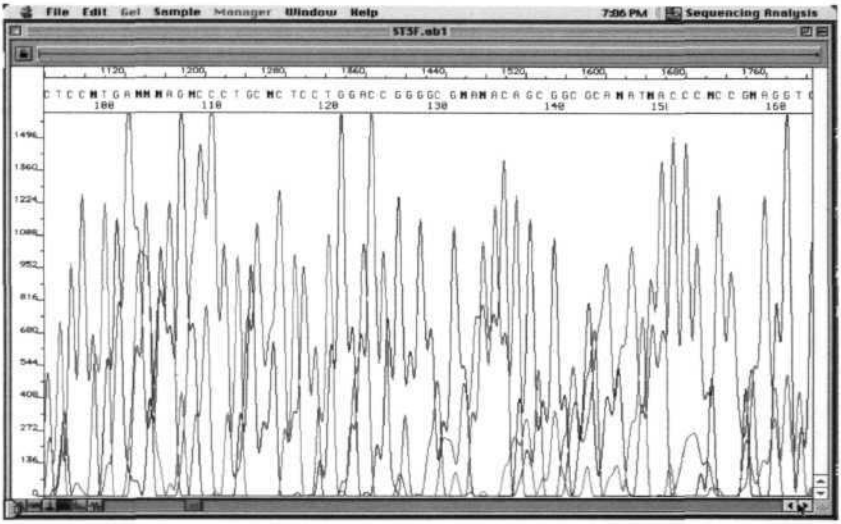
The amplicons generated using *a,* and a_2 specific primers were analyzed for SSCP. 8 out of 10 Gir forest lions (G1 to G10) and 2 out of 3 Hybrid lions (H1 to H3) showed smear like pattern, while the captive bred lions (C1 to C3) showed a single band. M - Molecular weight marker (100 bp ladder)

detected in PCR single-strand conformation polymorphism (SSCP) assays. In the present study, an intriguing observation of smear like appearance of bands was noticed in many samples of wild lions and also hybrid lions. Yamamoto *et al.* (1996) also reported appearance of such smear like bands in SSCP analysis of T-cell receptor messenger signals, when a heterogeneous T cell population was used. On the other hand, a single T cell clone was found to exhibit a clear band in their study. The appearance of smear like pattern in wild and hybrid lions in the present study might be due to the existence of a number of MHC allelic entities in a single PCR amplicon as a result of a number of point mutations in the MHC sequence. Hence, a strategy for sequence based typing of the MHC amplicons of lions and other animals was designed in the present study.

4.4 Direct sequencing of PCR amplicons of MHC loci

When the gel extracted amplicons of lion and other samples amplified using en and α2 locus specific primers were attempted for direct sequencing in a ABI PRISM 3700 sequencing apparatus, crowded and overlapping peaks were observed between 185-206 bp in case of en locus and 80-100 & 185-200 bp in case of α2 locus (Fig. 12). Lobashevsky and Thomas (2000) had earlier designed a similar direct sequencing strategy wherein amplicons of rhesus monkey Mamu-A locus amplified using allele specific primers were gel purified and sequenced directly. They reported such direct sequencing method to be highly reproducible and capable of discriminating subset of class IA locus genes in rhesus monkeys. But in the present study, the observation of crowded and overlapping peaks is somewhat intriguing. One possible explanation for this might be that the

Fig. 12. Electropherogram showing overlapping peaks after direct sequencing



When the PCR amplicons of a, and a_2 were sequenced directly in an automated sequencer (ABI Prism® Sequencer), overlapping peaks were observed in all the lion samples

sequenced individuals might be heterozygous with respect to allelic constitution at the MHC class I region sequenced. But, Santamaria *et al.* (1992) demonstrated that the methodology of direct sequencing of amplicons for sequence based typing studies is useful for determination of allelic sequences in heterozygotes. Another explanation for overlapping peaks is that the sequencing template might be a heterogeneous population consisting of multiple copies of MHC alleles, which might have arisen due to simultaneous PCR amplification of several ai and a_2 copies existing at MHC class I in these lions. Yukhi and O'Brien (1994) reported that the cat possesses a minimum of 20 class I genes and 5 class II genes per haploid genome. Lobashevsky *et al.* (1999) in efforts to Identify DRB alleles in rhesus monkeys using polymerase chain reaction-sequence-specific primers (PCR-SSP) amplification reported the occurrence of multiplicity of Mamu-DRB1 alleles per haplotype.

Since no other published paper has reported observation of such crowded sequencing peaks in direct sequencing studies, in the present study, the amplicons were first cloned in a blunt ended cloning vector pMOSB/ive and then the inserts in each of the clones were sequenced as described in materials and methods. In a study by Zheng *et al.* (1999), ten pairs of primers, derived from the sequences of expressed B-L beta II family (class II MHC) alleles in chicken, were used in Sequence based typing analysis involving PCR amplification, cloning and subsequent sequencing of these family-specific amplicons. Murray and White (1998) also adopted a similar strategy for studying Sequence variation at the major histocompatibilitv complex DRB loci in beluga *(Delphinapterus leucas)* and

narwhal *(Monodon monoceros),* wherein the PCR amplification of DRB loci was carried using allele-specific primers designed for amplification of exon-2, and the PCR amplicons were cloned and sequenced. Hence in the present study also a strategy was designed for cloning of PCR amplicons and sequencing them using vector-specific primers.

4.5 Large scale PCR amplification, gel elution and cloning of amplicons

Since, direct sequencing of PCR amplicons revealed only crowded overlapping peaks and clear sequencing peaks could not be obtained, a strategy was designed for large scale PCR amplification, gel elution of PCR amplicons and cloning of the amplicons in a blunt ended cloning vector, pMOSB/ue for subsequent sequencing using vector specific primers T7 and U19. After amplification in 50 ^l reactions, the amplified products were loaded in 1% agarose gels and eluted using GFX® gel elution columns. A recovery of 50-70% of PCR amplicons was observed after gel elution. When the gel-eluted amplicons were cloned in the vector using *pMOSBlue* competent cells and screened for positive colonies based on blue-white screening using X-Gal, the transformation efficiency in terms of percentage of positive colonies (white) was very low (5-10%). One reason for this low transformation frequency might be that the viability of these competent cells supplied by the manufacturer was low and might have lost its competency during storage/transportation. When DH5a and DH1 OB competent cells were used, the transformation efficiency was observed to be 30-40% and 60-80% respectively. When Blue and white colonies generated using DH5a and DH10B competent cells were checked for presence of insert

through colony PCR randomly, it was observed that many white colonies (-50%) were devoid of inserts in the clones generated using DH5a competent cells. Several reasons have been documented for appearance of such false positives (Murray *et* a/., 1993) including single base pair deletions, mutation in the *lacZ* promotor leading to lower transcription of the alpha peptide mRNA, loss of lacZ-Delta-M15 gene carried on the F episome of the *E .coli* strain DH5a, mutations in the alpha peptide gene, mutations in the first ATG or Shine-Dalgarno sequence of the alpha peptide sequence etc. The appearance of false positives in colonies generated using DH5a, might be due to any one of the above said reasons, but in the case of clones generated using DH10B cells, the number of such false positives were very less (-2%). Since the clones of DH5a were giving many false positives, DH10B competent cells were used for subsequent cloning efforts.

4.6 Sequencing of cloned amplicons

The PCR amplicons generated using genomic DNA from lions, tigers, leopards, domestic cats, dogs and humans were cloned in pMOSB/ue blunt ended cloning vector and the clones were sequenced using the T7 and U19 primers of the vector as described in materials and methods. The results of the sequencing of samples from the different animals under study are reported below. After removing vector sequences, ai & a2 insert sequences were aligned with respect to published sequence of domestic cat and checked for sequence polymorphism between the sequences of the clones of the same animal and also for polymorphism between the sequences of clones of different animals.

4.6.1 Sequence polymorphism at on locus in lions

When sequences were aligned with respect to published sequence of CM locus of domestic cat (from which the primers were designed in the present study). Sequence polymorphisms/mutations were observed between the domestic cat and lion. A few such sequence differences between domestic cat and lions, which were observed at 29, 31,124 bp, were identical in all the clones. It was observed that the clones of different lions had sequence polymorphism in the region between 160-200 bp (hot-spot region) of the 265 bp amplicon of en locus (Fig. 13). In rest of the sequence however, this polymorphism was comparatively very less. It was also observed that sequence polymorphism was concentrated in the 'hot-spot' region between the clones of different lions in all the three lion groups (wild, hybrid and captive).

Yukhi and O'Brien (1994) in their study on exchanges of short polymorphic DNA segments in feline major histocompatibility complex class I genes, reported that feline MHC class I alleles have highly mosaic structures with short polymorphic sequence motifs that are rearranged between alleles of individual MHC loci, between MHC class I genes within cat species, and between homologous MHC loci in different species.

Another intriguing observation in the present study was the presence o1 sequence polymorphism between clones of the same lion in the hot spot region with respect to ai locus-specific amplicon. The nature of polymorphism was similar to those observed between clones of different lions and lion groups (wild, hybrid and captive groups). One possible reason for appearance of such

sequence polymorphism between clones of the same lion (individual) might be because of PCR artifacts or due to the errors in PCR amplification by Taq polymerase (Kennedy *et al.,* 2002). But this seems unlikely in the present investigation, because a high fidelity Taq polymerase (AmpliTaq) that has negligible error rate was used for PCR amplification. In order to confirm this, the plasmids harboring the clones were used as template for amplification of the cloned insert using specific primers (ai or 012 specific primers). After amplification, the amplicons were once again cloned in the pMOSB/i/e cloning vector and 10-15 clones from each amplicon were randomly picked and sequenced. After sequencing, when the sequences of the reamplified clones were checked with the original sequence, it was observed that the randomly picked clones were exactly similar in sequence to that of the original clone. A similar such strategy was designed by Carol ef *al.,* (1998) to check the error rate of Taq polymerase. In this study, it was observed that Taq polymerase induced an error rate of 1 in 1369 bp. This testified that the sequence differences are not due to erroneous amplification by Taq polymerase. Moreover, if the sequence polymorphism is induced due to the erroneous amplification of Taq polymerase, then such mutations/polymorphism should be at random locations in the sequence. But this is not the case in the present study. The sequence polymorphism was found mainly at the 'hot-spot' region. Hence, in all likelihood, the observed polymorphism between the clones of the same individual and also between different individuals are due to the recombinations/conversion/mutations which might have occurred at the MHC loci in the somatic cells of the lions. Yukhi and

O'Brien (1990) have reviewed many such mechanisms, which can create such sequence polymorphism at MHC loci.

In Gir forest lions, the sequence polymorphism at ai locus between the clones of the same individual was observed to be in the range of 12-21% with an average of 14.8% (± 3.1) with a P value of <0.05, which is quite significant. The details of such sequence polymorphism at o^ locus between the clones of a single individual of Gir forest lions are given in Fig. 13.

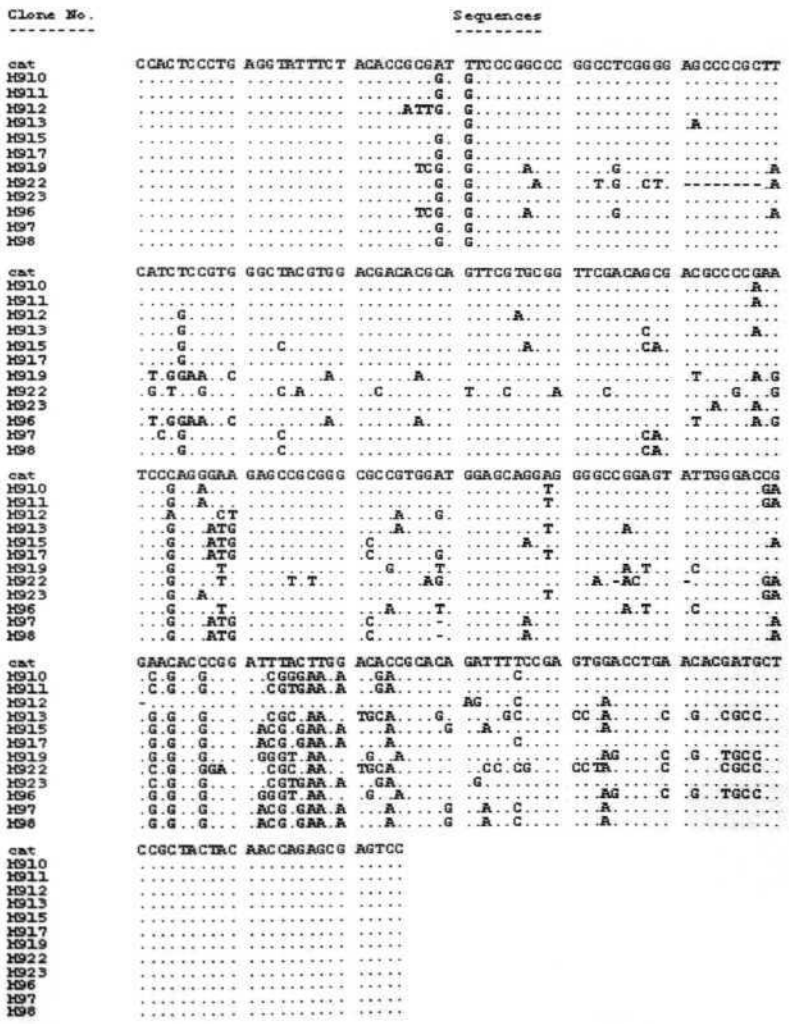
In Afro-Asian hybrid lions, the sequence polymorphism at ai between the clones of same individual was in the range of 20-23% with an average value of 21.0% (± 1.7). The sequence polymorphism at CM between the clones of a single individual of hybrid lion is given in Fig 14.

In captive lions, the sequence polymorphism at c^ between the clones of the same individual varied between 9-12% and the average value was observed to be 10.7% (± 1.5). Fig 15 shows the sequence polymorphism between the clones of a single individual of captive lion.

When all the clones of *ay* of the 10 Gir forest lions were aligned together and checked for extent of sequence variation, it was observed to be 28%. Similarly, between the different clones of the three hybrid lions the sequence variation was 32% and in the case of captive lions it was observed to be 15%.

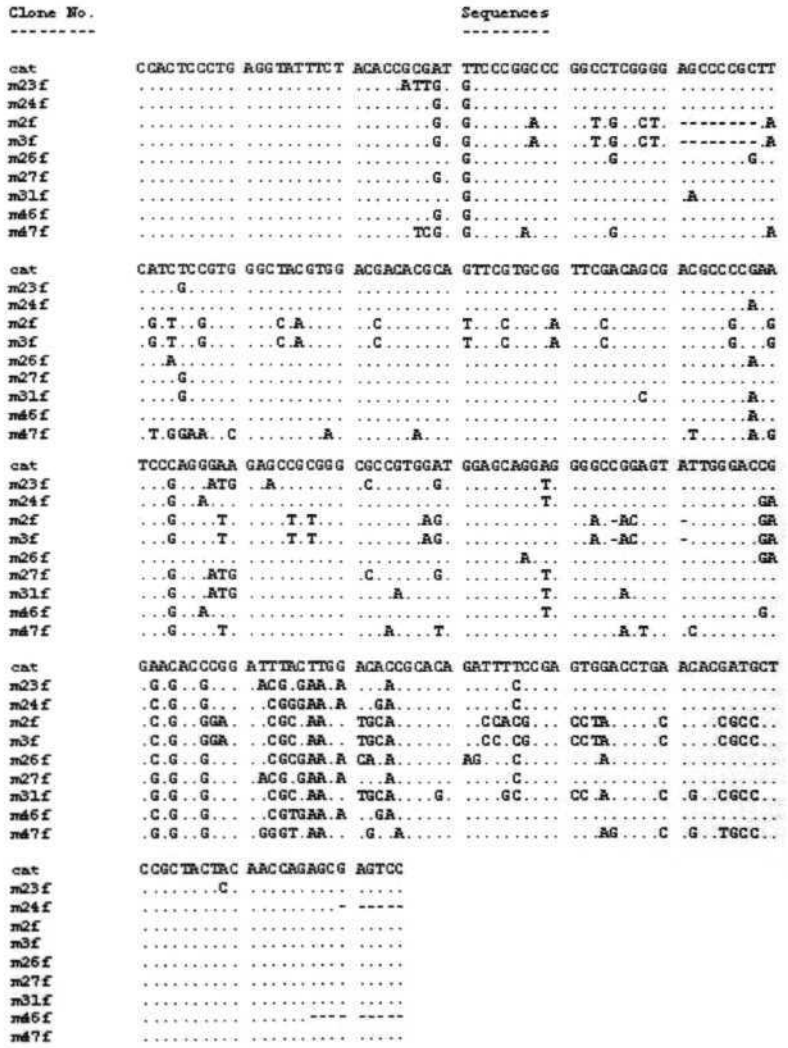
The sequence data of the 10 Gir forest lions, 3 hybrids and 3 captive-bred lions were calculated for heterozygosity value as per the procedure described in materials and methods. A heterozygosity of 23% was observed in the 10 Gir forest lions, 27% in hybrid lions and 21% in captive lions. The details of

Fig. 13. Sequence polymorphism between clones of a single Asiatic lion observed at a., locus



