CONSERVATION OF ENDANGERED ANIMALS

Submitted for funding to:

Central Zoo Authority of India
Ministry of Environment and Forests
New Delhi

(January, 2007)

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and

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CONTENTS

1. Title: CONSERVATION OF ENDANGERED ANIMALS IN INDIA

2. Background, introduction and definition of the problem

Human activities such as habitat destruction and poaching are known to fragment habitats and populations thus facilitating inbreeding of animals and genetic homogenization, which has negative effects such as poor reproductive performance, low fecundity, increased juvenile mortality and susceptibility to diseases. Thus, there is a need to arrest the accelerated depletion of the species.

India is considered a hot spot of biodiversity contributing about 7.6% of mammals, 12% of birds, 11% of fishes and 6% of flowering plants to the total world populations. But, unfortunately, India also has 172 animal species considered globally threatened or 2.9% of the world's total number of threatened species. Worldwide, extinction threatens 11% of birds, 25% of mammals and 34% of fish species. Given the current trends and pace of extinction, there is an urgent need to conserve and propagate species both *in situ* (in nature) and *ex situ* (in intensively managed programmes in captivity in zoos).

At the moment it is not clear whether the drastic reduction in lions and tigers from hundreds of thousands of animals in the beginning of 20th century to a few hundred lions and a few thousand tigers in the present century is due to industrialisation, urbanisation, aggressive agricultural activities or due to inbreeding. The tiger population fell precipitously from ~40000 in 1900 to about 3700 in 2002. The situation with respect to the Asiatic lion, which exists as a single wild population of about 350 animals in Gir forest, Gujarat, is even scary. The cheetah unfortunately is already extinct; the last animal fell victim to the barrel in 1952. A few of these charismatic animals would eventually be conserved but a good proportion, which is not charismatic, is bound to become extinct, and for many of them we do not even have census figures. But, considering that wildlife populations are fragmented and small, inbreeding could be a major deterrent in the development of the populations. The extinction of the cheetah in the absence of any major natural calamity hints at our insensitivity to factors that could phase out animals from their natural habitats.

The need for conservation is therefore immense. Habitat preservation and captive breeding are the best ways to conserve biodiversity. However, the reproduction process may be impaired in captivity due to space restriction, inadequate diet, health and husbandry problems, modified sexual behaviour or pair incompatibility, etc. so the only alternative is to develop new captive breeding strategies to improve the fertility status and the reproductive performance with the help of biotechnological approaches, which are better referred to as

assisted reproduction (AR). It involves application of techniques such as : semen collection, gamete and embryo cryopreservation, oestrus induction and artificial insemination (AI) and more complex methods such as oocyte pick up (OPU), *in vitro* Fertilization (IVF), *in vitro* production of embryos (IVP), intra-cytoplasmic sperm injection (ICSI), embryo transfer (ET) and cloning. Hand in hand with AR, molecular markers based on the genetic make up of the animals needs to be developed and applied to ascertain the extent of genetic polymorphism in the surviving wildlife populations. This would help in planning captive breeding programmes, which would further facilitate maintenance of genetic heterozygosity and prevent genetic homogenization, which leads to extinction.

The ultimate aim of this project would be to evaluate the genetic and fertility status of the endangered animals and develop technologies to boost their numbers.

It may be noted that the specific objectives in the present proposal were developed based on the suggestions made by CZA in their letter F.NO. 9-2/2005-CZA (M) dated 5-8-2005 and 23-11-2006 wherein it was suggested that CCMB should develop proposals on the following themes :

1. ***Monitoring of genetic variation by DNA fingerprinting***
2. ***Establishment of cell/ gene resource bank***
3. ***Assisted reproduction involving semen analysis, ovulation monitoring, artificial insemination, in vitro fertilization and cloning.***

It is also agreed upon that species to work on would be identified in consultation with CZA and wherever possible common non-endangeered species would be used as model systems for endangered species to develop and standardize techniques related to assisted reproduction.

3. Objectives

The three main objectives are :

1. DNA fingerprinting as a tool to monitor genetic variation in endangered animals
2. Cryobanking of cells, tissues and genes of endangered animals
3. Development of assisted reproductive technologies for the conservation of the endangered species

1. DNA fingerprinting as a tool to monitor genetic variation in endangered animals

Genetic variation or polymorphism is an accepted and reliable method for monitoring the genetic health of species and to understand the relationship among individuals within the population. For this purpose there is a need to develop species-specific probes, which could ultimately give us authentic data on the degree of polymorphism in the endangered species. These probes could also be used for wildlife forensics. Using this approach CCMB has already developed species-specific microsatellite markers for lions, tigers and spotted deer and used these markers to evaluate genetic diversity in these animals. **The specific tasks under this objective would be:**

A. **Genetic variation in leopards:** The geographic distribution of leopards (*Panthera pardus*) extends throughout Africa, central Asia, Southeast Asia and North to the Amur valley in Russia. The range of the Indian leopard has been reduced dramatically during the 20th century due to habitat loss, incessant hunting, elimination of prey base, poaching and a demand for body parts in Asian traditional medicines. It is, therefore, very important to assess the extent of genetic diversity present in the existing population and take corrective measures to maintain this genetic diversity.

In addition to the work on the leopards, it is proposed to collect samples from other endangered animals in a phased manner as advised by the CZA so as to establish genetic diversity of the other endangered animals including birds and reptiles.

B. **Evolutionary and molecular systematic studies in the Indian deer:** Of the 40 existing deer species in the world, nine occur in India. The Manipur brow-antlered deer (*Cervus eldi eldi*) is one of the critically endangered cervids in India. The present population of the Manipuri deer is approximately 150 individuals restricted to Keibul Lamjao National Park in Manipur. Because of the critically endangered status of Manipuri deer, the present study would be initiated to develop non-invasive methods for DNA based genetic characterization of Manipuri deer population and to study the genetic relationship of Manipuri deer with other related species/ subspecies

and its taxonomic status would be investigated using modern molecular genetic methods.

LaCONES would also extend its expertise to the Zoological Garden Alipore to carry out "Conservation breeding of Brow antlered deer, *Cervus eldi eldi*- Sangai at four selected Indian Zoos" a project sanctioned by CZA.

C. **Barcoding of Indian birds:** DNA barcoding is a technique which has been developed for the rapid identification of animal species based on the sequencing of short stretches of one or more conserved genes such cytochrome oxidase I gene of the mitochondria. In a recent study, Herbert *et al.* in 2004, studied 260 bird species from North America and based on the sequence differences in the cytochrome c oxidase (CO1) gene identified four new species of North American birds. The intent of DNA barcoding is to use large-scale screening of one or a few reference genes in order to: assign unknown individuals to a species, and enhance discovery of new species (Herbert et al., 2003; Stoeckle, 2003). Work on similar lines would be extremely relevant in India because of the vast biodiversity in the tropical ecosystem and also because of the rapid rate at which species are becoming extinct. We envisage the development of a comprehensive database of sequences, preferably associated with voucher specimens representing described species, against which sequences from sampled individuals can be compared.

2. **Cryobanking of cells, tissues and genes of endangered animals**

Cryopreservation of oocytes, semen, embryos, tissues and DNA of endangered species could serve as resources for the future. As of now techniques for cryopreservation have been developed at the CCMB for the semen of lion, tiger, leopard, deer and vulture. But there is scope to improve the methodology so as to increase the viability and motility of the post thaw semen. In addition, there is an urgent need to develop fibroblast cultures of all the endangered animals as and when tissues are available and freeze them for the future. **The specific tasks under this objective would be:**

A. **To improve protocols for cryopreservation of semen of lions, tigers, leopards, sangai deer and white backed vulture.**

B. **To establish fibroblast cultures of lions, tigers, leopards, sangai deer, white backed vulture and other endangered animals for which tissues are available due to natural or accidental death.**

C. **To establish a genome bank of all endangered species including animals, reptiles and birds.**

3. Development of assisted reproductive technologies for the conservation of the endangered species

For successful captive breeding, a basic understanding of the reproductive potential (fertility status, time of ovulation, optimal time for mating, pregnancy, time of spermatogenesis, etc.) of the animals is necessary. Reproductive potential of animals could be monitored based on the hormonal steroid profile of the animals using blood. But, repeated blood sampling is not advisable or encouraged in wild animals due to stress caused due to anesthesia. Therefore, non-invasive fecal steroid hormone analysis could be used as an alternative method for hormonal analysis. Therefore, studies would be undertaken to identify the major fecal steroid metabolites and develop a radioimmunoassay / ELISA for the Indian endangered animals to monitor reproduction function of endangered animals. However, despite proper reproductive cycling animals may not mate. Under such circumstances artificial insemination is one of the best alternatives when captive breeding programmes do not succeed in the zoos. For this purpose there is a need to develop methods for semen collection, standardize protocols for induction of ovulation, evaluate and develop methods for steroid analysis by non-invasive methods and develop methods for artificial insemination. Artificial insemination although well established in case of domestic animals, needs to be standardized for use in wild animals. As of now at the CCMB methods have been developed for collection of semen by electroejaculation from lions, leopards, tigers, spotted deer, black buck and vulture. Further protocols have also been developed for artificial insemination in the black buck and the deer and in the later case a young fawn was also born. Method has also been developed for steroid analysis from the feces of lions and deers. Nevertheless, there is a need to continue these studies on the other cats and deer species. **The specific tasks under this objective would be:**

A. Standardization of artificial insemination in lions, tigers, leopards, sangai deer, white backed vulture and nicobar pigeon.