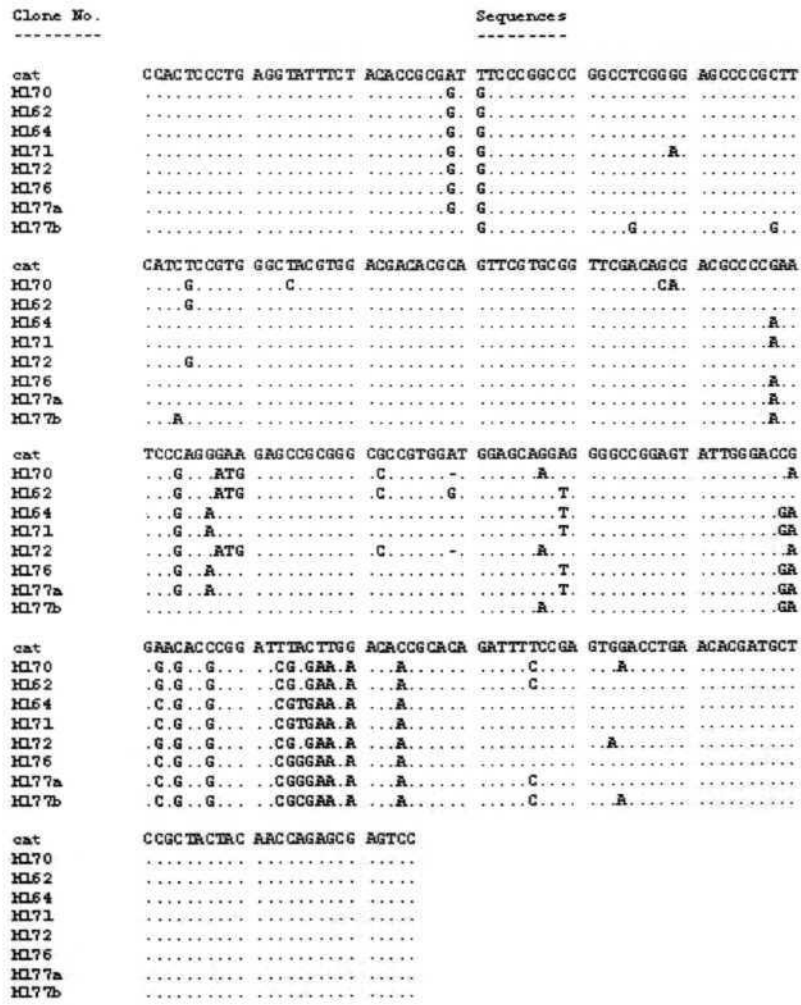
When the PCR amplicons of a, of a single Asiatic lion was cloned and the clones were sequenced individually, sequence polymorphism was observed between the clones mainly at a 'hot-spot' region lying between 160-200 bp.

Fig. 14. Sequence polymorphism between clones of a single hybrid lion observed at a, locus



Similar to the observations in Asiatic lions, the clones of a single Afro-Asiatic hybrid lion showed extensive nucleotide sequence polymorphism particularly in the 'hot-spot' region of the 265 bp amplicon of a_v

Fig. 15. Sequence polymorphism between clones of a single captive bred lion observed at a., locus



Even though sequence polymorphism was observed between clones of a single captive bred lion, the extent of polymorphism was found to be much less when compared to Asiatic and Hybrid lion

sequence variation between the clones of the different individuals of Gir forest lions, hybrid lions and Captive lions are given in Table 3.

A dendrogram (Fig. 16) was constructed based on similarity with respect to consensus sequences at <xi locus of each lion. It is observed that the hybrid lions did not cluster into a single group and were scattered. The three captive lions clustered into a single group with low sequence polymorphism among each other. As far as the Asiatic lions are concerned, even though they mostly clustered into a single group, the similarity between each lion varied widely from 70%-90%.

4.6.2 Sequence polymorphism at u_2 locus in lions

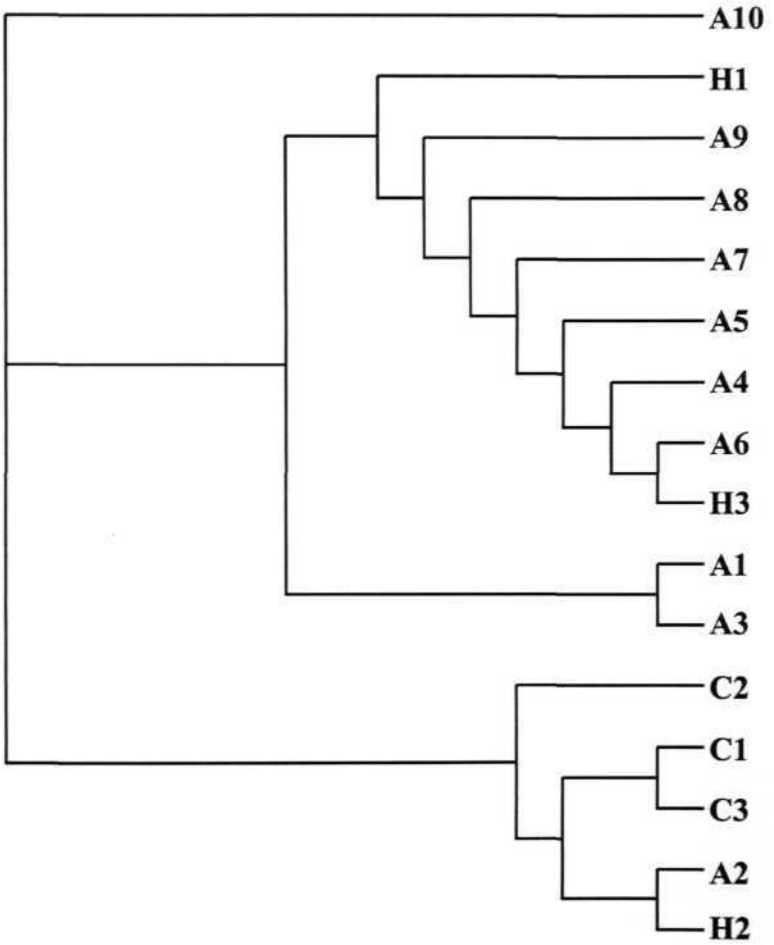
The PCR amplicons of a_2 locus in 10 Gir forest lions, three hybrid and three captive bred lions generated using consensus primers designed from published sequences of domestic cat were first cloned in pMOSB/ue cloning vector and the clones generated from amplicons of the different lions were sequenced as described in materials and methods. The vector sequences from the sequenced samples were removed and the sequences were aligned with respect to published sequence of a_2 locus of domestic cat from which the sequencing primers were designed. Few sequence differences were observed between the consensus sequence of domestic cats and lions. As observed in the case of CM locus, in a_2 also the different clones of the same lion showed polymorphism in all the three lion groups (viz., wild, hybrid and captive), but such polymorphisms were found scattered across the 246 bp amplicons instead of being concentrated in a specific 'hot-spot' region. One reason for this might be

Table-3: Summary of sequence polymorphism at ai locus in lions

Animal	Individual No.	Polymorphism % between clones
Asiatic lion (Gir forest lion)	A1	12
	A2	12
	A3	13
	A4	15
	A5	11
	A6	13
	A7	16
	A8	17
	A9	21
	A10	18
	Mean ± S.D	14.8 ±3.1
Hybrid lion	H1	20
	H2	23
	H3	20
	Mean ± S.D	21.0±1.7
Captive bred lion	C1	09
	C2	12
	C3	11
	Mean ± S.D	10.7±1.5

Table shows a comparison of nucleotide sequence polymorphism at *a-*, locus, between different groups of lions belonging to the different categories of wild (Asiatic), hybrid (Afro-Asiatic) and captive lions. The polymorphism extent was calculated by the use of Gene-tool software for each lion, as the sequence difference between 10 clones of the same animal.

Fig. 16. Dendrogram of sequence polymorphism at a_1 locus in lions



A dendrogram was constructed based on extent of nucleotide sequence polymorphism between consensus sequences of different lions at oc, locus. The hybrid lions (H1 to H3) did not cluster into a single group. The three captive bred lions (C1 to C3) clustered in a single group with low sequence polymorphism amongst each other. The 10 Asiatic lions of Gir forest (A1 to A10) were found to be scattered across the dendrogram

that α_2 region of the MHC molecule is involved in grabbing foreign peptides through side chain interactions of those amino acid residues, which are distantly placed in the MHC chain.

In Gir forest lions the polymorphism between different clones of the same individual varied between 12-20% with an average value of 14.5% (± 2.6) with a P-value of 0.02. The sequence polymorphism between clones of a single Gir forest lion is given in Fig. 17.

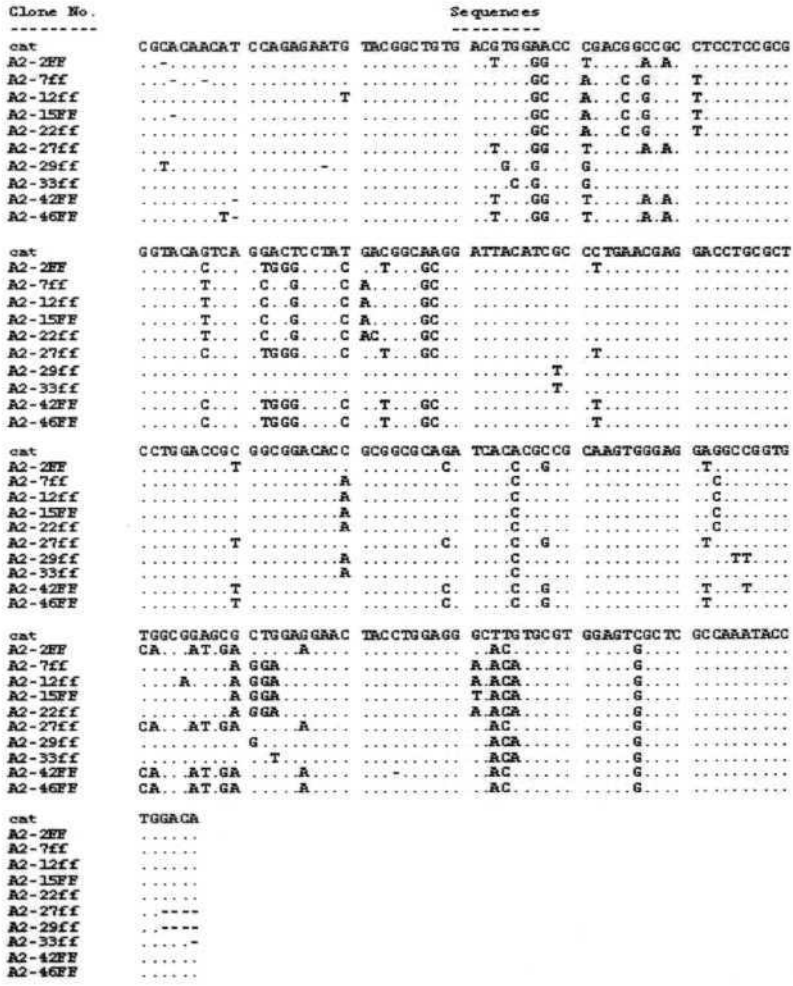
In the case of the three hybrid lions considered for the present study, the polymorphism at nucleotide sequence level between the clones of the same individual was observed to be in the range of 15-18% with an average of 16.7% (± 1.5). Fig. 18 depicts sequence polymorphism observed between clones of a_2 locus of a single hybrid lion.

The different clones of the same individual in case of the three captive lions exhibited sequence polymorphism in the range of 10-11% and the average value was observed to be 10.3% (± 0.5). The sequence polymorphism between clones of a single captive lion is given in Fig. 19.

When all the clones of the 10 Gir forest lions were aligned together and checked for nucleotide sequence variation, it was observed to be 25% and in the case of the three hybrid lion and three captive bred lions it was 29% and 15% respectively.

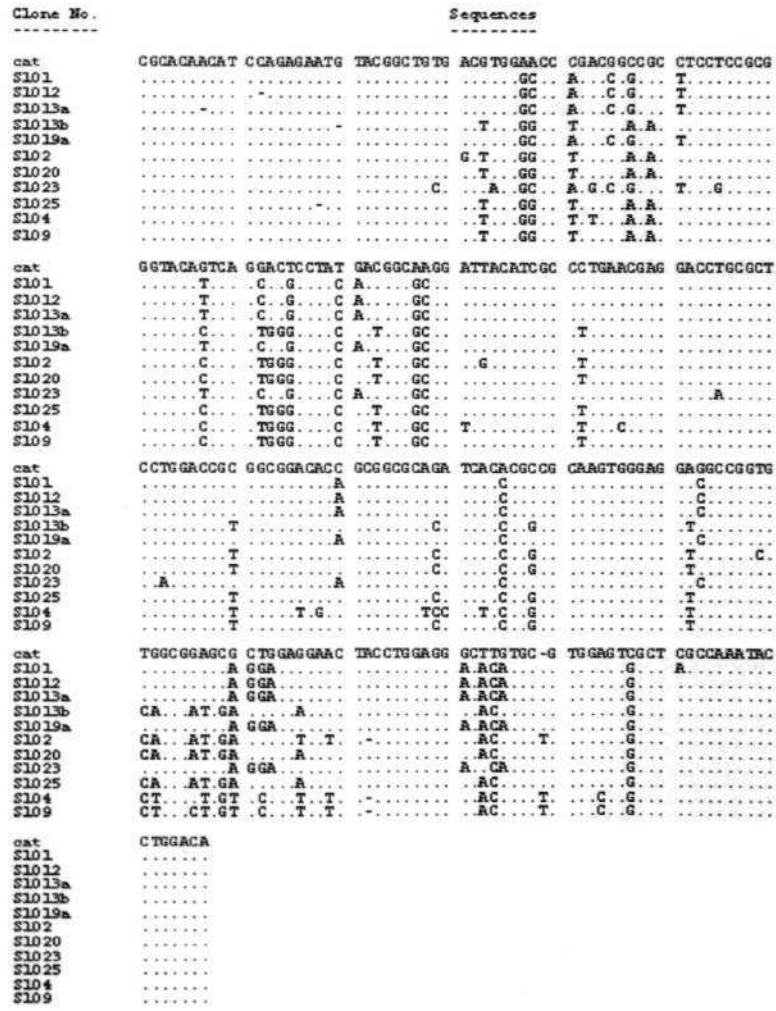
The Gir forest lions showed a heterozygosity of 22%, whereas hybrids and captive lions 25% and 15% respectively.

Fig. 17. Sequence polymorphism between clones of a single Asiatic lion observed at a_2 locus



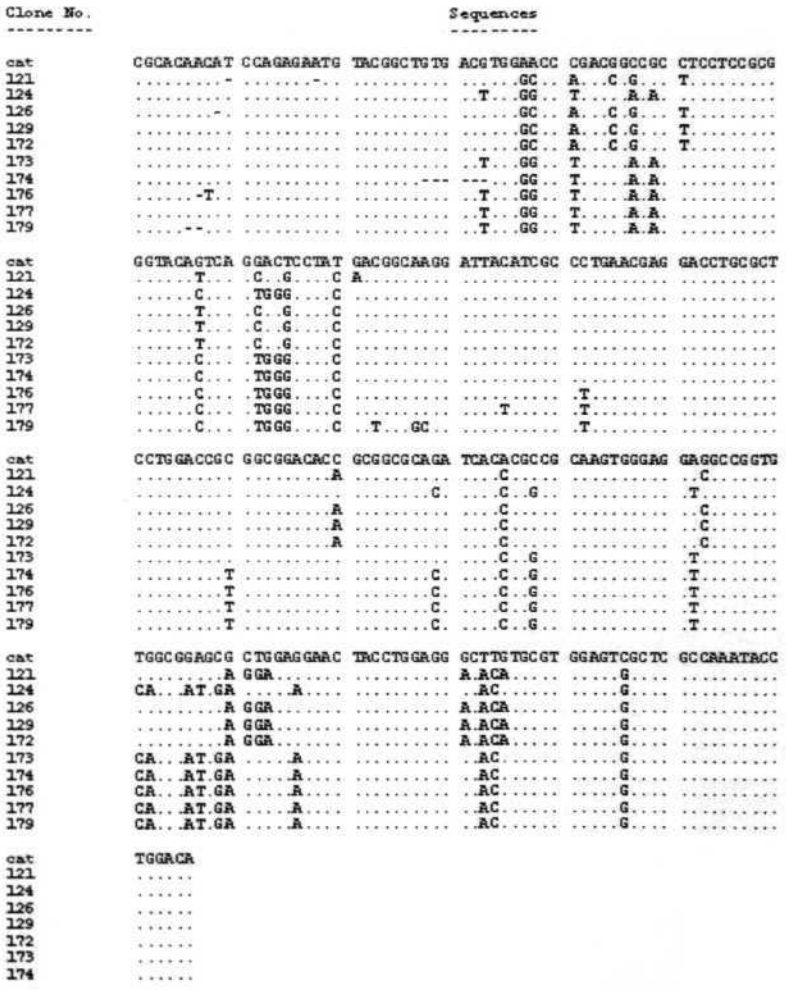
When the PCR amplicons of a_1 of a single Asiatic lion was cloned and the clones were sequenced individually, sequence polymorphism was observed between the clones. The polymorphism was found scattered throughout the 246 bp amplicon and not concentrated in single 'hot-spot

Fig. 18. Sequence polymorphism between clones of a single hybrid lion observed at a$_2$ locus



Similar to the observations in Asiatic lions, the clones of a single Afro-Asiatic hybrid lion showed extensive nucleotide sequence polymorphism scattered throughout the 246 bp amplicon of a$_2$.

Fig. 19. Sequence polymorphism between clones of a single captive bred lion observed at a_2 locus



Even though sequence polymorphism was observed between clones of a single captive bred lion, the extent of polymorphism was found to be less when compared to Asiatic and Hybrid lion

Table 4 list shows a summary of sequence -polymorphism at 012 locus in lions.

A dendrogram (Fig. 20) was constructed based on similarity between consensus sequences at a_2 loci of all the lions considered under the study. As observed in the case of dendrogram for OH locus, in a_2 also the hybrid lions were found to be scattered with a high polymorphism among each other. All the captive lions were clustered together with low sequence polymorphism between each other. The Asiatic lions, which exhibited moderate levels of sequence polymorphism, were found to be mostly concentrated as a single group.

4.6.3 Discussion: Sequence polymorphism at a-i and *0.2* locus in lions

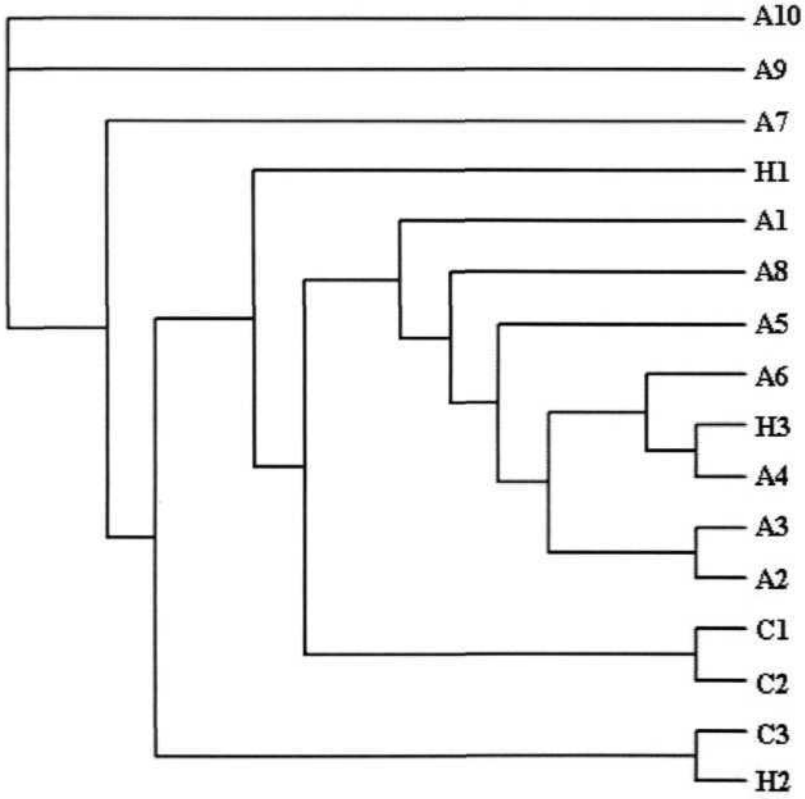
In a study using 10 representative polymorphic nuclear microsatellite markers, Singh *et al.* (2003) analyzed the genetic polymorphism in 6 Asiatic lions and 4 hybrid lions. A neighbor joining dendrogram, constructed based on the proportion of shared alleles distance measured, which showed differentiation in all the individuals analyzed. The Asiatic lions form a distinct cluster unlike the hybrids, which group with both Asiatic and African lions. In the present study also it was observed that the hybrid lions grouped along with pure Asiatic lions and captive lions. One postulated reason for this might be that when compared to the Asiatic lions and captive bred lions, hybrid lions do have more sequence polymorphism among each other, which is responsible for their not grouping in a single group. A similar observation was reported by Liu *et al.* (2002) in chickens, wherein hybrid lines possessed more sequence polymorphism at MHC and hence more disease resistance when compared to inbred chicken lines.

Table-4: Summary of sequence polymorphism at a_2 locus in lions

Animal	Individual No.	Polymorphism % between clones
Asiatic lion (Gir forest lion)	A1	15
	A2	13
	A3	12
	A4	13
	A5	13
	A6	13
	A7	15
	A8	13
	A9	20
	A10	18
	Mean ± S.D	14.5±2.6
Hybrid lion	H1	15
	H2	18
	H3	17
	Mean ± S.D	16.7±1.5
Captive bred lion	C1	10
	C2	11
	C3	10
	Mean ± S.D	10.3±0.5

Table shows a comparison of nucleotide sequence polymorphism at a_2 locus, between different groups of lions belonging to the different categories of wild (Asiatic), hybrid (Afro-Asiatic) and captive lions. The polymorphism extent was calculated by the use of Gene-tool software for each lion, as the sequence difference between 10 clones of the same animal.

Fig. 20. Dendrogram of sequence polymorphism at a_2 locus in lions



A dendrogram was constructed based on similarity between consensus sequences at a_2 loci of all the lions. The hybrid lions (H1 to H3) were found to be scattered with a high polymorphism amongst each other. All the captive lions (C1 to C3) clustered together with low sequence polymorphism between each other. The Asiatic lions (A1 to A10) were found to be mostly concentrated as a single group.

An interesting observation emerging out of the present study, which is significant from conservation point of view is that the Asiatic lions of the Gir forest exhibit a significantly high level of sequence polymorphism among the clones of the same individual (15% in case of ai and 14% in case of a_2 locus respectively) and between different individuals (28% in case of ai and 25% in case of 012 respectively). Wildt *et al.* (1987) based on a comparative study of the Asiatic lions of Gir forest and several African lions established a direct correlation between the lack of genetic variability in the Asiatic lion and high incidence of morphologically abnormal spermatozoa, low levels of the male steroid hormone-testosterone. They also predicted that the Asiatic lion has suffered a population bottleneck followed by inbreeding. But in this study, only three lions from the Serengeti ecosystem along with one from Gir forest have been considered, this number can be considered as very insignificant for such correlation studies. In another study, Shivaji *et al* (1998) analyzed 7 Asiatic lions from different zoos in India for sperm related traits and concluded that they do have good spermatozoa count; high percentage of motile spermatozoa and low incidence of abnormal spermatozoa, thus implying that inbreeding depression has not set in these animals. This observation is in direct contradiction with the observations of Wild *et al* (1987). In another study by Shankaranarayanan *et al.,* (1997), by using RAPD and microsatellite analysis of the Asiatic lions showed a significantly high heterozygosity of 25.82%. The results of the present study wherein the lions of Gir forest show a significantly higher level of sequence polymorphism at both *a,* and a_2 locus is in accordance to the observations of the above said studies. In a

study on evolution of MHC class II Ep Diversity within the genus *Peromyscus,* Richman *et al.* (2003), reported that intragenic recombination might efficiently regenerate allelic polymorphism following a population constriction. They have also proposed that population bottleneck(s) is responsible for both rapid evolutionary change and an episodic increase in the effective rate of recombination at the MHC loci in *Peromyscus.* Thus the observation of a significantly high level of diversity at MHC class I locus in the Gir forest lions in the present study, in spite of the population bottleneck these lions have undergone in their history, might be due to an enhanced rate of intragenic recombination and mutation at the immune loci. This explains the fact that the Gir forest lions still exist as a healthy group without much decline in their numbers in spite of excessive pressures exerted on their habitat by human interference.

The captive-bred lions show a lesser degree of sequence polymorphism at both ai and a_2, compared to wild lions of Gir forest and hybrid lions. A complete pedigree of these captive lions is not available and many such captive lions were actually rescued from circuses, wherein mating choices were limited to 4-5 lions. Hence sit is hypothesized that because of the severe inbreeding which they have undergone in captivity, the captive lions show a lesser sequence polymorphism at MHC class I loci when compared to wild lions and hence may be vulnerable to diseases

The hybrid lions exhibited higher sequence polymorphism at both en and α_2 loci when compared to Gir forest lions and captive bred lions. Earlier,

Shankaranarayanan and Singh (1998) in their study on mitochondrial DNA sequence divergence among lions through mitochondrial D loop sequencing revealed the presence of only one haplotypes among the Asiatic lions while the hybrids (Asiatic x African) exhibited a number of haplotypes which is in agreement with the observations of the present study. O'Brien *et al.* (1987) also observed that African x Asian hybrids bred well in captivity whereas a high rate of mortality was noticed in Asiatic lions. Menotti-Raymond and O'Brien (1993) hypothesized that the lions underwent a population bottleneck about 10,000 years ago at the end of Pleistocene ice age. Only a few haplotypes of the Asiatic lions might have survived this bottleneck, and the degree of polymorphism might have been reduced due to the severe inbreeding witnessed in the Asiatic lions. In contrast, the African lions, 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 and hence higher sequence polymorphism. But since results of the present study do not show reduced polymorphism, this hypothesis is no longer valid.

From conservation point of view, the observation of higher genetic polymorphism among the Gir forest lions when compared to captive bred lions is a matter of satisfaction because these show that the Asiatic lions of Gir forest are far away from the point of no return. This strongly contradicts the observations an conclusion drawn by Wildt *et al.* (1987), that the Asiatic lions have no genetic variation and therefore are heading for extinction. The results of the present study might also facilitate the identification of wild lions from the Gir forest with

the best sequence polymorphism at both o^ and 012 loci and such animals could be used in controlled breeding programmes to ensure propagation of genetic variability. The Gir forest lion A9 was observed to show a higher degree of polymorphism at MHC sequence with respect to both ai and a_2 (21% at <n and 20% at 012) when compared to other lions of Gir forest and hence can be used in such breeding programmes. Shivaji *etal.* (1998) also proposed a similar strategy wherein Gir forest lions with the best semen profiles were advocated for use in controlled breeding programmes. Many studies (Gomendio *etal,* 2000; Dahlbom *et al,* 1997; O'Brien *et al,* 1987) have shown a direct correlation between the degree of genetic variation and the quality of semen.

The values of heterozygosity observed in the Gir forest lions in the present study are much higher than the value of 10% observed by Yukhi and O'Brien *et al* (1990) based on Allozyme variation and RFLP studies. This may be because, in the present study MHC sequence was considered for analysis of heterozygosity while Yukhi and O'Brien considered allozyme and RFLP data which generally reveal lesser polymorphism. The heterozygosity values with respect to African lions as calculated by Yukhi and O'Brien based on RFLP values is comparable to values obtained in hybrid lions in the present study (27% in case of ai and 25% in case of (22).

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

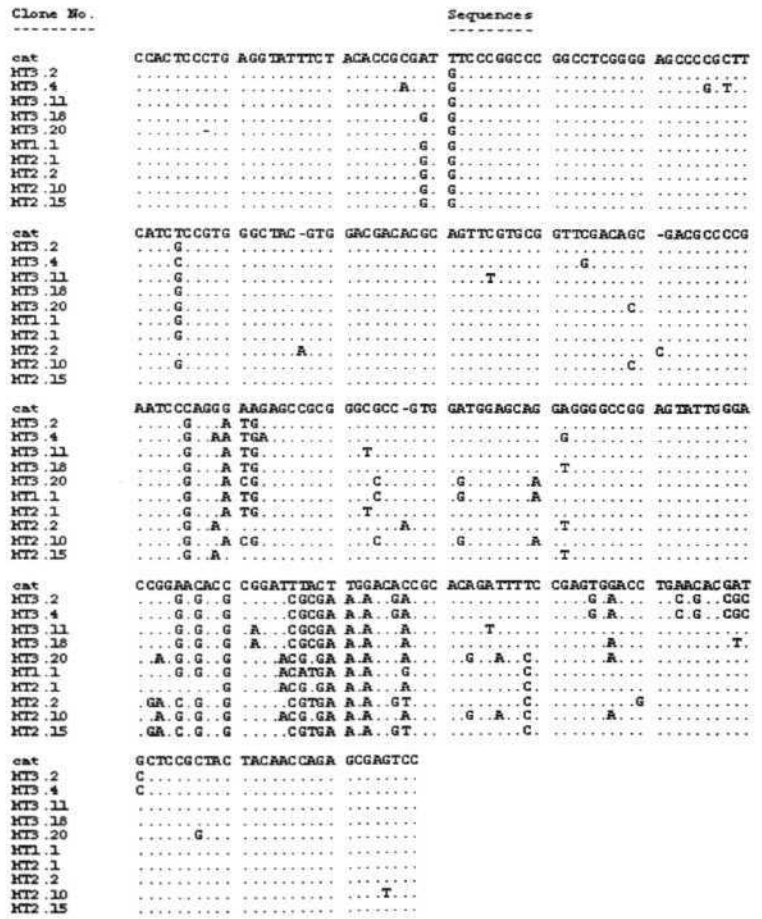
widely debated, it often leads to lower viability, that is, increased susceptibility to diseases and fecundity (inbreeding depression). It becomes imperative that conservation programs for small populations must devise methods to minimize, halt or even reverse the decline in genetic variability that occurs. Cluster analysis based on sequencing data of ai and ot2 locus shows that while most of the Gir forest lions and captive bred lions do cluster together, some individuals exist as outgroups and exhibit variation (Asiatic lions A9 and A2 based on sequence polymorphism) from the population. As discussed earlier, these can be used for conservation breeding programme to increase the genetic variability of the population.

4.6.4 Sequence polymorphism at «i locus in other animals and humans

In order to assess such sequence variation at ai in the case of other animals of Feline category like leopards, tigers & domestic cats and in other non-feline species like humans and dogs, the genomic DNAfrom these felid and non-felid species were amplified using the specific primers as described in materials and methods, cloned in *pMOSBlue* cloning vector and sequenced as done in the case of lions. In the case of all these animals, sequence polymorphism was observed between the clones of the same individual and also between different individuals.

In case of the three tigers considered in this study, the polymorphism at <*x*-\ locus between the clones of the same individual varied in range of 21-22% with an average value of 21.3%. Fig. 21 shows the sequence polymorphism between

Fig. 21. Sequence polymorphism between clones of a single tiger observed at a., locus



Similar to the observations in lions at a,, in tigers too, the sequence polymorphism was observed mainly at a 'hot-spot' region lying between 160-200 bp.

the clones of a single tiger. When the sequence variation between all the clones of the three tigers was calculated, it was observed to be 23%.

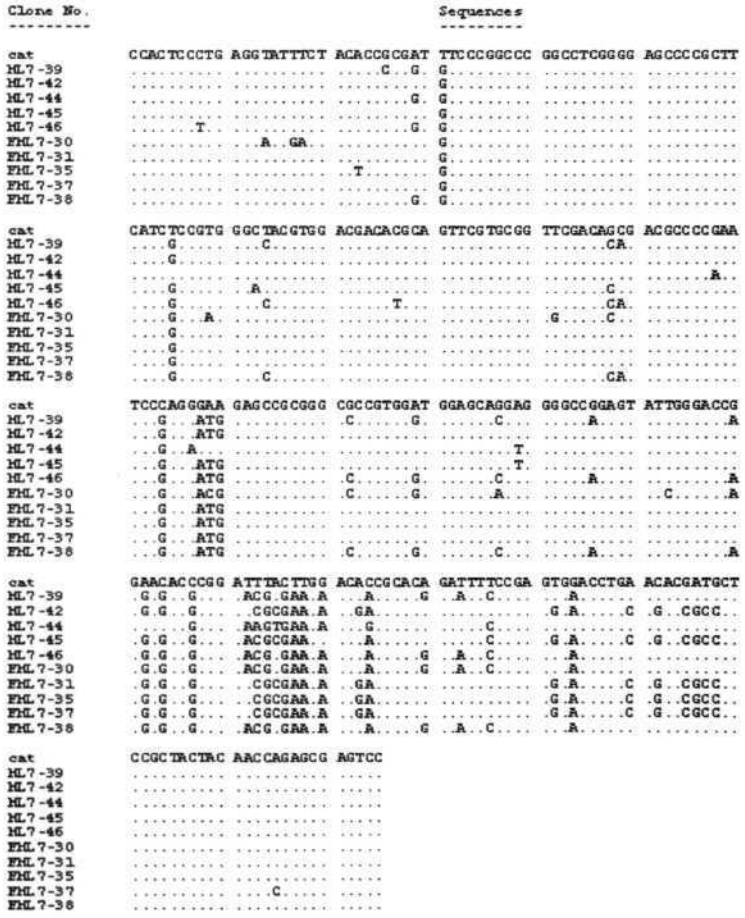
In case of Leopards, this polymorphism between the clones of the same individual was observed to be in the range of 23-27% with an average value of 25.3%. The sequence polymorphism at ai locus between the clones of a single leopard is given in Fig. 22. The extent of polymorphism between all the clones of the three leopards was 27%.

In case of the three domestic cats considered for such sequence polymorphism study at on locus of MHC, the intra-individual clone sequence polymorphism varied in the range of 17-18% with an average value of 17.7%. The sequence polymorphism at ai locus between the clones of a single domestic cat is given in Fig. 23. The extent of sequence variation extent between all the clones of the three domestic cats was observed to be 21 %.