B. Fecal steroid monitoring in lions, tigers, leopards, black buck and spotted deer.

C. Standardization of ultrasonography in lions, tigers, leopards, spotted deer, black buck and other Indian endangered animals so as to be able to follow follicular development and pregnancy.

Please note that for developing assisted reproductive technologies listed above the technique would be standardized in a common endangered species closely related to the endangered species.

3.1 National and International Status

I. Molecular genetic markers in conservation biology

Molecular genetic markers have increasingly become popular in conservation (Crandall et al., 2000; Sunnucks, 2000). Several indices of genomic diversity such as allozyme pattern, two-dimensional PAGE, skin graft and restriction fragment length polymorphism (RFLP) have been used to evaluate the effects of inbreeding on genetic diversity of cheetah, lion and panther (O'Brien et al., 1983; Wildt et al., 1987, Yuhki and O'Biren, 1990; Menotti-Raymond and O'Brien, 1995; O'Brien, 1994). More recently, microsatellites, which are short tandem DNA repeat sequences, found scattered throughout the eukaryotic genome, and which exhibit unusual degree of polymorphism (Valdes et al., 1993; Charlesworth et al., 1994) have been used to study genetic variation in wild animals. Their abundance, polymorphic nature and amenability to amplification by PCR make microsatellites ideal markers for studies on linkage mapping, forensics, population genetics and mating systems in the natural populations.

A. Development of microsatellite markers to study genetic variation in the big cats

The Asiatic lion (*Panthera leo persica*) once widespread throughout Southwest Asia is today restricted to a single location in the wild, the Gir forest in Gujarat. Genetic analysis of this species is, therefore, warranted in order to ascertain the degree of genetic polymorphism and to recommend steps for conservation of the population. Shankarnarayanan et al. (1997) used microsatellite loci originally developed in domestic cat[69], to study the genetic variation in the Asiatic lions. However, none of the microsatellites showed variation as all the Asiatic lions were found to be monomorphic and homozygous at the five loci analysed. Although a large number of cat microsatellite markers are already available, identification of polymorphic microsatellite markers of Asiatic lion would provide a more thorough way of gauging the intra- and inter-generic variation amongst big cat populations. CCMB developed microsatellite markers, from the genomic library of the pure Asiatic lion, and analysed the genetic variability in the present lion population (Singh et al., 2002). The results also suggest that the level of heterozygosity between pure Asiatic and hybrid lions was comparable. These markers have also shown a high degree of polymorphism in leopard and tiger populations (unpublished data). Further efforts are needed to generate more number of such polymorphic markers so as to accurately evaluate the extent of relatedness and level of inbreeding in big cats.

B. Conservation genetics of the Sangai Deer (Cervus eldi eldi) using sequence analysis of mitochondrial control region

The analysis of mitochondrial DNA (mt DNA) has revolutionised the evolutionary, conservation and population studies of a large number of species. The maternal inheritance,

the high copy number per cell, and the faster rate of sequence evolution, have rendered mt DNA a special value as compared to the nuclear or chromosomal DNA. mt DNA control region sequences were analysed to examine the genetic structure of Eld's deer populations (Balakrishnan et al., 2003). Population genetic parameters, including nucleotide diversity and haplotype diversity (Tajima, 1993; Schneider et al., 2000) were determined. These data indicated lack of genetic variation among the Sangai deer probably due to inbreeding and would thus benefit from the incorporation of new genetic material.

C. *Major Histocompatibility Complex (MHC) variation in Asiatic lions*

MHC is a large multigene family involved in the humoral and T-cell mediated immune responses of vertebrates. These molecules play an important role in immune recognition and defense and are extremely polymorphic in most species. Detailed sequence analysis of the gene encoding these molecules shows that the majority of the polymorphism is exhibited in the antigen-binding domain. Therefore, genetic variation in MHC loci of Asiatic lions was studied for identifying the reasons for susceptibility/resistance of these wild cats to diseases. Abundant polymorphism in MHC loci was observed not only between different individuals but also amongst the clones from the same individual in spite of the population bottlenecks these wild cats were being subjected to. Earlier RFLP studies (Yuhki and O'Brien, 1990) using MHC class-I probe failed to demonstrate variation in the immune loci of Asiatic lion. However, this seems unlikely, since the Asiatic lions still represent a healthy wild population, with wide genetic variability and still possess good polymorphism at MHC loci.

D. *Mitochondrial DNA and Wildlife identification : a forensic perspective*

Biodiversity protection and wildlife forensic identification are both linked to the stability of natural ecosystem by means of establishing identity of confiscated animal remains for wildlife law enforcement. Various approaches, which are either, based on morphological markers or biochemical traits, such as, the bile characteristics (Hagey et al., 1993), blood haem analysis (Espinoza et al., 1996, 1999) etc. have also been employed for establishment of identity of forensic samples. These approaches have various limitations for use in wildlife forensics since these markers are limited in number and cannot be practically applicable to mutilated remains with decomposed morphology and biochemical markers. We have developed a novel approach utilizing the immense potential of mitochondrial cytochrome b gene to reveal the identity of an unknown sample to the level of family, genus and species using a pair of novel universal primers mcb398 and mcb869 (Verma and Singh, 2003). The universal nature and high performance of the CCMB primers amongst a vast range of animal genera has been validated. The international patent for these primers has already been filed (International publication number under PCT: WO 02/077278 A1) and the approach is being used to resolve the forensic investigation of the cases forwarded by various crime investigation agencies and wildlife curators.

II. Assisted reproduction and conservation biology

The objectives of assisted reproduction (AR) are to overcome or improve fertility of endangered animals, which exhibit a reduction in reproductive performance under captivity. The various methods of AR are :

1. Semen collection – by electroejaculation
2. Semen evaluation with respect to sperm morphology, sperm motility, sperm acrosome reaction, sperm fertilizing ability, etc.
3. Induction of ovulation and detection especially by non-invasive techniques suitable for wildlife
4. Artificial insemination (AI) or intrauterine insemination (IUI)
5. *In vitro* fertilization (IVF)
6. Sperm / oocyte cryopreservation
7. Embryo transfer (ET)
8. Cloning

A. Semen collection

Electroejaculation (EE), first employed by Weisbroth and Young (1965), has become the standard semen collection technique in wild species though it has been possible to collect semen from hand-reared cheetah, chimpanzees and Gorillas (Fussell et al., 1973) without electroejaculation. EE has been used in India to induce electroejaculation in lions (100%), tigers (83%), leopards (85%) and spotted deer (100%) (Shivaji et al., 1998, 2003; Patil et al., 1998; Jayaprakash et al., 2001). In avian species, manual massage technique is commonly practiced for semen collection in domestic birds (Sontakke et al., 2004) and with slight modifications in uncooperative non-domestic birds like cranes (Gee, 1983), budgerigar (Samour et al., 1986) and pheasants. We have been successful in collecting ejaculates consistently from the Blue rock pigeon (*Columba livia*) and the White backed vulture (*Gyps bengalensis*) by this manual massage technique (Gee, 1994; Umapathy et al., 2005; Sontakke et al., 2004).

B. Semen evaluation

Parameters of semen quality such as ejaculate volume, sperm motility, concentration and morphology have been used to assess the fertility status of mega cats of India and other wild animals (Shivaji et al., 1998; Jayaprakash et al., 2001). The ejaculate volumes, percent motile spermatozoa and sperm concentration of tigers and leopards were less than that observed in lions (Jayaprakash et al., 2001; Shivaji et al., 1998). Our studies in spotted deer (*Cervus axis axis*) demonstrated that the ejaculate volume ranged from 0.2 to 7 ml, percentage motility range was 35 to 80% with the sperm concentration showing a wide range (4 to 4000 million sperm per ml), whereas the sperm abnormalities ranged from 15% to 50% (Unpublished). Similar studies have been conducted in birds such as the Blue rock

pigeon (*Columba livia*) and White-backed vulture (*Gyps bengalensis*) to study semen characteristics (Sontakke et al., 2004; Umapathy et al., 2005)

A close relationship seems to exist between genetic diversity and sperm pleiomorphism as reported in cheetah, where high proportion of pleiomorphic spermatozoa (>60%) (Wildt et al., 1987, 1988) correlated with low levels of genetic polymorphism (Wildt et al., 1987; O'Brien et al., 1983, 1985). Earlier studies by Wildt *et al.* (1987) showed that the Asiatic lions (Sakkarbaugh Zoo, Gujarat) are genetically monomorphic and there is a significant decrease in motile spermatozoa per ejaculate and an increase in pleiomorphic spermatozoa to 66%. Further, it was concluded that the Asiatic lion, which has experienced a severe population bottleneck and has been inbreeding ever since it was isolated as a small population, is a highly endangered species. However, our studies on Asiatic lions showed that the mean percentage of abnormal spermatozoa in lions was significantly lower (23%) than that reported by Wildt *et al.* (1987). In our study spermatozoal pleiomorphism in tiger is about 25% (Shivaji et al., 1998) and is similar to the observations made by Wildt *et al.* (1992) whereas in leopard, it was about 28%. Thus, the percentage of pleiomorphic spermatozoa in Indian lions, tigers and leopards is not alarming. A study carried out by our group on genetic variation showed that the Asiatic lions and Indian tigers showed 25.82% and 22.65% heterozygosity respectively (Shakaranarayanan et al., 1997). These results were comparable to the genetic variability in 50 to 125-year-old skin samples from museum specimens of Indian tigers (21.01%). Thus, it can be concluded that the low genetic variability observed in the mega cats could be an inherent feature of these species and not the consequence of prolonged inbreeding (Shakaranarayanan et al., 1997).