In case of dogs, the polymorphism extent was observed to be in the range of 29-31% with an average value 30.0%. The extent of sequence variation between all the clones of the three dogs was observed to be 38%.

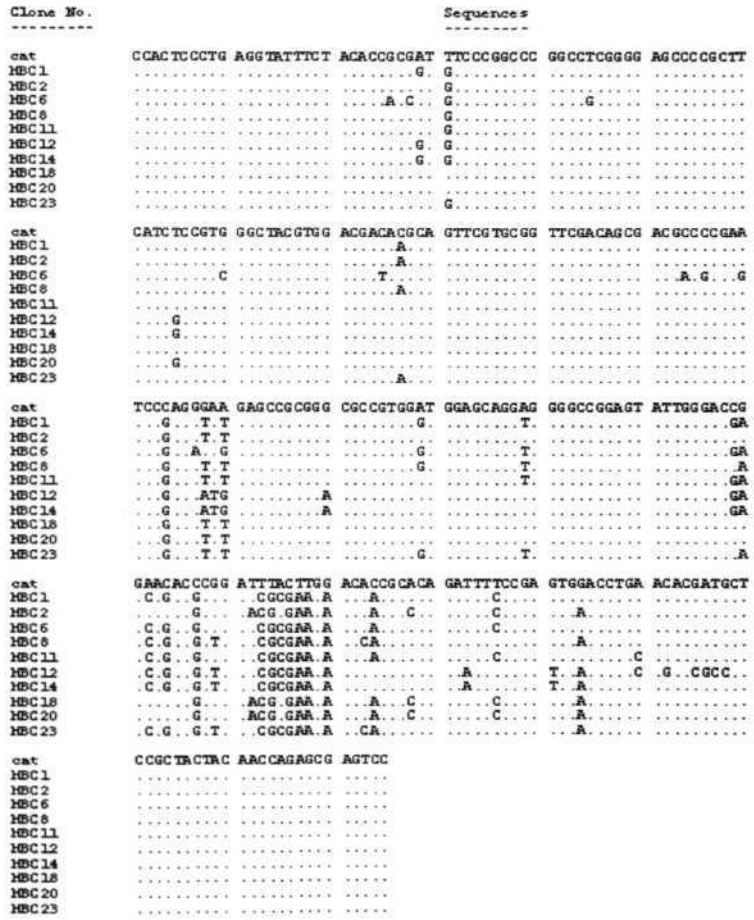
When three human samples were considered for sequence polymorphism study at cti, the extent of sequence variation between the clones of a! amplicons of the three human samples was in the range of 15-19% and the average value was 16.7%. The extent of sequence polymorphism between the clones of all the three human samples when considered together was observed to be 23%.

Fig. 22. Sequence polymorphism between clones of a single leopard observed at GL, locus



Similar to the observations in lions and tigers at a_v sequence polymorphism was observed mainly at a 'hot-spot' region lying between 160-200 bp in case of leopards. But the extent of polymorphism was slightly higher in leopards when compared to tiger

Fig. 23. Sequence polymorphism between clones of a single domestic cat observed at a_1 locus



Sequence of a few clones derived from amplicons of a_1 locus of domestic cat revealed extensive nucleotide sequence polymorphism at 'hot-spot' region between 160-200 bp, much similar to the observations made in lions, tigers and leopards. But the extent of polymorphism in cats was slightly lower when compared to big wild cats

A summary of sequence polymorphism observed between the clones of ai locus specific amplicons of the different lions, tigers, leopards, domestic cats, dogs and humans are given in Table 5.

4.6.5 Sequence polymorphism at u_2 locus in other animals and humans

To observe sequence polymorphism between the clones of a_2 amplicons of the 3 tigers, 3 leopards, 3 domestic cats, 3 dogs and 3 human samples, the amplicons generated using specific primers designed were cloned into the cloning vector and sequenced as described in the materials and methods.

In case of the three tigers considered in this study, the polymorphism at a_2 locus between the clones of the same individual varied in range of 23-24% with an average value of 23.7%. Fig. 24 shows the sequence polymorphism between the clones of a single tiger. When the sequence variation between all the clones of the three tigers was calculated, it was observed to be 25%.

In case of Leopards, the polymorphism between the clones of the same individual was found to be in the range of 24-28% with an average value of 26.7%. The sequence polymorphism at a_2 locus between the clones of a single leopard is given in Fig. 25. The extent of polymorphism between all the clones of a_2 of the three leopards was observed to be 30%.

In case of the three domestic cats considered for such sequence polymorphism study at a_2 locus of MHC, the intra-individual clone sequence polymorphism varied in the range of 12-15% with an average value of 13.7%. The sequence polymorphism at a_2 locus between the clones of a single domestic

Table-5: Summary of sequence polymorphism at on locus of other animals and humans

Animal	Individual No.	Polymorphism % between clones
Tiger	**T1**	21
	T2	22
	T3	21
	Mean ± S.D	21.3±0.6
Leopard	**L1**	23
	L2	26
	L3	27
	Mean ± S.D	25.3±2.0
Domestic cat	Ca1	17
	Ca2	18
	Ca3	18
	Mean ± S.D	17.7±0.6
Dog	**D1**	31
	D2	30
	D3	29
	Mean ± S.D	30.0±0.7
Human	Hu1	15
	Hu2	19
	Hu3	16
	Mean ± S.D	16.7±2.0

Table shows a comparison of nucleotide sequence polymorphism at a, locus, between different animals. The polymorphism extent was calculated for each animal by the use of Gene-tool software, as the sequence difference between 10 clones of the same animal.

cat is given in Fig. 26. The sequence variation extent between all the clones of the three domestic cats was observed to be 18%.

In case of dogs, the extent of polymorphism was observed in the range of 24-26% and the average value observed was 25.3%. The extent of sequence variation between all the clones of the three dogs observed was 38%.

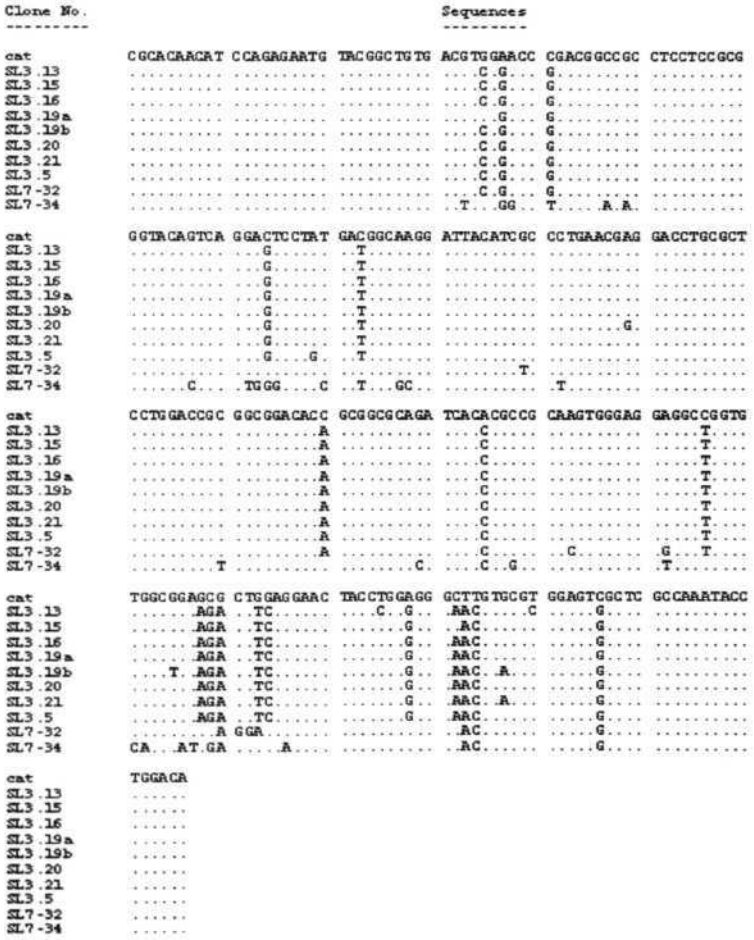
When three human samples were considered for sequence polymorphism study at a_{2I} the extent of sequence variation between the clones of a_2 amplicons of the three human samples observed was in the range of 20-23% with an average value of 21.3%. The extent of sequence polymorphism between the clones of all the three human samples when considered together was observed to be 25%.

A summary of sequence polymorphism observed at a_2 locus in other animals and humans is given in Table 6.

4.6.6 Discussion: Sequence polymorphism at ai **and a_2 locus in other animals and humans**

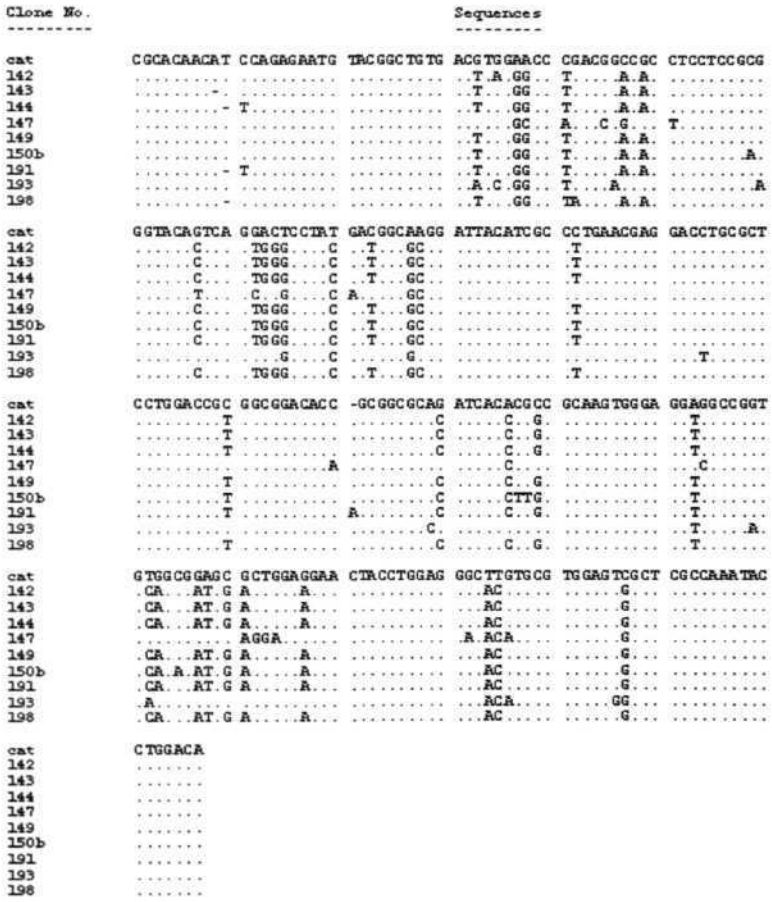
In the present study, similar to the observation in lions, in tigers also the clones of the same individual revealed polymorphism amongst each other. The clones exhibited polymorphism with respect to nucleotides in the range of 21-22% and 23-24% with respect to on and a_2 respectively. An average Heterozygosity value of 19% and 20% was observed among the tigers with respect to ai and a_2. All the three tigers considered in the present study were from different zoological parks. Shankaranarayanan and Singh (1998) did a detailed analysis of mitochondrial DNA sequence divergence among tigers and

Fig. 24. Sequence polymorphism between clones of a single tiger observed at a_2 locus



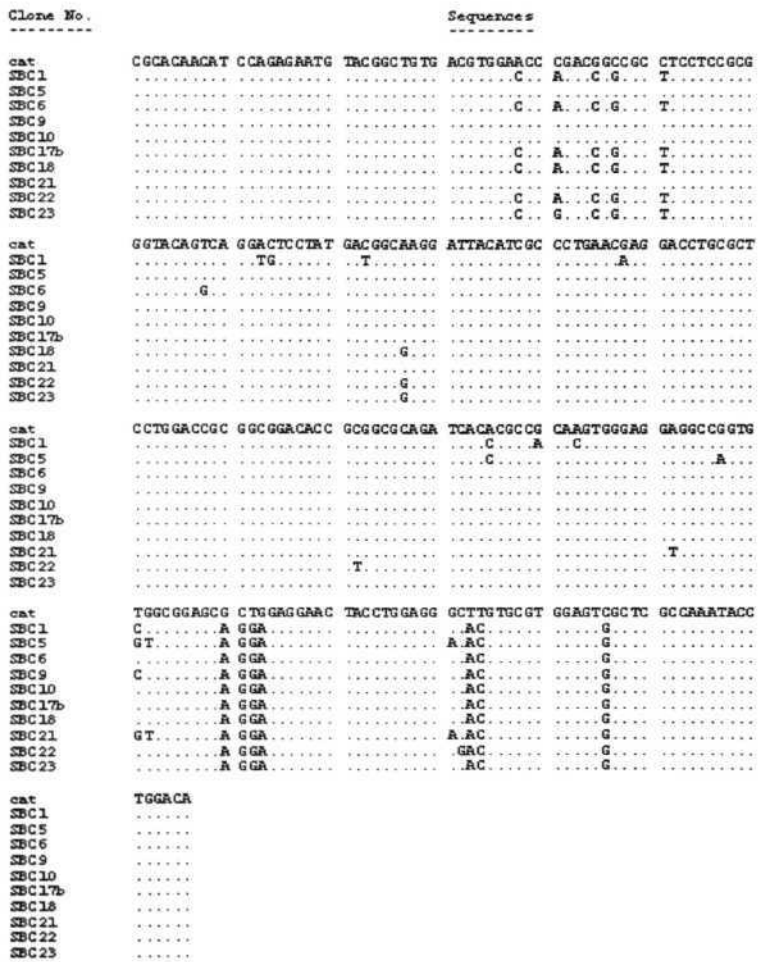
Unlike a,, the clones derived from amplicons of a_2 of tigers exhibited scattered sequence polymorphism throughout the 246 bp amplicon

Fig. 25. Sequence polymorphism between clones of a single leopard observed at a_2 locus



The extent of sequence polymorphism between the clones of a single leopard with respect to a_2 was found to be higher when compared to tigers and lions

Fig. 26. Sequence polymorphism between clones of a single domestic cat observed at α_2 locus



The extent of nucleotide sequence polymorphism between the different clones of a single domestic cat with respect to a_2 was found to be significantly lower when compared to tigers and leopards

Table-6: **Summary of sequence polymorphism at a_2 locus of other animals and humans**

Animal	Individual No.	Polymorphism % between clones
Tiger	**T1**	**23**
	T2	**24**
	T3	**24**
	Mean ± S.D	**23.7±0.6**
Leopard	**L1**	**24**
	L2	**28**
	L3	**28**
	Mean ± S.D	**26.712.9**
Domestic cat	Ca1	**12**
	Ca2	**14**
	Ca3	**15**
	Mean ± S.D	**13.7±1.5**
Dog	**D1**	**26**
	D2	**24**
	D3	**26**
	Mean ± S.D	**25.3±1.1**
Human	Hu1	**21**
	Hu2	**23**
	Hu3	**20**
	Mean ± S.D	**21.3±1.6**

Table shows a comparison of nucleotide sequence polymorphism at a_2 locus, between different animals. The polymorphism extent was calculated for each animal by the use of Gene-tool software, as the sequence difference between 10 clones of the same animal.

their hybrids and observed low levels of polymorphism in tigers. They also observed separate haplotypes for Indian and Siberian tigers. In a study of genetic variation among Indian tigers using RAPD markers (Shankaranarayanan *et al.,* 1997), heterozygosity values of 20-28% with an average of 23.48% was observed, which is similar to the observations of the present study. Similarly, Shivaji *et al.* (1998) analyzed the testosterone levels and semen characteristics in 16 Indian tigers and observed that majority of animals exhibited good spermatozoal number, high percentage of motile spermatozoa and low incidence of abnormal spermatazoa unlike inbred animals, thus implying that inbreeding depression has not yet affected the tigers under study.

In the case of leopards, the sequence polymorphism between clones was observed to be 23-27% and 24-28% with respect to o^ and a_2 respectively. Like the tigers considered in the present study, the three leopards used in the study were also from zoological parks. Even though leopards are not highly endangered as lions and tigers, their existence is threatened due to human interventions. The observation of a high genetic polymorphism at MHC loci in leopards is good from conservation point of view, since it shows that they do possess a healthy immune system.

When an analysis was performed with respect to polymorphism at sequence level at c^ and a_2 in the three domestic cats under study, it was found that the clones of the same cat exhibited polymorphism in the range of 17-18% and 12-15% with respect to <xi and a_2. Even the comparison of sequence polymorphism with respect to all the clones was observed to be low (21% with

respect to both ai and 012). Yukhi *et ai.* (1989) did a detailed sequence analysis of MHC cDNA clones in domestic cat. DNA sequence analysis of eight clones revealed that they all fell into one of two internally identical allelic groups, which differed, by 9% of their nucleotide sequences. The occurrence of only two allelic cDNA clones is consistent with the expression of a single heterozygous functional class I gene in the studied cell line despite the occurrence of more than 20 class I copies estimated to be present in the cat genome. Comparison of the FLA class I coding sequence with other class I genes from other species revealed that the domestic cat genes display 81 to 82% sequence identity with human, and 73 to 79% sequence identity with mouse class I genes. In a study on DNA variation in domestic cats at MHC loci through RFLP, an intermediate level of polymorphism was observed (Yukhi and O'Brien, 1990a). A heterozygosity level of 28.9% was observed in domestic cat in the study by Yukhi and O'Brien (1990), which is not consistent with that observed in the present study (23% with respect to both on and a_2). One possible reason for this might be is that all the three domestic cats used in the present study were from the same family, whereas the 16 domestic cats used by Yukhi and O'Brien were from random sources. Smith and Hoffman (2001) designed a strategy similar to the one adopted in the present study wherein PCR was performed using genomic DNA isolated from different cat species to amplify exons 2 and 3, PCR amplicons cloned and individual clones were sequenced. In this study, more than 7 new alleles were defined for the loci sequenced.