C. Testosterone : an indicator of male fertility

Testosterone, the male sex hormone, is essential for normal spermatogenesis in mammals and reduced amounts would impair spermatogenesis. It has been observed that the serum testosterone level significantly correlated with semen characteristics in various species as in the African elephant (Howard et al., 1984), domestic cat (Howard et al., 1990) and Eld's deer (Monfort et al., 1993). Wildt et al. (1987) reported that the inbred lions in Gir forest, which exhibited more than 60% abnormal spermatozoa had serum testosterone levels three-fold lower (<1 ng/ml) as compared to the outbred lions (~ 1.5 ng/ml) from the Serengeti population. However, in a recent study by us, it was observed that the mean serum testosterone level in the Asiatic lions was higher (1.85 ng/ml) compared to the report of Wildt et al. (1987). In fact, in most of the Asiatic lions, the levels of testosterone, the spermatozoal concentration, the percentage of motile spermatozoa were normal and the incidence of morphologically abnormal spermatozoa was low (< 25%). These results were comparable to the out-bred population of Serengeti, thus, implying that they are not inbred.

In tigers, mean testosterone level was comparable with the lions (1.72 ng/ml) (Shivaji et al., 1998), but in leopards (from Indian zoos), it was lower than those in lions and tigers (0.89 ng/ml), but comparable to the North Chinese leopard (Wildt et al., 1986).

D. Computerized evaluation of semen

Computer assisted semen analysis (CASA) is routinely used to eliminate subjective nature of routine semen evaluation and to facilitate rapid analysis of various motility parameters of spermatozoa which are normally very difficult to quantify by visual examination. Apart from determining the sperm count and the number of motile spermatozoa, CASA provides data for various motility characteristics of spermatozoa. CASA (HTM-IVOS, Version 10, Hamilton Thorne Research Inc. Danvers, MA, USA) has been standardized to evaluate the spermatozoal motlity parameters of wild cats (tiger, lion and leopard) (Jayaprakash et al., 2001; Patil et al., 1998) and such studies formed the basis for evaluating male fertility in wild animals. Similarly, CASA was also used for semen analysis of the Blue rock pigeon as a model for avian species and the motility parameters and motility pattern of fresh- and cryopreserved-spermatozoa of pigeon were studied (communicated).

E. Hamster zona-free oocyte penetration test

The hamster zona-free oocyte penetration is extensively used to explore the fertilizing capacity of spermatozoa. This heterologous sperm penetration assay has been developed for widespread use in the prediction of IVF success in humans (Yanagimachi et al., 1976) and also in numerous wild species including dolphin (Fleming et al., 1981), rhesus macaque (Boatman and Bavister, 1984), budgerigar (Samour et al., 1986) and tiger (Shivaji et al., 1998). This test may find its greatest usefulness in the evaluation of various cryopreservation protocols.

Our studies revealed that the spermatozoa of lion, tiger and leopard were capable of binding and penetrating zona-free hamster oocytes. The higher percentage of penetration of tiger spermatozoa could be due to less percentage of pleiomorphic spermatozoa (10%), as against 40% in lions (Shivaji et al., 1998). Earlier studies had indicated that in domestic cats and leopards with more than 60% normal spermatozoa, a greater number of zona-free hamster oocytes or zona-intact cat oocytes were penetrated compared to ejaculates of teratospermic cats which had more than 60% pleiomorphic spermatozoa (Howard and Wildt, 1990; Howard et al., 1981), thus indicating that teratospermia affects gamete interaction. IVF studies in puma and tiger also showed that teratospermia may be responsible for poor fertilizing ability of spermatozoa (Miller et al., 1990; Donoghue et al., 1990). In our

experiments, it was observed that both the neat and cryopreserved spermatozoa of lions, tigers and leopards successfully penetrated the zona-free hamster oocytes (Shivaji et al. 1998; Jayaprakash et al. 2001)

F. Cryopreservation of semen

Semen cryobanking is a great boon to the conservation and management of wildlife, especially endangered species. Preserved semen could be used as and when needed and more importantly to preserve genetic heterozygosity of a rare population held in captivity. Although cryopreservation protocols of cattle and human spermatozoa are very well established, protocol developed for one species is not universally applicable to all animals and needs to be modified and standardized for each species (Howard et al., 1986; Brotherton, 1990). These protocols depend on various factors such as semen diluent, cryoprotectant, the freezing regime and the method of storage (straws, ampules or pellets) (Howard et al., 1981, 1986). Studies have been carried out to standardize protocols for cryopreserving the semen of lions, tigers, and leopards (Patil et al., 1998; Shivaji et al., 1998; Jayaprakash et al., 2001). The method followed is essentially the same as that used for human semen with minor modifications. The extender used was Tris-Egg Yolk buffer (TYB) and the cryoprotectant was glycerol. The data indicated a decrease of 15 - 50% in percentage motility of tiger, lion and leopard spermatozoa following freeze-thawing, which is in agreement with the reports of Donoghue *et al.* (1992) and Byers *et al.* (1989) in the tiger. Moreover, cryopreservation of spermatozoa also results in damage to the acrosome of spermatozoa, thus affecting its fertilizing ability (Shivaji et al., 1998; Jayaprakash et al., 2001). Successful attempts have also been made to cryopreserve the semen of spotted deer, Blue rock pigeon and White backed vulture (Sontakke et al. 2004; Umapathy et al. 2005).

G. Non-invasive artificial insemination

Induction of oestrus and ovulation in Mega cats: Our standardized protocol for induction of oestrus and ovulation in big cats involves two doses of eCG 24 hours apart followed by hCG 80 hours later. Approximately 40 hours to 45 hours post hCG, the females were inseminated transcervically. This protocol induced behavioral oestrus in 80% of females and faecal progesterone data suggested that 75% of animals successfully ovulated following gonadotropic treatment (unpublished data).

Induction of oestrus in spotted deer: Oestrus synchronization has been widely used in ungulates with variable success (Monfort et al., 1993; Asher et al., 1992). However, the exact time of ovulation has been very difficult to assess (Schiewe et al., 1991; Wildt et al., 1992). Moreover, behavioural cues are not reliable indicators of oestrus detection in

these animals. Commonly acceptable procedure for oestrus synchronization in ungulates involves the use of CIDR intravaginal devices, MAP-pessaries or $PGF_{2\alpha}$. Attempts are in progress to synchronize oestrus in spotted deer using Crestar ear implant (Intervet), a progestin slow-releasing device followed by administration of eCG at the time of implant removal.

Transcervical artificial insemination in mega cats, deer and blue rock pigeon: Incompatibility between male and female preventing the natural mating and aggression towards female are common behavioural problems in mega cats and in such cases AI seems promising. The technique of AI, although routinely practiced in domestic animals, has not been commonly applied to wild animals. Success has been reported using laparoscopic intrauterine (surgical) insemination in some of the wild animals such as tiger (Donoghue et al., 1993), cheetah (Howard et al., 1992), puma (Barone et al., 1994), snow leopard (Roth et al., 1997) and Eld's deer (Monfort et al., 1993). In our laboratory, attempts are being made to standardize non-surgical (non-invasive) AI following exogenous gonadotropins in lion and leopard. However, no pregnancy has been achieved so far. Our studies suggest that the failure of AI is probably not due to lack of ovulation but due to either the unavailability of spermatozoa at the site of fertilization or a lack of proper management after insemination. Our attempts in AI have been successful in the blue rock pigeon (Sontakke et al., 2004) and the spotted deer.

H. Detection of ovulation by estimation of progesterone in faeces

Monitoring ovarian cyclicity is a prerequisite for success of captive breeding programmes and this is normally monitored by estimating steroid hormones. However, regular blood sampling is impractical, since, most of the wild animals need to be restrained chemically. The only alternative is the detection of hormonal metabolites in faeces and has been established as an aid for captive breeding programs in various wild animals such as leopard (Brown et al., 1994) and red wolf. At the CCMB we have recently standardized methods to monitor steroid hormones in lions and deers (unpublished).

G. Molecular sexing in monomorphic bird species

Sexing in birds is often a difficult task due to the fact that more than half of the existing bird species are monomorphic, i.e. males and females are phenotypically identical. This problem can hinder assisted breeding of wild bird species. DNA-based sexed identification provides a solution (Griffiths et al., 1998). In birds, the heterogametic sex is the female (WZ), while males are homogametic (ZZ). The test is based on two-conserved CHD (chromo-helicase-DNA-binding) genes located on the avian sex chromosomes of most of the bird species (Griffiths and Tiwari, 1995, 1996; Griffiths et al., 1996). This Polymerase Chain Reaction (PCR) test employs two primers, which anneal to conserve the exonic regions and

amplify across an intron, which usually differs in length between the CHD-W and CHD-Z genes (Griffiths et al., 1998). This method thus yields one amplified DNA fragment in males and two in females as seen by gel electrophoresis. As a part of our ongoing research in assisted reproductive techniques in birds this test was successfully used for the sexing of the Blue rock pigeon (*Columba livia*) and the White-backed vulture (*Gyps bengalensis*) (Reddy et al., 2006).

3.2 Work accomplished at CCMB

The Centre for Cellular and Molecular Biology during the last 10 years, attempted to ascertain the genetic variability and phylogenetic position (where needed) of endangered animals like lions, tigers, leopards, wolves, deers by DNA fingerprinting, chromosomal analysis, RAPD analysis, microsatellite DNA analysis and mitochondrial DNA sequencing. These studies initially were carried out with blood samples (Shankaranarayanan et al., 1997). During last two years we have also standardized methods of DNA isolation from scat and hair samples (Shankaranarayanan et al., 1997; Shankaranarayanan and Singh, 1998a, 1998b; Singh et al., 2002, 2003; Gaur et al., 2003; Shivaji et al., 2003; Sachdev et al., 2005). **These non-invasive methods will be of great importance to study genetic variation in various endangered species and need to be continued and extended to cover the animals in all zoos.** Recently, scat samples of tigers were used as a source for DNA and for arriving at population numbers of the animals in the wild (Bhagavatula and Singh, 2006). Further a number of cases related to wild life forensics have also been resolved. Simultaneously, methods were standardized for collection of semen from lion, tiger, leopard, hyena, wolf, deer, etc. by electroejaculation and the semen samples were analysed with respect to various semen characteristics such as volume, pH, sperm concentration, % motile sperms, % viable sperms and motility characteristics (Shivaji et al., 1998, 2003; Patil et al., 1998; Jayaprakash et al., 2001; Sontakke et al., 2004; Umapathy et al., 2005). Further, a computer aided semen analyzer (CASA) was used to objectively assess the motility of spermatozoa to ascertain the velocity of movement, the frequency of tail beat and the amplitude of the lateral head displacement. Studies have also been carried out to cryopreserve the semen of big cats and to evaluate their fertilizing ability under *in vitro* conditions (Patil et al., 1998; Sontakke et al., 2004; Umapathy et al., 2005). Protocols have been developed for non-invasive analysis of sex steroids. Sexing of vultures, collection of semen from vultures and artificial insemination in deer leading to the birth of a fawn and are some of the recent studies (Reddy et al., 2006; Umapathy et al., 2005).

All the above studies were carried out in collaboration with the Nehru Zoological Park, Hyderabad, Sakkarbaug Zoo, Junagadh; Nandankanan Biological Park,

Bhubaneshwar; Darjeeling Zoo, Darjeeling; Indira Gandhi Zoological Park, Visakhapatnam; and S V Zoological Park, Tirupathi.

Papers published in the area of Conservation of Endangered Species from CCMB

1. Shankaranarayanan, P., Banerjee, M., Kacker, R.K., Aggarwal, R.K. and Singh, L. (1997) Genetic variation in Asiatic lions and Indian tigers. *Electrophoresis*, 18: 1693-1700.
2. Shivaji, S., Jayaprakash, D. and Patil, S.B. (1998) Assessment of inbreeding depression in big cats: Testosterone levels and semen analysis. *Current Science*, 75 : 923-930.
3. Shankaranarayanan, P. and Singh, L. (1998) A rapid and simplified procedure for isolating DNA from scat samples. *Current Science*, 75 : 883–884.
4. Shankaranarayanan, P. and Singh, L. (1998) Mitochondrial DNA sequence divergence among big cats and their hybrids. *Current Science*, 75 : 919–923.
5. Patil, S.B., Jayaprakash, D. and Shivaji, S. (1998) Cryopreservation of semen of tigers and lions : Computerized analysis of the motility parameters of spermatozoa. *Current Science*, 75 : 930-935.
6. Jayaprakash, D., Patil, S., Navin Kumar, M., Muzumdar, K.C. and Shivaji, S. (2001) Semen characteristics of the captive Indian leopard. *Journal of Andrology*, 22 : 25-33.
7. Singh, A., Shailaja, K., Gaur, A. and Singh, L. (2002) Development and characterization of novel microsatellite markers in Asiatic lion (*Panthera leo persica*). *Molecular Ecology Notes*, 2 : 542 – 543.
8. Singh, A., Gaur, A., Shailaja, K., Satyarebala, B. and Singh, L. (2003) A novel microsatellite (STR) marker for forensic identification of big cats. *Forensic Science International*, 141 : 123-127.
9. Gaur, A., Singh, A., Arunabala, V., Umapathy, G., Shailaja, K. and Singh, L. (2003) Development and charecterisation of ten novel microsatellite markers from Chital deer (*Cervus axis*) and their cross-amplification in other related species. *Molecular Ecology Notes*, 3 : 607–609.
10. Balakrishnan, C.N., Monfort, S.L., Gaur, A., Singh, L. and Sorenson, M.D. (2003) Phylogeography and conservation genetics of Eld's Deer (*Cervus eldi*). *Molecular Ecology*, 12 : 1–10.
11. Shivaji S., Kholkute, S.D., Verma, S.K., Gaur, A., Umapathy, G., Singh, A., Sontakke, S., Shailaja, K., Reddy, A., Monika, S., Sivaram, V., Jyotsna, Bala, S., Ahmed, S., Bala, A., Chandrashekar, B.V.N., Gupta, S., Surya Prakash and Singh, L. (2003) Conservation of wild animals by assisted reproduction and

Molecular marker technology- A review. *Indian Journal of Experimental Biology*, 41 : 710 – 723.

12. Verma, S.K. and Singh, L. (2003) Novel universal primers establish identity of enormous number of animal species for forensic application. *Molecular Ecology Notes*, 3 : 28-31.
13. Aggarwal, R.K., Ramadevi, J. and Singh, L. (2003) Ancient origin and evolution of the Indian wolf : Evidence from mitochondrial DNA typing of wolves from Trans-Himalayan region and Peninsular India. Genome Biol. 4 : P6.
14. Verma, S.K., Prasad, K., Nagesh, N., Sultana, M. and Singh, L. (2003) Was elusive carnivore a panther? DNA typing of faeces reveals the mystery. *Forensic Science International,* 137 : 16-20.
15. Sontakke, S.D., Umapathy, G., Sivaram, V., Kholkute, S.D., Shivaji, S. (2004) Semen characteristics, cryopreservation, andsuccessful artificial insemination in the Blue rock pigeon (*Columba livia*) *Theriogenology*, 62 : 139-153.
16. Aggarwal, R.K., Velavan, T.P., Udaykumar, D., Hendre, P.S., Kartik Shanker, Choudhury, B.C. and Singh, L. (2004) Development and characterization of novel microsatellite markers from the olive ridley sea turtle (*Lepidochelys olivacea*). Mol. Ecol. Notes, 4 : 477-479.
17. Dutta, S.K., Vasudevan, K., Chaitra, M.S., Shanker, K. and Aggarwal, R.K. (2004) Jurassic frogs and the evolution of amphibian endemism in the Western Ghats. Curr. Sci. 86 : 211-216
18. Verma, S.K., Sinha, R.K. and Singh, L. (2004) Phylogenetic position of *Platanista gangetica* : insights from the mitochondrial cytochrome b and nuclear interphotoreceptor retinoid-binding protein gene sequences. Molecular Phylogenetic & Evolution, 33 : 280-288.
19. Aggarwal, R.K. (2004) Ancient frog could spearhead conservation efforts. Nature Pub. Group, 428 : 467.
20. Shanker, K., Ramadevi, J., Choudhury, B.C., Singh, L. and Aggarwal, R.K. (2004) Phylogeography of olive ridley turtles (*Lepidochelys olivacea*) on the east coast of India : Implications for conservation theory. Mol. Ecol. 13 : 1899-1909.
21. Gaur, A., Reddy, A., Annapoorni, S., Satyarebala, B. and Shivaji, S. (2005) The origin of Indian Star tortoises (*Geochelone elegans*) based on nuclear and mitochondrial DNA analysis: a story of rescue and repatriation. Conservation Genetics, In press.
22. Umapathy, G., Sontakke, SD, Reddy, A., Ahmed, S. and Shivaji, S. (2005) Semen Characteristics of the Captive Indian White-Backed Vulture (*Gyps bengalensis*). Biology of Reproduction, 73(5) : 1039-45.