With respect to the three dogs considered in the present study, a high sequence polymorphism range of 29-31% and 20-23% were observed among clones of the same individual with respect to ai and a_2. Similarly the extent of polymorphism when all the clones were considered was observed to be very high (38%) in case of both CM and a_2. Kennedy *et al.* (2002b) through DNA sequence based typing analysis of DLA-DRB1, DQA1 and DQB alleles, studied MHC Alaskan Husky dogs and Brazilian mongrel dogs and compared them with a panel of 568 European dogs and 40 Alaskan gray wolves. Within these dogs, 22 previously undescribed DLA class II alleles were identified: 10 DRB1, 5 DQA1 and 7 DQB1 alleles. All these alleles were found in more than one animal and in some cases, as a homozygote. Several alleles initially observed in Alaskan gray wolves were found in these dogs. The observations of Kennedy *et al.* (2002b) at MHC II is consistent with the observations of the present study wherein a high polymorphism was observed at MHC class I compared to other animals.

In the present study, three human samples were also considered for polymorphism analysis at MHC class I at ai and a_2 domains. The clones of the same individuals revealed polymorphism range of 15-19% and 20-23% with respect to ai and a_2 with Heterozygosity values of 23%and 25% respectively. In a study through RFLP analysis, Janer and Geraghty (1998) observed that polymorphic loci were dispersed over the class I region and many of the loci exhibited typical size variability, which is consistent with the present observations. Janer and Geraghty (1998) also observed that extensive portions of HLA class I are conserved among mammals. Turner *et al.* (1998) performed a

detailed sequence based typing analysis of HLA-C loci (MHC class I) using 1823 samples. In this study, they observed the presence of 19 new alleles. These new HLA-C alleles result from 29 nucleotide substitutions of which 4 are silent, such that coding substitutions concentrated in about the Antigen-binding groove. In the present study also, many nucleotide substitutions were witnessed among the clones of the three human samples under study and many of these substitutions were observed to be non-synonymous indicating a role for MHC polymorphism in survival against infectious diseases and other stresses. Maintenance of functional polymorphism in humans is important with respect to survival against diseases. Since MHC molecules are directly involved in antigen-binding and T-cell stimulation, the observation of polymorphism amongst clones of the same individual and also different individuals is significant. Malhotra ef *al.* (2001) indicated that maintenance of functional polymorphism at class II MHC in humans helps in HIV-1 suppression and cellular immunity following antiretroviral treatment

4.7 Analysis of putative translated products

The raw nucleotide sequence data of the clones of amplicons of the lions and other animals and human samples considered in this study were converted to putative translated products using computer software as described in materials and methods. In all the cases, putative translated sequences showed higher degree of polymorphism between the clones of the same individual as well as between different individuals in case of all the animals and humans considered in this study compared to the polymorphism in nucleotide sequence. Majority of the

mutations were non-synonymous, which is significant in terms of MHC protein structure. When these nucleotide sequences were translated into putative amino acid sequences, a number of pseudo-alleles (with internal stop codons) were also observed in many of the clones.

4.7.1 Polymorphism in the translated product at on locus in lions

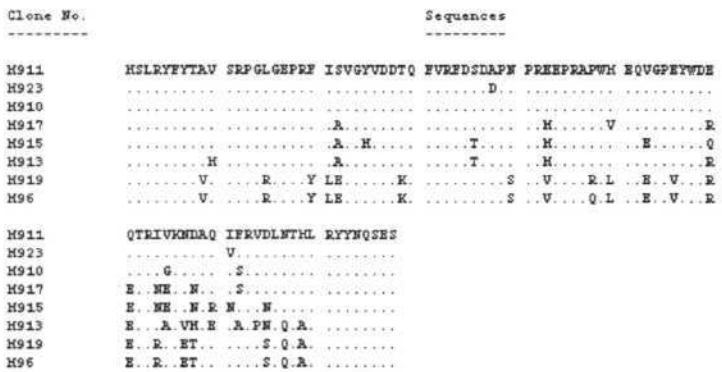
Similar to the observation with respect to nucleotide sequence polymorphism amongst the clones of a single lion and also between different lions, the polymorphism with respect to the putative translated amino acid sequence of the clones was observed to be concentrated in a 'hot-spot' region between the amino acid residues 59-72.

In case of 10 Gir forest lions considered in the study, polymorphism in the putative amino acid sequences of the clones of a single lion varied between 15-34% with an average value of 25.1%. The polymorphism in the putative amino acid sequence between different clones of a single Gir forest lion is given in Fig. 27.

In case of the 3 hybrid lions, the polymorphism with respect to putative amino acid sequence of the clones of a single lion observed was 28-35% with an average value of 31.0%. Fig. 28 depicts such putative amino acid sequence variation between clones of a single hybrid lion.

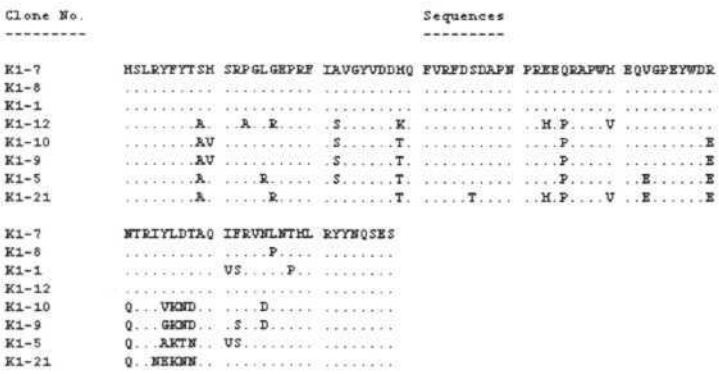
Similarly the putative amino acid sequences, coded by the clones of cti locus of the 3 captive lions, exhibited polymorphism in the range of 16-18% with an average value of 17.0%. The polymorphism in the putative amino acid

Fig. 27. Putative amino acid sequence polymorphism between clones of a single Asiatic lion observed at GL, locus



The extent of putative amino acid sequence polymorphism between different clones of a single Asiatic lion was calculated based on nucleotide sequence using computer software as described in materials and methods. The polymorphism was observed to be concentrated in a 'hot-spot' region between the amino acid residues 59-72.

Fig. 28. Putative amino acid sequence polymorphism between clones of a single hybrid lion observed at a., locus



Much similar to the observations in Asiatic lions, in Afro-Asiatic hybrid lions also, polymorphism was concentrated in a 'hot-spot' region which is a part of the antigen binding site of MHC class I

sequence between different clones of a single captive-bred lion is shown in Fig. 29.

A summary of amino acid sequence polymorphism at ai locus in lions is given in Table 7.

4.7.2 Polymorphism in the translated product at u_2 locus in lions

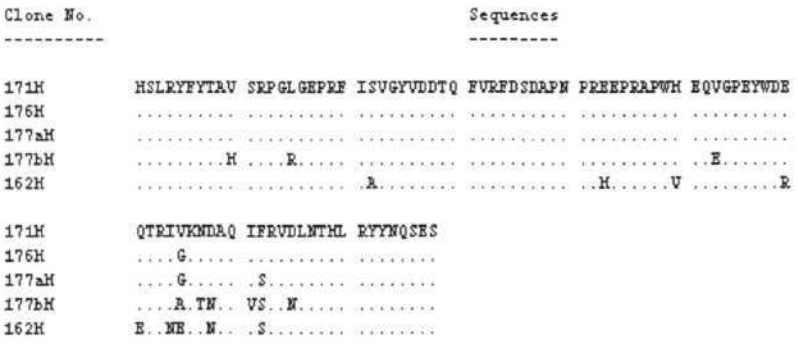
Much similar to the observations made with respect to the study on nucleotide sequence polymorphism between clones of 012 specific amplicons of the different lions, the polymorphism with respect to translated amino acid sequence was also observed to be scattered all across the 83 amino acid protein product.

In case of 10 Gir forest lions, the polymorphism between clones of a single lion with respect to putative amino acid sequence (as inferred by software using the nucleotide sequences of these clones) was observed to be in the range of 22-34% with the average value of 26.4%. An example of such amino acid sequence polymorphism at a2 locus between the clones of a single Gir forest lion is shown in Fig 30.

In case of 3 hybrid lions, the polymorphism with respect to the putative amino acid sequences for d2 locus for the clones of a single lion varied in the range of 31-34% with an average value of 32.3%. Fig. 31 depicts one such amino acid sequence variation between the clones of a single hybrid lion with respect to d2 locus.

In case of 3 captive lions the polymorphism with respect to the putative amino acid sequences for 012 loci for the clones of a single lion was observed to

Fig. 29. Putative amino acid sequence polymorphism between clones of a single captive bred lion observed at a_1 locus



When compared to Asiatic and hybrid lions, the extent of amino acid sequence polymorphism at o^ was found to be significantly lower in captive bred lions

Table-7: Summary of amino acid sequence polymorphism at on locus (putative translated product) in Lions

Animal	Individual No.	Polymorphism % between clones
Asiatic lion (Gir forest lion)	**A1**	**26**
	A2	**30**
	A3	**24**
	A4	**15**
	A5	**17**
	A6	**30**
	A7	**28**
	A8	**27**
	A9	**34**
	A10	**20**
	Mean ± S.D	**25.1±6.1**
Hybrid lion	**H1**	**35**
	H2	**28**
	H3	**30**
	Meant S.D	**31.0±3.6**
Captive bred lion	**C1**	**17**
	C2	**16**
	C3	**18**
	Mean ± S.D	**17.0+1.0**

Table shows a comparison of amino acid sequence polymorphism at a-, locus, between different groups of lions belonging to the different categories of wild (Asiatic), hybrid (Afro-Asiatic) and captive lions. The polymorphism extent was calculated by the use of Pep-tool software for each lion, as the sequence difference between 10 clones of the same animal.

be in the range of 20-24% with the average value of 22.0%. The amino acid sequence polymorphism between the clones of a single captive-bred lion with respect to 012 locus is given in Fig. 32.

A summary of amino acid sequence polymorphism at a_2 locus in lions is given in Table 8.

4.7.3 Polymorphism in the translated product at u-, locus in other animals and human samples

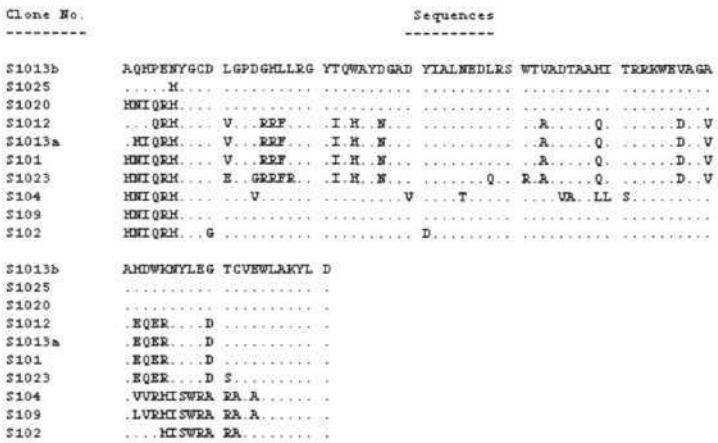
As done in the case of the lions, the nucleotide sequence of the clones of *ai* locus specific amplicons of 3 tigers, 3 leopards, 3 domestic cats, 3 dogs and 3 human samples were converted to putative amino acid sequence and checked for polymorphism between the clones of the same animal/human and also between the clones of different animal/human samples.

In case of 3 tigers considered in the study, polymorphism between the clones of a single tiger in terms of putative amino acid sequences was observed in the range of 23-29% with an average value of 26.3%. Fig. 33 depicts the amino acid sequence polymorphism between the clones of a single tiger.

In case of leopards, the variation between clones of a single leopard with respect to the putative amino acid sequences was observed to be between 28-34% with an average value of 30.7%. The amino acid sequence polymorphism between clones of a single leopard is shown in Fig. 34.

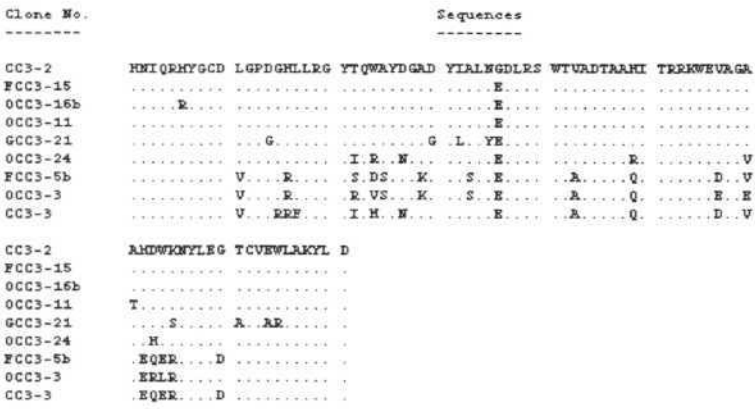
The clones of ai locus specific amplicons of the 3 domestic cats considered in the study with respect to putative amino acid sequences varied in

Fig. 30. Putative amino acid sequence polymorphism between clones of a single Asiatic lion observed at α_2 locus



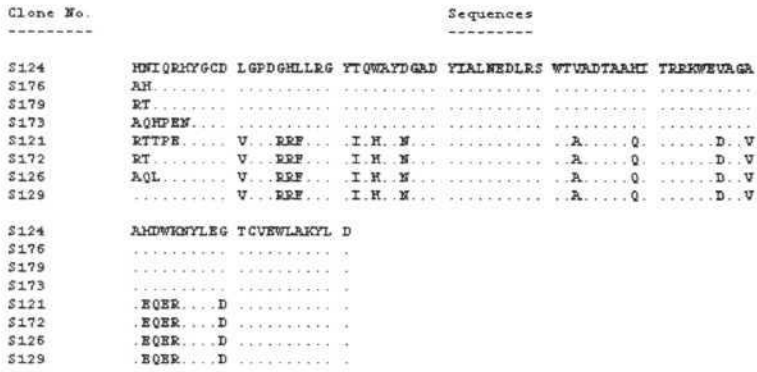
The extent of putative amino acid sequence polymorphism between different clones of a single Asiatic lion with respect to a_2 locus revealed that the polymorphism was observed to be concentrated in a 'hot-spot' region between the amino acid residues 59-72.

Fig. 31. Putative amino acid sequence polymorphism between clones of a single hybrid lion observed at a_2 locus



When the clones of single hybrid lion with respect to a_2 locus were sequenced and the nucleotide sequences converted to putative amino acid sequences, it was observed that the polymorphism is scattered across the putative protein

Fig. 32. Putative amino acid sequence polymorphism between clones of a single captive bred lion observed at α_2 locus



When compared to Asiatic and hybrid lions, the extent of amino acid sequence polymorphism at c^ was found to be significantly lower in captive bred lions

Table-8: Summary of amino acid sequence polymorphism at 02 locus (putative translated product) in Lions

Animal	Individual No.	Polymorphism % between clones
Asiatic lion (Gir forest lion)	**A1**	**24**
	A2	**33**
	A3	**28**
	A4	**26**
	A5	**25**
	A6	**20**
	A7	**28**
	A8	**24**
	A9	**34**
	A10	**22**
	Mean ± S.D	**26.4±4.5**
Hybrid lion	**H1**	**31**
	H2	**32**
	H3	**34**
	Mean ± S.D	**32.3±1.5**
Captive bred lion	**C1**	**20**
	C2	**22**
	C3	**24**
	Mean ± S.D	**22.012.0**

Table shows a comparison of amino acid sequence polymorphism at a_2 locus, between different groups of lions belonging to the different categories of wild (Asiatic), hybrid (Afro-Asiatic) and captive lions. The polymorphism extent was calculated by the use of Pep-tool software for each lion, as the sequence difference between 10 clones of the same animal.

the range of 21-23% with an average value of 22.0%. Fig. 35 depicts the amino acid sequence polymorphism between clones of a single domestic cat.

In case of 3 dog samples, the polymorphism for the putative amino acid sequences for the clones of a single dog varied in the range of 36-39% with the average value of 38.0%. Fig. 36 depicts the amino acid sequence polymorphism between clones of a single dog.

In case of the 3 humans samples, polymorphism for the translated amino acid product with respect to ai locus for the clones of a single human sample was observed in the range of 20-25% with the average value of 22.3%. The amino acid sequence polymorphism between the clones of a single human sample is given in Fig. 37.

A summary of amino acid sequence polymorphism in other animals and human samples at ai locus is given in Table 9.