23. Sachdev, M., Sankaranarayanan, R., Reddanna, P., Thangaraj, K. and Singh, L. (2005) Major histocompatibility complex Class I polymorphism in Asiatic lions. Tissue Antigens, 66 : 918.

24. Gupta, S.K., Verma, S.K. and Singh, L. (2005) Molecular insight into a wildlife crime : A case of peafowl slaughter. Forensic Sci. Int. 154 : 214-217.

25. Janke, A., Gullberg, A., Hughes, S., Aggarwal, R.K. and Arnason, U. (2005) Mitogenomic analyses place the Gharial (*Gavialis gangeticus*) on the crocodile tree and provide pre-K/T divergence times for most crocodilians. J. Mol. Evol. 61 : 620-626.

26. Umapathy, G., Sontakke, SD., Srinivasu, K., Thomas Kiran, S.D. Kholkute, S. Shivaji 2006 Estrus behavior and fecal steroid profiles in the Asiatic lion (*Panthera leo persica*) during natural and gonadotropin-induced estrus. *Animal Reproduction Science,* In press.

27. Ajay Gaur, K. Shailaja, Anju Singh, V. Arunabala, B. Satyarebala and Lalji Singh 2006. Twenty polymorphic microsatellite markers in the Asiatic lion (*Panthera leo persica*). Conservation Genetics 7 (6):1005-1008.

28. Ajay Gaur, Anuradha Reddy, S. Annapoorni, B. Satyarebala and S. Shivaji (2006). The origin of Indian Star tortoises (*Geochelone elegans*) based on nuclear and mitochondrial NDA analysis: A story of rescue and repatriation. Conservation Genetics 7 (2): 231-240.

29. Bhagavatula J, Singh L. 2006. Genotyping faecal samples of Bengal tiger *Panthera tigris tigris* for population estimation: a pilot study. BMC Genet. 2006 Oct 17;7:48.

30. Sontakke, S.D., Reddy, A., Umapathy, G., Shivaji. S., 2006. Anesthesia induced by administration of xylazine hydrochloride alone or in combination with ketamine hydrochloride and reversal by administration of yohimbine hydrochloride in captive Axis deer (*Axis axis*). *American Journal of Veterinary Research* (In press)

31. Reddy, A., Prakash, V. and Shivaji, S. (2006) A rapid, non-invasive, PCR-based method for the identification of the sex of the endangered Old World Vultures (white-backed and long-billed vultures) - Implications for captive breeding programs. Curr. Sci. In press.

32. Aggarwal, R.K., Kivisild, T., Ramadevi, J. and Singh, L. (2006) Mitochondrial DNA coding region sequences support the phylogenetic distinction of two Indian wold species. J. Zool. Syst. Evol. Res. In press.

Patent filed

1. Verma S. K., Singh, L. Universal primers for wildlife identification. Patent filed in US and PCT (Patent application Filing number 158/01; US 28-3-2001 and 39/01 PCT; WO-28-3-2001).

4. DETAILED WORK PLAN

4.1 Methodology

1. **Collection of blood**: Blood samples of a number of male and female leopards, and deer would be collected from various zoos under sterile conditions. For DNA isolation, blood samples would be brought in frozen condition and stored at –70°C at LaCONES.

2. **DNA from scats, feather, hair, etc**: DNA would also be isolated from scat of leopards and deer and from feathers of birds according to protocols described by us earlier (Shankaranarayanan et al., 1997; Shankaranarayanan and Singh, 1998a, 1998b; Singh et al., 2002, 2003; Gaur et al., 2003; Shivaji et al., 2003; Sachdev et al., 2005). Hair samples would also be used as and when available as a source to isolate DNA. This non-invasive method of sample collection is of special use in wildlife for monitoring population size and study of evolution.

3. **Development of species-specific molecular probes** : The standard approach of isolation of microsatellites is construction and screening of a partial genomic library in the focal species, sequencing the positive clones obtained and designing of markers based on the flanking regions (conserved between distantly related species) as described earlier (Singh et al., 2002, 2003; Gaur et al., 2003; Shivaji et al., 2003; Sachdev et al., 2005). The markers would serve as invaluable tool for genetic individualization, parentage assessment and gene mapping, and population monitors of genetic diversity, especially in the area of molecular ecology and breeding programmes.

4. **Mitochondrial D-loop sequencing :** Specific PCR primers already reported for mitochondrial D-loop analysis of leopards and deer would be synthesized and the amplified products would be sequenced using ABI 377 automated sequencer (Gaur et al., 2003).

5. **Barcoding of Indian birds :** DNA barcoding is a technique which has been developed for the rapid identification of animal species based on the sequencing of short stretches of one or more conserved genes such cytochrome oxidase I gene of the mitochondria (Herbert et al., 2004). The work will progress through the following steps –

    1. A random collection of fresh feathers (2-3) from Indian birds at the Nehru Zoological Park, Hyderabad, both from the caged birds and the birds from

the surrounding areas. This will be a preliminary study to standardize the techniques.

2. Collection of samples from parks and reserved areas in and around Hyderabad along with GPS locations and information on the canopy cover and topography so as to establish an inventory of the birds.

6. **Collection and evaluation of semen** : To improve protocols for cryopreservation of semen of endangered cats, deers and vultures, semen would be collected by electroejaculation or manual massage as in the case of birds and analysed with respect to semen parameters such as its appearance, consistency, volume, pH, total count of spermatozoa in the semen, percentage of motile spermatozoa, viability, percentage abnormal spermatozoa, type of abnormal spermatozoa according to established protocols (Shivaji et al., 1998, 2003; Patil et al., 1998; Jayaprakash et al., 2001; Sontakke et al., 2004; Umapathy et al., 2005). Motility assessment would be done using a computer aided sperm analyzer (Girija Devi and Shivaji, 1994). The morphology of the spermatozoa would be assessed by phase contrast microscopy and following staining with Giemsa, Papanicolaou and Bryan-Leishman stain. Viability of the spermatozoa would be evaluated by Eosin Y method. Ejaculates showing good semen profiles would then be used for sryopreservation.

7. **Selection of an optimum buffer for semen analysis** : Various buffers such as Tyrodes buffer, modified Tyrodes buffer (Bavister, 1989), Ham's F-10 medium, Kreb Ringers Bicarbonate buffer, Biggers, Whitten and Whittingham (BWW) medium, phosphate buffered saline etc. would be used for washing, dilution and maintenance of mammalian sperm. These buffers would be evaluated with respect to the maintenance of viability and motility of spermatozoa.

8. **Computerized analysis of the motility parameters of spermatozoa** : Computer aided motility analysis of spermatozoa facilitates rapid analysis of various motility parameters which was hitherto not possible or was subjective. Apart from determining the sperm count and the number of motile spermatozoa, the analyzer would be used to ascertain data with respect to seven other characteristics (Girija Devi and Shivaji, 1994). The other characteristics of sperm motility which would be determined include VCL or curvilinear velocity (which is the track speed of the sperm obtained by dividing the total distance traveled by the sperm during an acquisition by the time elapsed), VSL or progressive velocity (which is the straight line distance between the beginning and end of a sperm track divided by the time elapsed), VAP or path velocity (which is the track speed

along the average path of each sperm), STR or straightness (VSL/VAP), LIN or linearity (VSL/VCL), ALH or the amplitude of lateral head displacement (which refers to the mean width of the sperm head oscillation along the sperm track as it swims), and BCF or beat cross frequency (which is the frequency with which the track crosses the path in either direction).

9. **Cryopreservation of spermatozoa** : Cryopreservation methods vary from species to species with respect to the cryoprotectants, the extenders, and the cooling regime. Therefore ther is a need to standardize the method depending on the wild animal to be studied. Recent studies have indicated that tiger spermatozoa could be cryopreserved using a solution containing 20% egg yolk, 11% lactose and 4% glycerol (Donoghue et al., 1992). In the present study semen samples with more than 70% motility would be diluted 1:1 either in TALP [Tyrode medium supplemented with albumin, lactate and pyruvate (Sontakke et al., 2004)]; or in Tris-citrate medium containing 20% egg yolk and 4% or 8% glycerol to a final concentration of 200-250 million spermatozoa/ mL at 37°C. Aliquots of 0.25 mL would be packed into straws (IMV, France) and frozen in a programmable cryogenic unit (Consarctic, Gottingen Germany) with the following temperature regime: 24^0 C to 4^0 C @ 1^0 C /min and subsequently from 4^0 C to –80^0C @ 6^0 C/min. Finally, the straws would be plunged and stored in liquid nitrogen. Two to three days later, the straws would be thawed in a 37^0 C water bath for one minute and evaluated for post thaw sperm motility

10. **Cryobanking of fibroblast cultures of endangered animals:** Fibroblast cultures from endangered animals such as the big cats, deer and vultures would be set up using standard protocols of tissue culture using tissues from animals obtained at an opportune time of an accidental or natural death of the animals. Once the fibroblast culture line has set up it would be cryopreserved for future use. Cryobanking of tissues is now considered a significant conservation effort since it opens up avenues for future studies on the genetics and resurrection of the species.

11. **Genome banking of endangered animals:** A genome bank consisting of DNA isolated from endangered species would be maintained at LaCONES in duplicate at –70^0C. LaCONES already has a bank of almost hundred different endangered animals.

12. **Induction of ovulation in big cats and ungulates:** A commercially available kit, CRESTAR (Intervet, Boxmeer, The Netherlands) (Ptak et al., 2002) would be used to induce estrus in the adult female deer. CRESTAR consists of an ear implant containing 3 mg of norgestomet and an injection of 3 mg norgestomet and 5 mg oestradiol valerate. The implant would be inserted intra-dermally in the ear for 10 days and on the day of implant removal, 200 IU of PMSG (Pregnant mare serum gonadotropin) would be administered intramuscularly. The deer would then be artificially inseminated at 48 and 57 h after implant removal under a surgical plane of anesthesia using a combination of ketamine hydrochloride (1.5 mg/ kg body weight) and xylazine hydrochloride (1.0 mg/kg body weight), which was injected intramuscularly using a blowpipe. For insemination, a speculum with a fiber-optic light source (Caprine, Brockville, Ontario, Canada) would be inserted into the vaginal canal to visualize the os cervix. A cattle artificial insemination (AI) sheath containing semen would then be inserted into the os cervix and manipulated gently through the cervical canal until the catheter no longer moved forward and then semen would be slowly deposited. Immediately after insemination the animals would be revived by an i.v. injection of yohimbine hydrochloride (0.2 mg/kg body weight).

To evaluate the efficacy of gonadotropin-induced estrus the big cats would be administered 750 IU of PMSG (Folligon Intervet, the Netherlands) on day one of the experiment followed by 750 IU of human chorionic gonadotropin (hCG; Chorulon, Intervet The Netherlands) 80 hours after the PMSG injection (Brown et al., 1995; Graham et al., 2000, 2006). Gonadotropins would be administered intra-muscularly using a blowpipe. Subsequently, the animals would be monitored for estrus signs based on behavioral cues and hormone assays. The doses of the gonadotropins may also be changed depending on the response of the animals.

13. **Ultrasonographic monitoring of ovarian follicular response to various hormone regimens :** It is now an established fact that the basic knowledge about the reproductive processes such as ovarian cycle, follicular development, and ovulation timing is very essential for the successful application of any assisted reproductive technique in an endangered species. Ultrasonography is the only technique, which provides information on structural morphology and the functional status of reproductive organs in a live animal. Using this technique, the ovarian follicular response to the various hormone-regimens would be evaluated in big cats and ungulates. Based on the effectiveness of the protocols; the best hormone-treatment protocol would be used for estrus induction in big cats and wild ungulates.

14. **Artificial insemination in big cats, ungulates and vultures:** Based on the ovarian status and ovarian follicular response to the hormone treatment as monitored by ultrasonography, the females will be inseminated transcervically using freshly collected semen as well as with frozen-thawed semen. The semen will be deposited in the cervix using an insemination catheter with the help of a vaginal speculum. Our thrust has been to develop a non-surgical and field friendly insemination technique in wild animals. In vultures insemination would be attempted only during the breeding season.

15. **Analysis of fecal steroids to monitor cyclicity of the animals :** Fecal samples would be collected on alternate days from animals subjected to gonadotropin induced ovulation and naturally ovulating animals and would be stored in absolute methanol at 4°C. Prior to extraction (Brown et al., 1994)., undigested pieces of meat and strands of hair if present would be removed and the samples dried at 65° C. About 0.2 to 0.3 g of the dried, mixed and pulverized fecal sample was would be boiled in 5 ml of 90% aqueous ethanol for 20 min. After centrifugation at 1000 rpm for 10-15 min, the supernatant would be recovered and the pellet resuspended in 5 ml of 90% aqueous ethanol, vortexed for 1 min and re-centrifuged to recover the supernatant. Both the ethanol supernatants would then be combined dried completely (in an oven at 40° C), suspended in 1 ml of methanol, vortexed for 1 min and sonicated for 30 s (Branson Ultrasonics 250, CT, USA) to free particles adhering to the test tube's wall. The sonicated sample would then be used as the source of the steroid metabolites from the fecal samples.

16. **Steroid Hormone assays :** Estradiol and progesterone (metabolites) would be measured in duplicate using commercial RIA kits (Estradiol Coat-A-Count, Progesterone Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA) as per the manufacturer's protocol. The sensitivity of the assay for progesterone was 3 pg/ml for serum and 160 pg/g for fecal samples. The assays would be validated by demonstrating parallelism between standard curves and serial dilution of fecal extracts.

5. JUSTIFICATION

India holds a tremendous wealth of mega-biodiversity. Unfortunately, the very existence of this wealth is threatened due to habitat destruction, resulting in fragmentation of habitat, a condition known to push animals towards inbreeding depression and ultimately leading to their extinction. The problem is aggrevated by several factors such as farming and cattle-grazing by forest-dwellers who live in the sanctuaries and create a biotic pressure and competition for forest resources between man and animal. In addition, many a times a threat to the wildlife from within the sanctuaries grows because of encroachment and expanding agricultural activities on the periphery. Therefore, human intervention to facilitate conservation of the wild animals of our country both by habitat protection and assisted reproductive technologies is the need of the hour.

Continuous inbreeding, in general, leads to sterility at times making a species biologically unfit for survival in the long run. Therefore, it is of great importance that pedigree of each species, bred in captivity, is meticulously maintained and as far as possible, inbreeding is avoided. Today, using the powerful techniques of molecular biology, one can accurately evaluate the degree of biological relatedness, level of inbreeding and gross genetic defects in any given species. Therefore, these studies would help greatly in redesigning the captive breeding programmes and better management practices for the endangered species in our zoological parks.

A project of this nature which is aimed at understanding the biology of wild animals, development of molecular probes in ascertaining genetic variation, and development of assisted reproductive technologies would go a long way in conserving the rich wildlife of India.

REFERENCES

Asher, G.W., Morrow, C.J., Jabbour H N, Mulley R C, Weldhuizen F A & Langridge M. (1992) Laparoscopic intra-uterine insemination of fallow der with frozen thawed or fresh semen after synchronization. New Zealand Vet J, 40 : 8.

Balakrishnan C N, Monfort S L, Gaur A, Singh L & Sorenson M D (2003) Phylogeography and conservation genetics of Eld's Deer (*Cervus eldi*). *Molecular Ecology*, 12 : 1-10.

Barone, M.A., Wildt, D.E., Byers, A.P., Roelke, M.E., Glass, C.M. and Howard, J.G. (1994) Gonadotropin dose and timing of anaesthesia foe laparoscopic artificial insemination in the puma (Felis concolor). *J Reprod Fertil*, 101 : 103.

Bavister, B.D. (1989) A consistently successful procedure for *in vitro* fertilization of Golden hamster eggs. Gamete Res. 1989 23 : 139-158.

Bhagavatula J, Singh L. 2006. Genotyping faecal samples of Bengal tiger *Panthera tigris tigris* for population estimation: a pilot study. BMC Genet. 2006 Oct 17;7:48.

Boatman D E & Bavister B D. (1984) Stimulation of rhesus monkey sperm capacitation by cyclic nucleotide mediators. *J Reprod Fertil*, 71 : 357.

Brotherton J. (1990) Cryopreservation of human semen. Arch Androl, 25 : 181-195.

Brown J L, Wasser S K, Wildt D E & Graham L H. (1994) Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biol Reprod, 51: 776-786.

Brown, J.L., Wildt, D.E., Graham, L.H., Byers, A.P., Collins, L., Barrett, S., Howard, J.G., 1995. Natural versus chorionic gonadotropin induced ovarian responses in the clouded leopard (*Neofelis nebulosa*) assessed by fecal steroid analysis. Biol. Reprod. 53, 93-102.

Byers A P, Hunter A G, Seal U S, Binizik J A, Graham E F, Rendl N J & Tilson R L (1989) In vitro induction of capacitation of fresh and frozen spermatozoa of the Siberian tiger (*Panthera tigris*). J Reprod Fertil 86 : 599-607.

Charlesworth B, Sniegowski P & Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature, 371 : 215-220.

Crandall K A, Bininda-Emonds O R, Mace G M & Wayne R K (2000) Considering evolutionary processes in conservation biology. Trends Ecol Evol. 15 : 290-295.

Donoghue A M, Johnston L A, Seal U S, Armstrong D L, Tilson R L, Wolf P, Petrini K, Simmons L G, Gross T & Wildt D E (1990) In vitro fertilization and embryo development in vitro and in vivo in the tiger (Panthera tigris). Biol Reprod, 43 : 733-744.