4.7.4 Polymorphism in the translated product at α_2 locus in other animals and human samples

The nucleotide sequence of the clones of α_2-locus specific amplicons of 3 tigers, 3 leopards, 3 domestic cats, 3 dogs and 3 human samples were converted to putative amino acid sequence using software as described in materials and methods and checked for polymorphism in amino acid sequence between clones of the same animals and human and also between different animals and human samples.

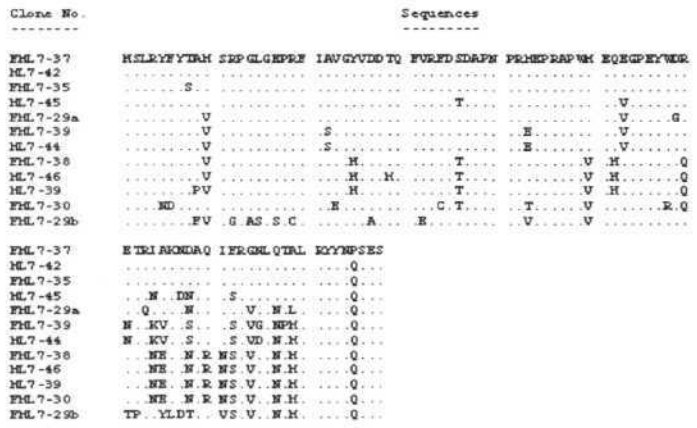
Among the 3 tigers considered in the study, polymorphism between the clones of a single tiger in terms of putative amino acid sequences of α_2 locus was

Fig. 33. Putative amino acid sequence polymorphism between clones of a single tiger observed at OL, locus

Clone No.	Sequences							
MT1-1	HSLRYFYTAV	SRPGLGEPRF	IAVGYVDDTQ	FVRFDSDAPN	PRMEPR-APW	VEQEGPEYWD		
MT2-10	....	....	....	.....T....	..T.......	....		
MT2-1	....	....	....	....	.......V..	H.........		
MT2-15	....	....	.S........	....	..E.......	H..V......		
MT2-2	....	....	.S...SGRMA	VRAVRQPT.R	I.EKS.GRQ.	H..V......		
MT1-1	RETRNHKNSA	QISRVDLNTH	LRYYNQSES					
MT2-10	Q....E..N.	RN...N....	.......D.					
MT2-1	.N...E..N.	....	....					
MT2-15	EQ..IV..V.	....	....					
MT2-2	EQ..IV..V.	......V...	....					

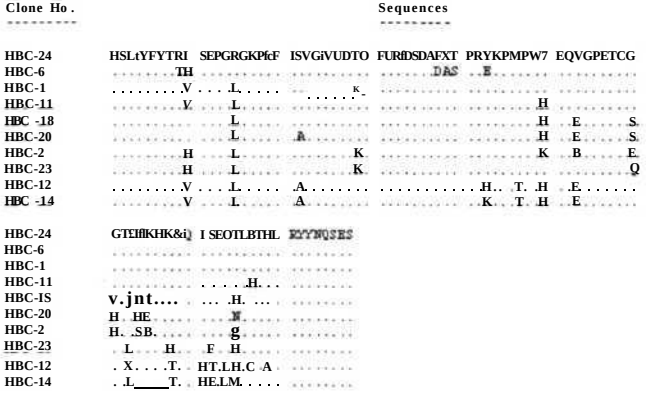
Similar to the observations made in lions with respect to putative translated products of a_v in tigers also, extensive amino acid sequence polymorphism was observed in the region involved in antigen binding

Fig. 34. Putative amino acid sequence polymorphism between clones of a single leopard observed at a_1 locus



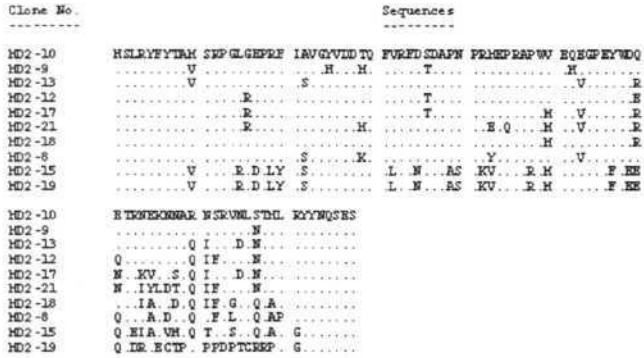
The extent of putative amino acid sequence polymorphism was found to be significantly higher in leopards compared to lions and tigers with respect to <*! locus

Fig. 35. Putative amino acid sequence polymorphism between clones of a single domestic cat observed at a_1 locus



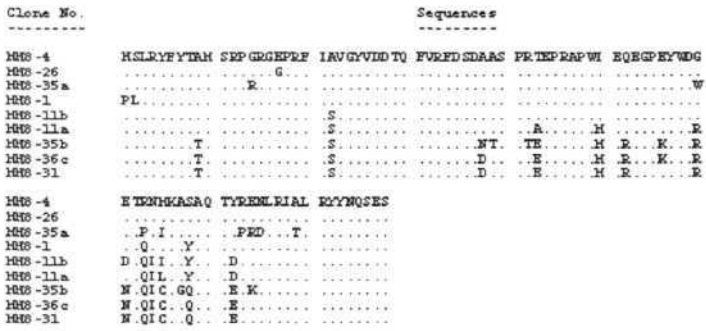
Among the clones of domestic cat, the extent of sequence polymorphism was found to be lesser when compared to big wild cats

Fig. 36. Putative amino acid sequence polymorphism between clones of a single dog observed at GL, locus



With respect to extent of amino acid sequence polymorphism between clones, dogs exhibited the highest level of polymorphism (-38%) compared to all other animals

Fig. 37. Putative amino acid sequence polymorphism between clones of a single human sample observed at a., locus



The extent of sequence polymorphism with respect to putative amino acids at a_1 was found to be less in humans and was comparable to that observed in domestic cat (-22%)

Table-9: Summary of amino acid sequence polymorphism at a-i locus (putative translated product) in other animals and humans

Animal	Individual No.	Polymorphism % between clones
Tiger	**T1**	**23**
	T2	**29**
	T3	**27**
	Mean ± S.D	**26.3±3.0**
Leopard	**L1**	**34**
	L2	**28**
	L3	**30**
	Mean ± S.D	**30.7±3.0**
Domestic cat	Ca1	**22**
	Ca2	**23**
	Ca3	**21**
	Mean ± S.D	**22.0±1.0**
Dog	**D1**	**39**
	D2	**36**
	D3	**39**
	Mean ± S.D	**38.011.7**
Human	Hu1	**20**
	Hu2	**25**
	Hu3	**22**
	Mean ± S.D	**22.3±2.5**

Table shows a comparison of amino-acid sequence polymorphism at *a,* locus, between different animals. The polymorphism extent was calculated for each animal by the use of Pep-tool software, as the sequence difference between 10 clones of the same animal.

observed in the range of 26-29% with an average value of 27.3%. Fig. 38 depicts the amino acid sequence polymorphism between the clones of a single tiger.

In case of leopards, the variation between clones of a single leopard with respect to the putative amino acid sequences was observed to be between 31-35% with an average value of 32.3%. The amino acid sequence polymorphism between the clones of a single leopard is shown in Fig. 39.

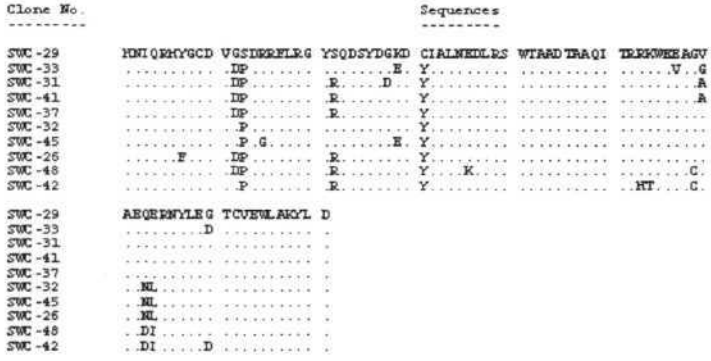
The clones of a_2 locus-specific amplicons of the 3 domestic cats considered in the study with respect to putative amino acid sequences varied in the range of 14-17% with an average value of 15.7%. Fig. 40 depicts the amino acid sequence polymorphism between the clones of a single domestic cat.

In case of 3 dog samples, the polymorphism for the putative amino acid sequences for the clones of a single dog varied in the range of 29-32% with an average value of 30.3%. Fig. 41 represents the amino acid sequence polymorphism between the clones of a dog.

For the 3 humans samples considered in the study, the polymorphism for the translated amino acid product with respect to a_2 locus for the clones of a single human sample was observed to be in the range of 23-29% with the average value of 26.7%. Fig. 42 represents the amino acid sequence polymorphism between the clones of a single human sample.

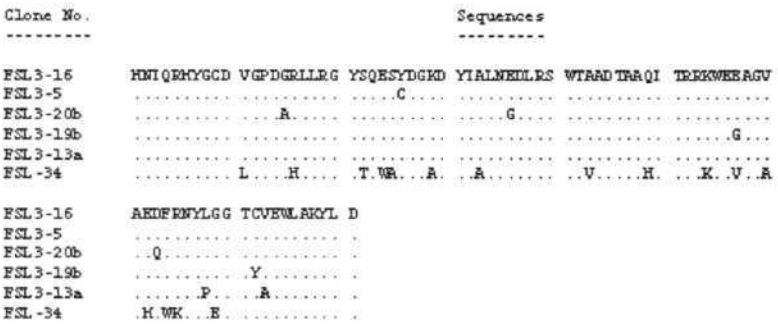
A summary of amino acid sequence polymorphism at a_2 locus in other animals and humans is given in Table 10.

Fig. 38. Putative amino acid sequence polymorphism between clones of a single tiger observed at a_2 locus



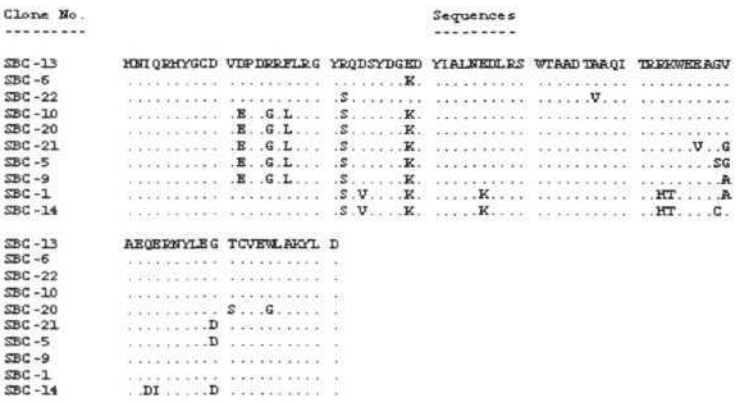
The putative amino acid sequence polymorphism in tigers with respect to a_2 locus was slightly lesser as compare to those observed in Asiatic lions

Fig. 39. Putative amino acid sequence polymorphism between clones of a single leopard observed at a_2 locus



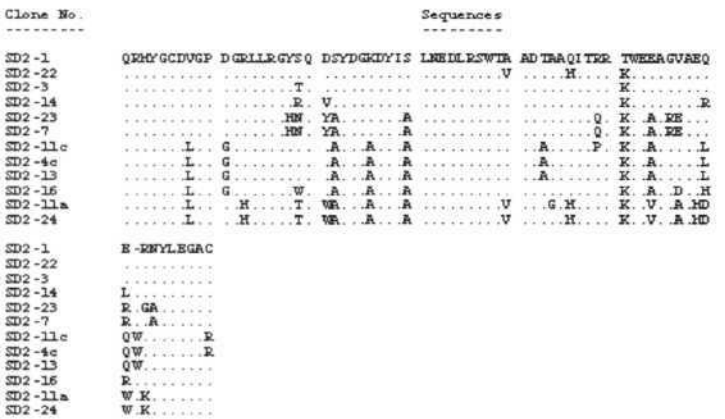
The extent of amino acid sequence polymorphism was found to be significantly higher in leopard compared to tigers and lions.

Fig. 40. Putative amino acid sequence polymorphism between clones of a single domestic cat observed at α_2 locus



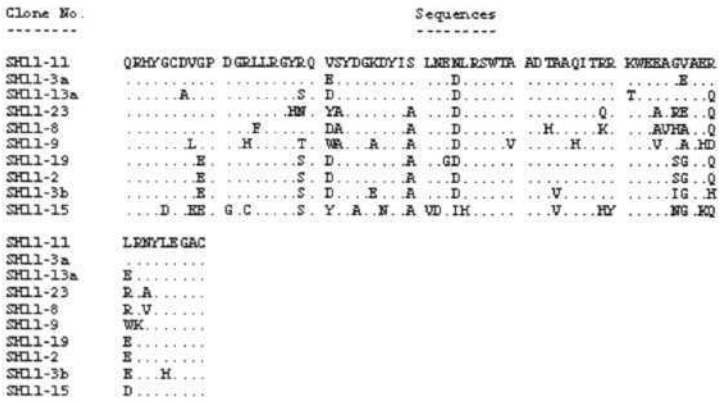
Cats exhibited the least polymorphism (-15%) with respect to putative amino acid sequences of a_2 locus when compared to other animals

Fig. 41. Putative amino acid sequence polymorphism between clones of a single dog observed at a_2 locus



The extent of sequence polymorphism in dogs with respect to translated amino acid sequences for a_2 locus was observed to be ~30%

Fig. 42. Putative amino acid sequence polymorphism between clones of a single human sample observed at a_2 locus



The extent of sequence polymorphism in different clones of human sample with respect to translated amino acid sequences for a_2 locus was observed to be -28%, which is significantly higher than those observed with respect to *a,* locus (22%)

Table-10: Summary of amino acid sequence polymorphism at a_2 locus (putative translated product) in other animals and humans

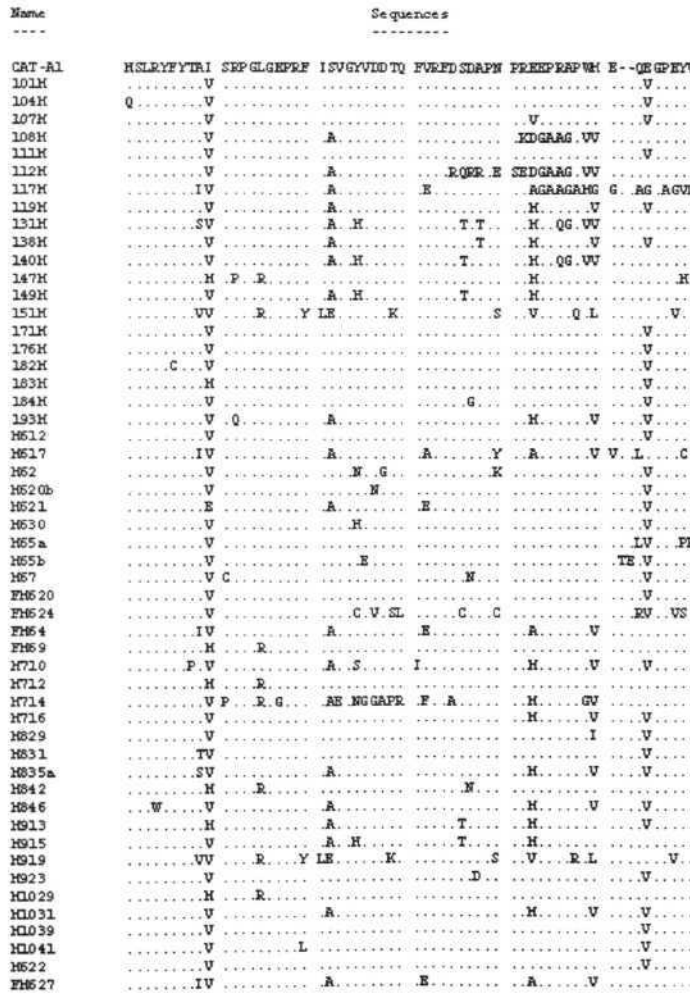
Animal	Individual No.	Polymorphism % between clones
Tiger	**T1**	**26**
	T2	**29**
	T3	**27**
	Mean ± S.D	**27.3±1.5**
Leopard	**L1**	**35**
	L2	**31**
	L3	**31**
	Mean ± S.D	**32.3±2.3**
Domestic cat	Ca1	**16**
	Ca2	**17**
	Ca3	**14**
	Mean ± S.D	**15.7±1.5**
Dog	**D1**	32
	D2	30
	D3	29
	Mean ± S.D	**30.3±1.5**
Human	**Hu1**	29
	Hu2	23
	Hu3	28
	Mean ± S.D	**26.7±3.2**

Table shows a comparison of amino-acid sequence polymorphism at a_2 locus, between different animals. The polymorphism extent was calculated for each animal by the use of Pep-tool software, as the sequence difference between 10 clones of the same animal.

4.8 Structural Analysis of amino acid polymorphism in lions

To do structural analysis of amino acid polymorphism in lions, randomly few clones were checked for identity with published sequence of HLA-A2. Selected clones for modeling showed 70.5% and 68% identity with HLA-A2 for ai and (X2 domains respectively, which is quite significant to overlap the structures. To built the overlapping structural model and to understand the 3D structural changes, 3D-JIGSAW (Protein Comparative Modeling server- http://www.bmm.icnet.uk/~3djigsaw) server was used; this server builds three-dimensional models for proteins based on homologues of known structure. Therefore obtained PDB files (Brookhaven Protein Databank format- http://www.rcsb.org, proposed by Saqi and Sayle, 1994) of both ai and 012 domains were used to make overlapping model and analyzed with respect to published HLA-A2 structure (Gao *et ai,* 1997) using software 'O' (Jones *et al.,* 1991), these structures overlapped each other completely, which was quite reliable to do the mutational analysis also. Many amino acid residues as given in Table 11 were found to be conserved and polymorphic between domestic cat and lions. The amino acid residue nos. 63-77 and 149-163 were found to be highly polymorphic in case of Hybrid lions and Asiatic lions and moderately polymorphic in captive bred lions. These residues, as per Yukhi and O'Brien (1994), are concerned with antigen binding/recognition in the overall structure of MHC. Alignment of deduced amino acid sequences of all the Asiatic lions, showed 52 different alleles at on locus (Fig. 43) and 51 different alleles at a_2 locus (Fig. 44). The alignments of deduced amino acid sequences also showed

Fig. 43. Polymorphism of putative translated product polymorphism at a_1 observed between clones of all Asiatic Lions



(Continued in next page)

Fig. 43 (Continued from pre-page)...

Name	Sequences
CAT-A1	DRNTRIYLDT AQIFRVDLNT H--LEYYNQS ES
101H	.EQ...GKND ...S..... ....
104H	.EQA..GKND ...S..... ....
107H	.EQ...GKND ...S..... ....
108H	.E..NEKNN .RNS..N... ....
111H	.EQ...VKND ..V..... ....
112H	.QE..NEKNN .RNS..N... ....
117H	GP..... ..VS..N... ....
119H	.E..NEKNN ...S..... ....
131H	.QE..NEKNN .RNS..N... ....P..... ..
138H	.E..NEKNN ...S..... ....
140H	.QE.QNEKNN .RNS..N... ...V..... ..
147H	.EQ...K.N .....S.QN T..R..... ..
149H	.QE..KEKNN .RNI..KV.. ...H..... ..
151H	.E..RVKE. .....S.Q. A..... ..
171H	.EQ...VKND ....
176H	.EQ...GKND ....
182H	.EQ...GKND ...S..... ....
183H	.EQ...VKND ....
184H	.EQ...GKND ..TS..... ....
193H	.E..NEKND ...S..... ....
M512	.EQ...GKND ....N... ..
M517	.AR.L.... ..VS..N.RS ....
M52	.EQ...GKND ...S..... ....
M520b	.EQ...GKND ....
M521	.EQ...VKND ....N.... ..
M530	.EQ...VKND ..... T..P..... ..
M55a	.EQ...GHND ...S..... ....
M55b	.EQ...GKND ...S..... .ET..... ..
M57	.EQ...GKND ...S..... ....
FM620	.EQ...GKND ..... T..... ..
FM624	.ER...GHND ...S....SS ....
FM64	..TPGSN... ..VS..N... T..R..... ..
FM69	.EQ...AKND ...S..N... ....
M710	.E..NEKNN ...S..... ....
M712	.EQ...AKTN ..VS..N... ....
M714	.QM..NEKNN .RNS..N.S. ....
M716	.E..NEKNN ...S..... ....
M829	.EQ...VKND ....
M831	.EQ...VKND ....
M835a	.E..NEKNN ...S..... ....
M842	.EQ...AKTN ..VS..N... ....
M846	.E..NEKNN ...S..... ....
M913	.E...AKVH .E.A.PN.Q. A..... ..
M915	.QE..NEKNN .RN...N... ....
M919	.E..RVKE. .....S.Q. A..... ..
M923	.EQ...VKND ..V..... ....
ML029	.EQ...AKTN ..VS..S.. ....
ML031	.E..NEKTN ...S..... ....
ML039	.EQ...VKND ....
ML041	.EQ...VKND ....
M522	.EQ...VKND ....LQPE RV
FM627	..TPGS.... ..VS..N... ....

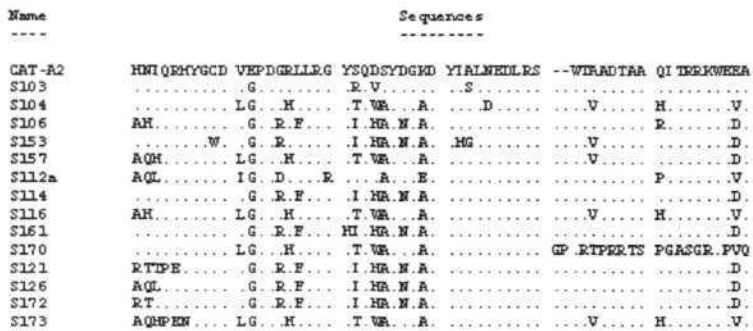
Alignment of deduced amino acid sequences of all the Asiatic lions, showed 52 different alleles at a_1 locus. The alignments of deduced amino acid sequences also showed precise homology with domestic cat for both a_1 and a_2 sequences, permitting the identification of functional residue

precise homology with domestic cat for both on and a_2 sequences, permitting the identification of functional residues (Bjorkman ef a/., 1987). A similar such homology analysis was carried out by Yukhi and O'Brien (1987), wherein they compared the putative amino acids coded by cDNA clones of MHC class I with respect to cheetah, domestic cat and ocelot, and reported clear homology between the three different felid species.

When a comparison was made of amino acid residues, which were conserved and/or highly polymorphic between the different lions and domestic cat at both a-i and a_2 domains, it was observed that the amino acid residues at certain positions on the polypeptide chain were highly conserved and a few others were highly polymorphic. A list of such residues, which were found highly conserved among the different lions and domestic cat have been mentioned in Table11. But at the same time some of the residues were found highly polymorphic in lions, which were reported conserved in domestic cats, also given in Table 11. The comparison was made from the amino acid residues mentioned in the published report of Yukhi and O'Brien (1994).

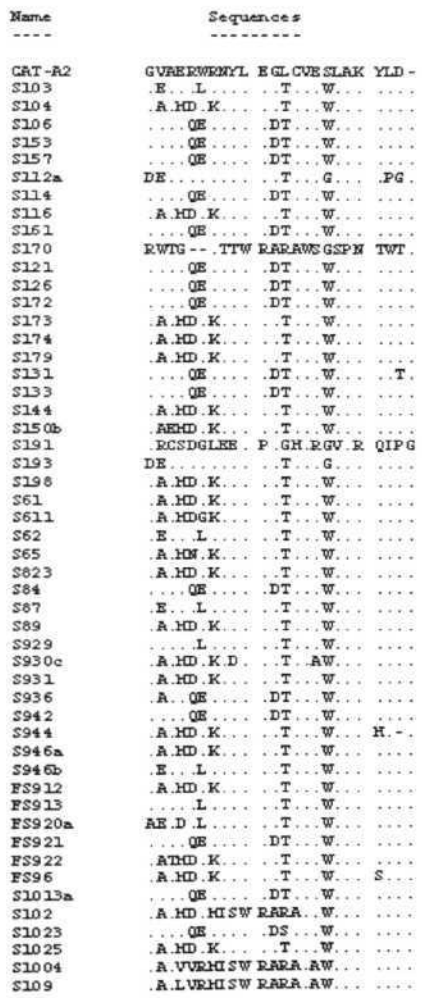
From table 11 it is clear that a few amino acid residues are polymorphic in lions, which have been reported as conserved residues in cats, some of these residues are functionally significant. Certain residues are conserved in both lions and domestic cats (S4, L5, Y7, Y9, P15, G16, G18, P20, R21, G26, R35, F36, Q32, P41, G56, A71, R75, Y99, P105, R111, Q115, Y118, G120, D122, L126, E128). As per Yukhi and O'Brien (1994), the amino acid residues which are highly conserved in domestic cats are involved in inter domain (fc & CD4a)

Fig. 44. Polymorphism of putative translated product polymorphism at a_2 observed between clones of all Asiatic Lions



(Continued in next page)

Fig. 44 (Continued from pre- page)..



Name	Sequences
CRT-A2	GVAERWRMYL EGLCVESLAK YLD-
S103	.E..L.... ..T...W... ....
S104	.A.HD.K... ..T...W... ....
S106	....QE.... .DT...W... ....
S153	....QE.... .DT...W... ....
S157	....QE.... .DT...W... ....
S112a	DE..... ..T...G... .PG.
S114	....QE.... .DT...W... ....
S116	.A.HD.K... ..T...W... ....
S161	....QE.... .DT...W... ....
S170	RWTG--.TTW RARAWSGSPN TWT.
S121	....QE.... .DT...W... ....
S126	....QE.... .DT...W... ....
S172	....QE.... .DT...W... ....
S173	.A.HD.K... ..T...W... ....
S174	.A.HD.K... ..T...W... ....
S179	.A.HD.K... ..T...W... ....
S131	....QE.... .DT...W... ..T.
S133	....QE.... .DT...W... ....
S144	.A.HD.K... ..T...W... ....
S150b	.AEHD.K... ..T...W... ....
S191	.RCSDGLKE. P.GH.RGV.R QIPG
S193	DE..... ..T...G... ....
S198	.A.HD.K... ..T...W... ....
S61	.A.HD.K... ..T...W... ....
S611	.A.HDGK... ..T...W... ....
S62	.E..L.... ..T...W... ....
S65	.A.HM.K... ..T...W... ....
S823	.A.HD.K... ..T...W... ....
S84	....QE.... .DT...W... ....
S87	.E..L.... ..T...W... ....
S89	.A.HD.K... ..T...W... ....
S929	....L.... ..T...W... ....
S930c	.A.HD.K.D. ..T..AW... ....
S931	.A.HD.K... ..T...W... ....
S936	.A..QE.... .DT...W... ....
S942	....QE.... .DT...W... ....
S944	.A.HD.K... ..T...W... H.-.
S946a	.A.HD.K... ..T...W... ....
S946b	.E..L.... ..T...W... ....
FS912	.A.HD.K... ..T...W... ....
FS913	.....L.... ..T...W... ....
FS920a	AE.D.L.... ..T...W... ....
FS921	....QE.... .DT...W... ....
FS922	.ATHD.K... ..T...W... ....
FS96	.A.HD.K... ..T...W... S...
S1013a	....QE.... .DT...W... ....
S102	.A.HD.HISW RARA..W... ....
S1023	....QE.... .DS...W... ....
S1025	.A.HD.K... ..T...W... ....
S1004	.A.VVRHISW RARA.AW... ....
S109	.A.LVRHISW RARA.AW... ....

Alignment of deduced amino acid sequences of all the Asiatic lions, showed 51 different alleles at a_7 locus.

Table-11: List of amino acid residues conserved and polymorphic between cats and lions

Criteria	Positions
ai	
Residues found conserved in both cats and lions	S4, L5, Y7, Y9, P15, G16, G18, P20, R21, G26, Q32, R35, F36, P41, G56, P57, D61, A71, R75, L78, Y84, N86, Q87, S88
Residues conserved in cat but polymorphic in lions (polymorphism restricted to only one amino acid change)	17L _ I,L; 46E _, E,G; 47P _ P,A; 49A _»A,G
Residues conserved in cat but polymorphic in lions (more than one amino acid residue change)	22F ., **F.Y.L;** 58E _ E,G,V; 59Y _ Y,V,H,C,P,S; 64T _ T,A,R,P;72Q ^Q,R,E
α_2	
Residues found conserved in both cats and lions	Y99, P105, R111, Q115, Y118, G120, D122, L126, E128, V165, A169
Residues conserved in cat but polymorphic in lions (polymorphism restricted to only one amino acid change)	160L_L,W
Residues conserved in cat but polymorphic in lions (more than one amino acid residue change)	101C _ C,W; 143T _ T,A,H,S; 146K _ K,R,A,M; 150A _ A,Q,G; 154E _ E,M,G,S,D,V,L; 158H _ N,T,E,I; 159Y _ Y,T,E,D,S; 162G _G,D,A; 164C _>C,A,H

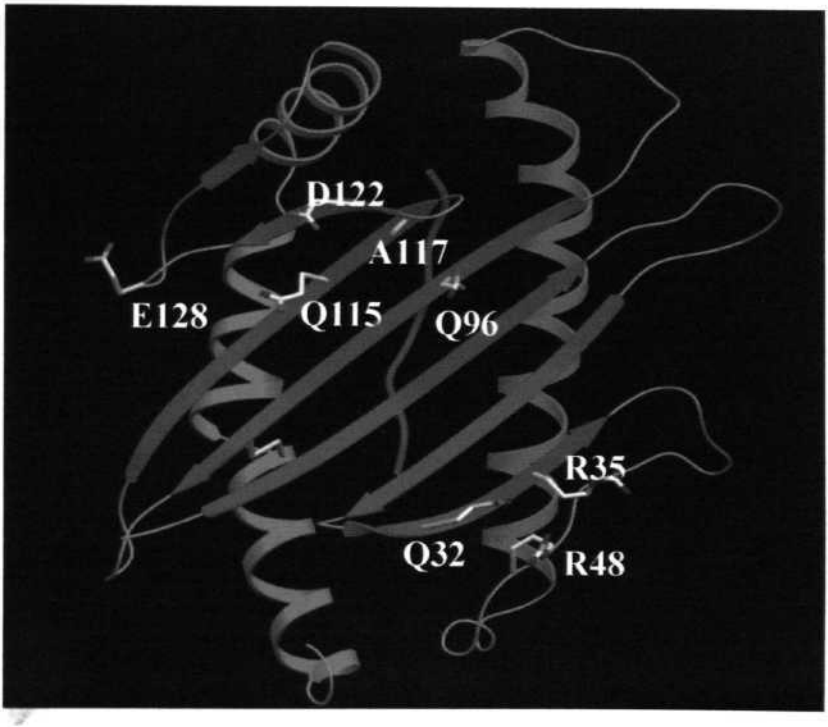
Table represents comparative analysis of conserved residues, between cats and Asiatic Lions. (->) represents amino acid substitutions.

hydrogen bonding through side chains of amino acids in the overall structural context of MHC (Fig. 45). Functionally important residues were also found highly conserved in the present study, when structure-based sequence alignment was done according to the published report of Gao *et al.* (1997). An analysis was also made with respect to amino acids that are polymorphic in lions (Table 12).

Yukhi and O'Brien (1990) in a study on sequence comparisons of seven distinct MHC class I cDNA clones identified that feline class I molecules have a remarkable similarity to human HLA genes in their organization of functional domains. MHC class I genes consist of eight coding exons which encode the following protein domains (Yukhi and O'Brien, 1990): (1) leader sequence (amino acid residues -24-0); (2) and (3) ai (amino acid residues 1-90) and a_2 (amino acid residues 91-182) polymorphic extracellular domains which are involved in antigen and T-cell receptor recognition; (4) a_3 extracellular domain (amino acid residues 183-274); (5) transmembrane domain (amino acid residues 275-306); and (6-8) three cytoplasmic domains (amino acid residues 307-338). The present study was restricted to analysis of structural implications of polymorphism at on and a_2 domains. With respect to o^, amino acid residues 3-90 were amplified and with respect to a_2, amino acid residues 93-173 were amplified in the present study because PCR primers were designed in such a manner to obtain a robust amplification.

Bjorkman *et al.* (1987) described 57 amino acid residues located in the o^ and a_2 domains that are involved in antigen recognition, termed as the antigen recognition site (ARS). Further, the ARS occurs in two a-helix stretches that are

Fig. 45. a., and a_2 domains of MHC, involved in inter-domain (p_2 & CD4a) binding



Highlighted residues are highly conserved as they are responsible for inter-domain (p_2 & CD4a) binding. Majority of these residues did not exhibit polymorphism in lions. Pictures were made with the help of software 'SETOR' (Evans, 1993), original HLA structure was downloaded for analysis (Garrett *et a/*, 1989).