Donoghue, A. M., Johnston, L. A., Seal, U. S., Armstrong, D.L., Simmons, L.G., Gross, T., Tilson, R. L. and Wildt, D.E. (1992) Ability of thawed tiger (Panthera tigris)

spermatozoa to fertilize conspecific oocytes and bind and penetrate domestic cat eggs in vitro. *J Reprod Fertil*, 96 : 555.

Donoghue, A.M., Johnston, L.A., Armstrong, D.L., Simmons, L.G. and Wildt, D.E. (1993) Birth of a Siberian tiger cub (Panthera tigris altaica) following laparoscopic intrauterine artificial insemination. *J Zoo Wildl Med*, 24 : 185.

Espinoza E A, Kirms, M A & Filipek M S (1996) Identification and quantitation of source from hemoglobin of blood and blood mixtures by high performance liquid chromatography. *Journal of Forensic Sciences,* 5 : 804-811.

Espinoza E O, Lindley N C, Gordon K M, Ekhoff J A & Kirms M A (1999) Electrospray ionization mass spectrometric analysis of blood for differentiation of species. Analytical *Biochemistry,* 15 : 252-261.

Fleming A D, Yanagimachi R & Yanagimachi H (1981) Spermatozoa of the Atlantic bottlenosed dolphin, Tursiops truncatus. J Reprod Fertil 63 : 509-514.

Fussell E N, Franklin L F & Frantz R C (1973) Collection of chimpanzee semen with an artificial vagina. Lab Anim Sci, 23 : 252-255.

Gaur, A. Anju Singh, V. Arunabala, G. Umapathy, K. Shailaja and Lalji Singh (2003). Development and charecterisation of ten novel microsatellite markers from Chital deer (*Cervus axis*) and their cross-amplification in other related species. *Molecular Ecology Notes*, **3**: 607 – 609.

Gee G F, Artificial Insemination and Cryopreservation of Semen from Non-domestic Birds, paper presented to the Proceedings First International Symposium on the Artificial Insemination of Poultry, University of Maryland, College Park, June 1994 pp. 262.

Gee G F, Avian Artificial Insemination and Semen Preservation, paper presented at Jean Delacour/IFCB Symposium on Breeding Birds in Captivity, N Hollywood, C. A. 1983 pp. 375.

Girija Devi, L. and Shivaji, S. (1994) Computerized analysis of the motility parameters of hamster spermatozoa during maturation. *Mol. Reprod. Dev.* 38: 94-106.

Graham, L.H, Byers A.P., Armstrong, D.L., Loskutoff, N.M., Swanson, W.F., Wildt, D.E., Brown, J.L., 2006. Natural and gonadotropin-induced ovarian activity in tigers (*Panthera tigris*) assessed by fecal steroid analyses. Gen Comp Endocrinol. In Press. doi:10.1016 / j.ygcen.2006.02.008.

Graham, L.H., Swanson, W.F., Brown, J.L., 2000. Chorionic gonadotropin administration in domestic cats causes an abnormal endocrine environment that disrupts oviductal embryo transport. Theriogenology, 54, 1117-1131.

Griffiths R & Tiwari B, in International patent publication no. WO9639505 (Isis Publication, Innovation Oxford) 1996.

Griffiths R & Tiwari B (1995) Sex of the last wild Spix's macaw. Nature, 375 : 454.

Griffiths R, Daan S & Dijkstra C, Sex identification in birds using two CHD genes, paper presented to Proceedings of the Royal Society of London B, 1996.

Griffiths R, Double M C, Orr K & Dawson R J G (1998) A DNA test to sex most birds. Mol Ecol 7 : 1071-1075.

Hagey I R, Crombie D L, Espinoza E O, Carey M C, Igimi H, Hofmann A F, Journal *of lipid Research,* 34 (1993) ursodeoxycholic Acids in the ursidae: Biliary Bile Acids of Bears, Pandas, and Related Carnivores., 1911-1918.

Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003a) Biological identi.cations through DNA barcodes. Proc R Soc Lond B Biol Sci 270: 313–321.

Hebert PDN, Ratnasingham S, DeWaard JR (2003b) Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc Lond B Biol Sci 270: S596–S599.

Howard J G, Brown J L, Bush M & Wildt D E (1990) Teratospermic and normospermic domestic cats: ejaculate traits, pituitary-gonadal hormones, and improvement of spermatozoal motility and morphology after swim-up processing. *J Androl*, 11 : 204-215.

Howard J G, Bush M & Wildt D In Current Therapy in Theriogenology, by D. A. Morrow (W. B. Sounders Co., Philadelphia, USA) 1986, 1047.

Howard J G, Bush M, de Vos V & Wildt D E (1984) Electroejaculation, semen characteristics and serum testosterone concentrations of free-ranging african elephants (Loxodonta africana). *J Reprod Fertil*, 72 : 187-195.

Howard J G, Pursel V G, Wildt D E & Bush M (1981) Comparison of various extenders for freeze-preservation of semen from selective captive wild ungulates. *J Am Vet Med Asso*, 179 :1157-1161.

Howard, J.G. and Wildt, D.E. (1990) Ejaculate-hormonal traits in the leopard cat (Felis bengalensis) and sperm function as measured by in vitro penetration of zona-free hamster ova and zona-intact domestic cat oocytes. *Mol Reprod Dev*, 26 : 163.

Howard, J.G., Donoghue, A.M., Barone, M.A., Goodrowe, K.L., Snodgrass, K., Starnes, D., Tucker, M., Bush, M. and Wildt, D.E. (1992) Successful induction of ovarian activity and laparoscopic intrauterine artificial insemination in the cheetah (Acinonyx jubatus). *J Zoo Wildl Med*, 23 : 288.

Jayaprakash, D., Patil, S.B., Navin Kumar, M., Majumdar, K.C. and Shivaji, S. (2001) Semen characteristics of the captive Indian leopard. *J Androl*, 22 : 25.

Menotti-Raymond, M. A. and O'Brien, S.J. (1995) Evolutionary conservation of ten microsatellite loci in four species of felidae. *Journal of Heredity*, 86 : 319.

Miller A M, Roelke M E, Goodrowe K L, Howard J G & Wildt D E (1990) Oocyte recovery, maturation and fertilization in vitro in the puma (Felis concolor). *J Reprod Fertil*, 88 : 249-258.

Monfort, S.L., Asher, G.W., Wildt, D.E., Wood, T.C., Schiewe, M.C., Williamson, L.R., Bush, M. and Rall, W.F. (1993) Successful intrauterine insemination of Eld's deer (Cervus eldi thamin) with frozen-thawed spermatozoa. *J Reprod Fertil*, 99 : 459.

O'Brien (1994) A role for molecular genetics in biological conservation. *Proc. Natl. Acad. Sci. USA*, 91 : 5748-5755.

O'Brien, S.J., Roelke, M.E., Marker, L., Newman, A., Winkler, C.A., Meltzer, D., Collly, L., Evermann, J.F., Bush, M. and Wildt, D.E. (1985) Genetic basis of species vulnerability in the cheetah. *Science*, 227 : 1428.

O'Brien, S.J., Wildt, D.E., Goldman, D., Merril, C.R. and Bush, M. (1983) The cheetah is depauperate in genetic variation. *Science*, 221 : 459.

Patil, S.B., Jayaprakash, D. and Shivaji, S. (1998) Cryopreservation of semen of tigers and lions: Computerized analysis of the motility parameters of spermatozoa. *Curr Sci*, 75 : 930.

Ptak G, Clinton M, Barboni B, Muzzeddu M, Cappai P, Tischner M, Loi P. Preservation of the Wild European Mouflon: The first example of genetic management using a complete program of reproductive biotechnologies. Biol Reprod 2002;66:796-801.

Reddy, A., Prakash, V. and Shivaji, S. (2006) A rapid, non-invasive, PCR-based method for the identification of the sex of the endangered Old World Vultures (white-backed and long-billed vultures) - Implications for captive breeding programs. Curr. Sci. In press.

Roth, T. L., Armstrong, D.L., Barrie, M.T. and Wildt, D.E. (1997) Seasonal effects on ovarian responsiveness to exogenous gonadotropins and successful artificial insemination in the snow leopard (Panthera uncia). *Reprod Fertil Dev*, 9 : 285.

Sachdev, M., Sankaranarayanan, R., Reddanna, P., Thangaraj, K. and Singh, L. (2005) Major histocompatibility complex Class I polymorphism in Asiatic lions. Tissue Antigens, 66 : 918.

Samour J H, Moore D & Smith C A (1986) Avian spermatozoa penetrate zona-free hamster oocytes in vitro. *J Exp Zool* 239 : 295-298.

Schneider S, Roessli D & Excoffier L, in A software for population genetics data analysis (Genetics and Biometry Laboratory, University of Geneva, Switzerland) 2000.

Shankaranarayanan, P. and Singh, L. (1998a) A rapid and simplified procedure for isolating DNA from scat samples. *Current Science*, 75 : 883–884.

Shankaranarayanan, P. and Singh, L. (1998b) Mitochondrial DNA sequence divergence among big cats and their hybrids. *Current Science*, 75 : 919–923.