Table-12: Amino acid residues polymorphic in lions

Amino acid residue No.	In human HLA*	Amino acid residues polymorphic in lions observed in the present study
11	S	A, I,S, V, T
12	V	I.V, M, E
24	A	A, S, E
27	Y	Y, H, N,C, S
28	V	V, E, G
29	D	D, N. V, G
30	D	D, G, A
31	T	T, K, S, P
32	Q	Q, L, R
34	V	V, E, A, F
37	D	D, R, H
38	S	S,Q, T,C
39	D	D, R, G, N
40	A	A, R, T, D
42	S	N, E, S, Y, K, C
44	R	R, K, E
45	M	E, V, D, A, M
48	R	R, A, Q
50	P	P, A, Q, R
51	W	W, V, M, G
52	I	M, V, G, L, I
53	E	E, V, G
54	Q	Q, A, L, R
55	E	E, V, G

58	E	E, V, G
59	Y	Y, V, H, C, P, S
62	G	R, E, Q, P
63	E	N,Q, E, A, R, T, H
64	T	T, A, R, P
65	**R**	R.Q.G
66	**K**	I, N, K, R, L, S
67	V	Y, G, K, R, L, S
68	K	L, K, M
69	A	D, N, E, T, V
70	H	T, D, H, N
73	T	**I.W, V, T**
74	H	**F, S, I, A**
77	D	D, N,S, K
79	G	N, Q, R, S
80	T	T, N, S
81	L	M, T, A
82	R	L, V, R, M, P
93	H	H, A, R
94	T	N, H, Q, T, I
95	V	I, H, L, T
100	G	G, V, R
103	**V**	V, L, I, E
106	D	D, N, G, V
107	W	G, R, D.
110	L	L, H, R
113	Y	Y, H, F

114	H	S, R, T,I
116	Y	D, V, W, H, R
121	K	K,A, E
125	A	A, S,G, C
127	K	N, D, T
135	A	A, V, T
137	D	D, R, V
138	M	T, R, A
139	A	A, T, S
140	A	A, S, G
141	Q	Q, H, R, P, L
142	T	I, G, A, L
143	T	T, A, H, S
144	K	R, S, L, H
145	H	R. G. P
146	K	K, R, A, M
148	E	E, P, V, K
149	A	E, V, D, G
150	A	A, Q, G
151	H	G, D, R, A
153	A	A, T, E, C
154	E	E, M, G, S, D, V, L
155	Q	R, D, Q,N, V
156	L	W, L, E, G, R
157	R	R, K, L, M
158	A	N, T, E, I
159	Y	Y, T, E, D, S,

161	E	E, R, P
162	G	G, D, A
163	T	L, T, R, G, S
164	C	C, A, H
166	E	E, S, R, A
167	W	S, W, G
168	L	L, S, V

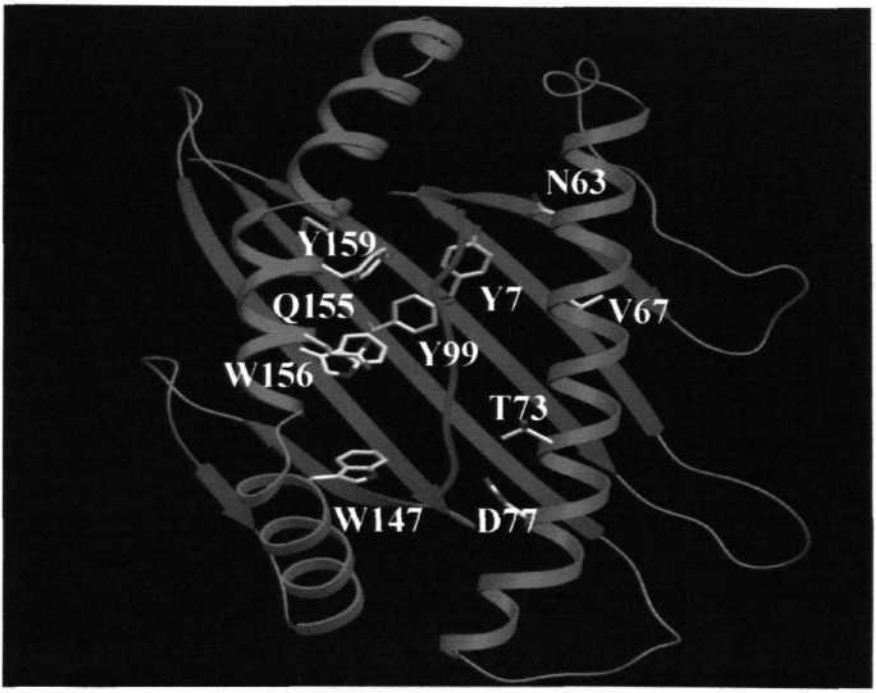
Table represents comparative analysis of polymorphic residues. The residue numbers mentioned, are as per the HLA structure mentioned in the published report of Gao *et al,* (1997)

separated by one platform of (3-pleated sheets in the class I molecule. Gao *et al.,* (1997) studied the interaction of HLA with HIV peptide and found that amino acid residue Nos 7, 63, 73, 77, 99, 147, 155 and 159 play a vital role in this interaction. When an analysis of amino acid polymorphism in lions with respect to these residues was made in the present study, it was observed that the residue Nos. 7 (Y), 99 (Y) and 147 (W) are conserved between both lions and human samples. But interestingly the amino acid residues 63 (E -» N, Q, E, A, R, T, H), 73 (T -> I, M, V, T), 77 (D -• D, W, S, K), 155 (Q ^ R, D, N, V) and 159 (Y -> Y, T, E, D, S) were found to be highly polymorphic in lions (Fig. 46). Careful structural analysis revealed that these conserved residues are responsible to interact with the main chain of foreign peptide whereas polymorphic residues interact with the side chains of foreign peptide.

An analysis was made with respect to amino acid residues which are polymorphic and which are concerned with antigen binding region. Table 13 shows a comparison of such polymorphism between wild and captive-bred lions.

Maintenance of polymorphism at functionally significant domains of MHC is vital for survival under wild. In the present study, a few such polymorphism have been observed, wherein the wild Gir forest lions had more polymorphism with respect to antigen binding region when compared to captive-bred lions. Hedrick *et al.* (2000) did a similar such analysis with respect to MHC polymorphism through SSCP in Arabian oryx. The Arabian oryx is one of the most endangered species in the world, extinct in the wild and surviving in only a few captive herds. The present day population of over 2000 descends from a

Fig. 46. Amino acid residues in *a,* and a_2 domains of MHC class I involved in binding with foreign peptide



The highlighted amino acid residues are more prone to mutations as they are involved in binding with different type of foreign antigens. Significantly, a majority of such residues showed more polymorphism in Asiatic lions of Gir forest compared to captive bred lions

Table-13: **Comparison of a few polymorphic amino acid residues between wild and captive bred- lions, which are associated with antigen binding**

Amino acid residue concerned with antigen binding	**Polymorphism in wild lions**	**Polymorphism in captive bred lions**
66	**I, M, K, R, L, S**	**I,** N
67	**Y, G, E, V, N, A**	E, A, G, V
70	**N, D, H, T**	N, D
77	**D, N, S, K**	D, N
154	**E, M, G, S, D, V, L**	M, E
155	**D, Q, V, R, N**	D, Q
157	**R, K, L, M**	**R, K**
159	**Y, T, E, D, S**	Y, S
163	**T, R, G, S**	T, G

Table represents comparative analysis between wild (Asiatic) and captive lions, of few polymorphic residues from antigen binding region. The residue numbers mentioned, are as per the HLA structure mentioned in the published report of Gaoefa/, (1997)

small number of founders and may have restricted genetic variation for important adaptive genes. They observed three very divergent alleles, which on average, differed by 24 nucleotides and 15 amino acids in the 236-bp fragment we examined. The average heterozygosity for the 22 amino acid positions involved in antigen binding was three times as high as that for the 56 amino acids not involved with antigen binding. A similar observation was made in the present study, wherein majority of the polymorphism in lions was restricted to antigen binding region.

Another study on similar lines was carried out by Liu *et* a/., (2002) in inbred and outbred chickens with respect to MHC polymorphism. The chicken MHC class I and class II were investigated as candidate genes for immune response to *Sal.monella enteritis (SE).* The complete MHC class I cDNA sequences were characterized for an outbred broiler line and four diverse highly inbred lines to define the allelic sequences within these lines, so that the association between particular MHC polymorphisms and response to SE can be studied. The Fi offspring of outbred broiler sires crossed with three inbred lines were evaluated as young chicks for either bacterial load in spleen and cecum after pathogenic SE inoculation or antibody level after SE vaccination. Alleles defined by a Lys(148)->Met(148) polymorphism in the MHC class I alpha(2) domain were associated with spleen bacterial load after SE challenge. These results suggest that particular MHC haplotypes may contribute to control of responses to SE, and that particular polymorphisms may serve as markers for genetic resistance to SE in the chicken. Another significant observation in their

study was that the outbred chickens were more polymorphic at MHC when compared to inbreds, similar to the observations in the present study.

4.9 Correlation of MHC polymorphism in conservation of endangered wild cats

Lions and tigers are our national heritage. Due to multivarious reasons their numbers have considerably decreased over the years and have reached a stage where deliberate interventions are required to maintain their numbers and save them from extinction. Khoshoo (1997) indicated that small populations like that of Asiatic lion lead to change in gene frequencies and become vulnerable to extinction on account of increasing inbreeding depression. He also stressed that data need to be generated on these aspects including the consequences of possible inbreeding degeneration. Further, he stressed the use of molecular methods to study the extent of genetic polymorphism in Asiatic lions. The present study has revealed abundant genetic polymorphism at MHC in Asiatic lions at both nucleotide sequence and amino acid sequence level, comparable to that of Afro-Asian hybrid lions, indicating that they still have the capability to survive stresses and hence represent a healthy population, which can be utilized in scientific captive breeding programmes. The present study revealed that Asiatic lion A2 and A9 have more polymorphism at both on and a_2 and hence these two can serve as sperm donors in captive breeding programmes. Shivaji *et a*l., (1998) in their study on testosterone levels and semen analysis in Asiatic lions, observed that certain individuals possessing best semen profiles could be used in controlled breeding programmes to ensure propagation and genetic

variability. Another interesting observation in the present study is the high levels of heterozygosity at both en and a_2 in case of Asiatic lions compared to captive-bred lions.

Genetic heterozygosity is thought to enhance resistance of hosts to infectious diseases. In particular, heterozygosity at MHC, the highly polymorphic loci that control immunological recognition of pathogens, is suspected to confer a selective advantage by enhancing resistance to infectious diseases. Doherty and Zinkernagel (1975) indicated that individuals heterozygous at particular MHC loci might be more resistant to particular infectious diseases than the corresponding homozygotes as heterozygotes could present a wider repertoire of antigens. O'Brien and Evermann (1998) suggested that organisms with low MHC variation, such as many endangered species, might have susceptibility to infectious diseases. Hedrick *et al.* (2002) in their study on MHC polymorphism in red wolves at amino acid level observed that the average heterozygosity at antigen binding site of MHC is 0.349, a value which is slightly higher to that observed in Asiatic lions in the present study. Penn *et al.* (2002) in their study on the role of MHC heterozygosity in mice observed that MHC heterozygotes had greater survival and weight than homozygotes and they were more likely to clear chronic *Sal.monella* infection than homozygotes. The moderate levels of heterozygosity with respect to nucleotide and amino acid polymorphism observed in Asiatic lions compared to hybrid lions might be because of the reduced population size and slight inbreeding experienced in the Asiatic lions of Gir forest. Still, compared to

captive-bred lions, the levels of heterozygosity in Gir forest lions were significantly higher.

The present study involved the analysis of variation at MHC class I, ai and α2 domains of Asiatic lions and other animals at both nucleotide and amino acid level. It is observed that there is a general correlation between the levels of nucleotide and amino acid polymorphism, even though in many cases, the nucleotide substitutions were observed to be synonymous. Hedrick *et al.* (2002) analyzed MHC variation in red wolves in a strategy similar to that adopted in the present study. They observed that the range of difference between pairs for nucleotides and amino acids was not large. Much similar to the observations in the present study, Hedrick *et al.* (2002) also observed lot of variation among the wolves at MHC at both nucleotide (62 out of 280 nucleotides) and amino acid (23 out of 93) levels.

The present study also revealed that in all the animals including Asiatic lions, some of the polymorphism at both ai and α2 were actually in antigen binding site of MHC, which is functionally significant. Polymorphism at antigen binding site of MHC helps in binding of MHC to a variety of target proteins of the pathogen, thus aiding in combating a repertoire of pathogenic infections. Hedrick *et al.* (2002) also observed such polymorphism at antigen binding site of MHC in red wolves and implicated a role for such polymorphism in the survival of the wolves against pathogenic infections.

In the present study, in addition to PCR based RFLP and Single Stranded Conformational Polymorphism, sequence based analysis at both nucleotide and

amino acid level was carried out to assess the polymorphism at MHC in Asiatic lions and other animals. Sequence based typing, despite the high costs involved in the analysis, is considered a more reliable strategy for genetic diversity studies, particularly in endangered wild animals. Kurz *et al.* (1999) have advocated the adoption of a sequence based typing strategy for large scale stem cell donor histocompatibility testing for fast molecular HLA-A matching. The strategy proposed by Kurz *et al.* (1999) involves the use of a limited number of primer-mixes for group-specific amplification of the HLA-A alleles from linked conserved polymorphic sites of the 5' untranslated regions and of intron 1 and 3. The strategy adopted in the present study is much similar to the one proposed by Kurz *et al.* (1999). The conserved regions of on and ◇2 of domestic cat were used for primer designing to amplify the corresponding regions in Asiatic lions. Considering the importance of such an approach, in future, refinements in primer designing may help in amplifying other functional domains of feline MHC and thus aiding in more precise assessment of functional MHC polymorphism in the wild cat family.

Embryo transfers have been repeatedly attempted for some members of the cat family Felidae, with the goal of using cats of related felid species as surrogate mothers for endangered wild cats like lions and tigers, but success rates remain low (Pope *et al.,* 1993). The possible roles of the major histocompatibility complex (MHC) proteins in a materno-fetal immunological response, for intra-specific as well as inter-specific pregnancies were implicated (Thellin *et al.* 2000). Smith and Hoffman (2001) explored the extent of allelic

diversity at a single class I MHC locus across many cat species, as well as among domestic cats used in transplant experiments, in order to look for patterns of variability that may affect immune system compatibility between surrogates and fetuses. Their study revealed high levels of allelic diversity at MHC class I in domestic cat, thus arguing against their involvement in transplantation and embryo transfer experiments. In the present study also, significant sequence differences were observed at both ai and a_2 loci between domestic cats and Asiatic lions and hence domestic cats considered in the present study might not be suitable for as surrogate mothers for Asiatic lions. However, a detailed analysis of MHC proteins expressed by the fetuses of other domestic and wild cats and genotyping of natural intra-specific pregnancies, may reveal information on whether allele matching at the feline MHC class I locus through sequence based typing can increase the success of inter-specific embryo transfers in felids.

5. Conclusions

The present study was designed to study the genetic polymorphism at MHC class I loci in Asiatic lions of Gir forest (which are highly endangered), Afro-Asiatic hybrid lions and captive-bred lions in order to assess the survivability/vulnerability of these mega wild animals. Based on the observations of the present study, the following conclusions can be drawn:

- The PCR primers designed from published MHC sequences of domestic cat for amplification of ai and a_2 regions of MHC class I gave good amplification in lions, tigers, leopards and domestic cats with amplicons of the size of 265 bp for ai and 246 bp for a_2 respectively.
- The PCR primes designed from published sequences of domestic cat amplified ai region of dog and humans but the a2-specific primers did not amplify the a_2 region in these samples.
- Special primers were developed for amplification of 012 region in dogs and human samples based on published MHC sequences in humans and dogs and these primers gave amplicons of size 210 bp.
- In PCR Based RFLP (PBR) analysis of on and a_2 amplicons of different lion samples, he restriction enzymes DpnII, DdeI, HinfI, HaeII, HhaI revealed PBR in Asiatic lions but not in captive-bred lions.
- Single Stranded Conformational Polymorphism (SSCP) analysis of on and a_2 amplicons did not reveal any polymorphism in captive-bred lions. In case of Asiatic lions, smear like pattern was observed.

- When the amplicons specific to ai and ct_2 sequences of all the lion samples were subjected to direct sequencing, crowded and overlapping peaks were observed at certain points (between 185-206 in case of ai-specific amplicons and 80-100 bp & 186-200 bp in case of a_2-specific amplicons).
- When the amplicons were cloned in pMOSB/ue blunt-ended cloning vector and the clones sequenced using vector-specific primers, clear sequencing peaks were obtained.
- In all the samples considered in the present study, which included lions, tigers, leopards, domestic cat, dog and human samples, in addition to clones of different individuals, the clones of the same individual also revealed polymorphism at both CM and a_2.
- In case of lions, sequencing of clones specific to ai revealed polymorphism at 'certain hot-spot' in the region between 160-200 bp and in the case of a_2, the polymorphism was scattered across the 246 bp amplicon.
- The Afro-Asiatic hybrid lions exhibited highest sequence polymorphism at both ai and $a_2$followed by Asiatic lions of Gir forest and the captive-bred lions showed very less sequence polymorphism
- In a dendrogram constructed based on similarity of nucleotide sequences, in case of both en and a_2, the captive-bred lions were found to be clustered together, whereas the Afro-Asiatic hybrid lions were found scattered in between the Asiatic lions.

- When the nucleotide sequences of CM and a_2 specific clones were converted to putative translated amino acid sequences, both synonymous and non-synonymous substitutions were observed.
- The non-synonymous substitutions were found to be concentrated in the antigen-recognition region of MHC.
- The Asiatic lions of Gir forest exhibited higher amino acid sequence polymorphism in antigen-recognition region of MHC when compared to captive-bred lions.
- A few of the amino acid sequence polymorphism in antigen-binding region of MHC were found to be critical from structural point of view, which might be increasing the specificity of binding of different antigenic peptides.
- The Asiatic lions of Gir forest, A2 and A9, which were observed to be genetically distant and possess high MHC polymorphism based on nucleotide and amino acid sequence polymorphism, can be used in controlled breeding programmes to ensure propagation and genetic variability.
- The present study revealed unequivocally the existence of sufficient genetic diversity in Asiatic lions, Indian tigers and leopards in contradiction to the earlier reports. This is good news for the future of Indian big wild cats.

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