Shankarnarayanan, P., Banerjee, M., Kacker, R.K., Aggrawal, R.K. and Singh, L. (1997) Genetic variation in Asiatic lions and Indian tigers. *Electrophoresis*, 18 : 1693.

Shivaji, S., Jayaprakash, D. and Patil, S.B. (1998) Assessment of inbreeding depression in big cats: Testosterone levels and semen analysis. *Curr Sci*, 75 : 923.

Shivaji, S., Kholkute, S.D., Verma, S.K., Ajay Gaur, Umapathy, G., Anju Singh, Sontakke, S., Shailaja, K., Anuradha Reddy, Monika, S., Sivaram, V., Jyotsna, B., Satyare Bala, Shakeel Ahmed, M., Aruna Bala, Chandrasekhar, B.V.N., Sandeep Gupta, Surya Prakash and Lalji Singh (2003) Conservation of wild animals by assisted reproduction and molecular marker technology. Ind. J. Experi. Biol. 41 : 710-723.

Singh, A., Gaur, A., Shailaja, K., Satyarebala, B. and Singh, L. (2003) A novel microsatellite (STR) marker for forensic identification of big cats. *Forensic Science International*, 141 : 123-127.

Singh, *A.*, Shailaja, K., Gaur, A. and Singh, L. (2002) Development and characterization of novel microsatellite markers in the Asiatic lion (*Panthera leo persica*). *Molecular Ecology Notes*, 2 : 542.

Sontakke SD, Umapathy G, Sivaram V, Kholkute SD, Shivaji S. Semen characteristics, cryopreservation, and successful artificial insemination in the Blue rock pigeon (*Columba livia*). Theriogenology 2004;62:139-53.

Sontakke, S.D., Umapathy, G., Sivaram, V., Kholkute, S.D. and Shivai, S. (2004) Semen characteristics, cryopreservation and successful artificial insemination in the Blue rock pigeon (*Columbia livia*). Theriogenology, 62 : 139-153.

Stoeckle M (2003) Taxonomy, DNA and the bar code of life. BioScience 53: 2–3.

Sunnucks P (2000) Efficient genetic markers for population biology. *Trends Ecol Evol* 15 : 199-203.

Tajima F, in Mechanisms of Molecular Evolution.Introduction to Molecular Paleopopulation Biology by Takahata N and Clark A G, 1993, 37.

Umapathy, G., Sontakke, S., Reddy, A., Ahmed, S. and Shivaji, S. (2005) Semen characteristics of the captive Indian White-backed Vulture (*Gyps bengalensis*). Biol. Reprod. 73 : 1039-1045.

Valdes A M, Slatkin M & Freimer N B (1993) Allele frequencies at microsatellite loci : the stepwise mutation model revisited. *Genetics*, 133 : 737-749.

Verma, S.K. and Singh, L. (2003). Novel universal primers establish identity of enormous number of animal species for forensic application. *Mol Ecol Notes*, 3: 28-31.

Weisbroth S & Young F A, (1965) The collection of primate semen by electroejaculation. *Fertil Sterility*, 16 : 229-235.

Wildt D E, Donoghue A M, Johnston L A, Schmidt P M & Howard J G, *Symp Zool Soc London*, 64 (1992) 45.

Wildt D E, Howard JG, Hall L L & Bush M (1986) Reproductive physiology of the clouded leopard: I. Electroejaculates contain high proportions of pleiomorphic spermatozoa throughout the year. *Biol Reprod*, 34 : 937-947.

Wildt D E, Seal U S & Rall W F, In Genetic Conservation of Salmonid Fishes by Cloud J. and Thorgaad G (Plenum Publishing Corp, New York) 1992, 85.

Wildt, D.E., Bush, M., Goodrowe, K.L., Packer, C., Pusey, A.E., Brown, J.L., Joshin, P. and O'Brien, S.J. (1987) Reproductive and genetic consequences of founding isolated lion populations. *Nature*, 329 : 328.

Wildt, D.E., Philips, L.G., Simmons, L.G., Chakraborthy, P.K., Brown, J.L., Howard, J.G., Teare and Bush, M. (1988) A Comparative analysis of ejaculate and hormonal characteristics of the captive male cheetah, tiger, leopard and puma. *Biol Reprod*, 38 : 245.

Yanagimachi R, Yanagimachi H & Rogers B J. (1976) The use of zona-free animal ova as a test-system for the assessment of the fertilizing. The capacity of human spermatozoa. *Biol Reprod*, 15 : 471-476.

Yuhki, N. and O'Brien, S.J. (1990) DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. *Proc Natil Acad Sci USA,* 87 : 836.

6. BUDGET ESTIMATES

A. Non-Recurring

(Rs. in lakhs)

S. No.	Item	Year 1 2007-2008	Year 2 2008-2009	Year 3 2009-2010	Year 4 2010 – 2011	Year 5 2011 - 2012	Total
1.	Minor equipment such as gel rocker, microwave oven, field microscope, pH meter, printer, vortex mixer, lap top computer, balance magnetic stirrer etc.	1.00	1.00	1.00	1.00	1.00	5.00
	Sub Total (A1)	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**5.00**

Total of A = Rs. 5.00 Lakhs

B. Recurring

B1. Manpower

S. No	Position (No.)	Scale	Year 1 2007-2008	Year 2 2008 – 2009	Year 3 2009 – 2010	Year 4 2010 – 2011	Year 5 2011 - 2012	Total
1.	Helper (1)	2,650-65-3,300-70-4,000	0.60	0.60	0.60	0.70	0.70	3.20
2.	Keeper (1)	2,650-65-3,300-70-4,000	0.60	0.60	0.60	0.70	0.70	3.20
3.	Research Associates (1)	11,000 + 15% HRA (1650)	2.30	2.30	2.40	2.40	2.40	11.80
4.	Sr. Research Fellows (3)	9,000 + 1350 HRA	3.75	3.75	3.75	3.75	3.75	18.75
5.	Jr. Research Fellows (3)	8,000 + 1200 HRA	3.32	3.32	3.75	3.75	3.75	17.89
	Total		**10.57**	**10.57**	**11.10**	**11.30**	**11.30**	**54.84**

Sub-Total (B1) = Rs. 54.84 Lakhs

B2. Consumables

Rs. in lakhs

S. No	Item	Year 1 2007 – 2008	Year 2 2008 - 2009	Year 3 2009- 2010	Year 4 2010 – 2011	Year 5 2011 - 2012	Total
1	Molecular biologicals	6.00	6.00	4.50	4.50	4.50	25.50
2	Animal feed	1.00	1.00	1.00	1.00	1.00	5.00
	Total	**7.00**	**7.00**	**5.50**	**5.50**	**5.50**	**30.50**

Sub-Total (B2) = 30.50 Lakhs

B3. Other Items

Rs. in lakhs

Item	Year 1 2007 – 2008	Year 2 2008 – 2009	Year 3 2009 - 2010	Year 4 2010 – 2011	Year 5 2011 - 2012	Total
Travel	1.50	1.50	1.50	1.50	1.50	7.50
Miscellaneous for stationary, postage, contingencies etc.	0.50	0.50	0.50	0.50	0.50	2.50
Total	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	**10.00**

Sub-Total (B3) = Rs. 10.0 Lakhs

Grand Total (A + B + C) = Rs. 100.34 Lakhs

Budget at a glance (for three years)

Rs. in lakhs

S. No	Item	Year 1 2007– 2008	Year 2 2008 – 2009	Year 3 2009 – 2010	Year 4 2010 – 2011	Year 5 2011 - 2012	Total
	Non-Recurring						
A1	**Equipment**	1.00	1.00	1.00	1.00	1.00	5.00
	Recurring						
B1	**Manpower**	10.57	10.57	11.10	11.30	11.30	54.84
B2	**Consumables and feed**	7.00	7.00	5.50	5.50	5.50	30.50
B3	**Travel and Miscellaneous expenses**	2.00	2.00	2.00	2.00	2.00	10.00
	Grand Total	**20.57**	**20.57**	**19.60**	**19.80**	**19.80**	**100.34**

Grand Total = Rs. 100.34 Lakhs

7. JUSTIFICATION FOR EACH HEAD AND SUB-HEAD OF THE BUDGET SEPARATELY MENTIONED IN THE ABOVE TABLES

A1. Minor Equipment

Many of the minor equipments have been in use for more than ten years and are malfunctioning and need to be replaced. These equipment have a short life and there is a need to replace them from time to time. It is difficult to say which among them would be replaced when but definitely a non- recurring cost of 1 lakh per year would be adequate for this purpose4.

B1. Justification for the staff in terms of broad work content/experience.

1. Helper (1 post)

Helper is required for the upkeep of the laboratories including cleaning and sterilization of the apparatus, handling of the animals in the laboratories and assisting scientists and technicians in the laboratory as well as in the field, and to maintain electrical, refrigeration and air-conditioning equipment.

2. Keeper (1 post)

Keeper is required for the cleaning and upkeep of the animal holding facility, feeding of animals and for assisting the Veterinarian in his various tasks.

3. Research Associates (1 post)

Research worker with M.Sc., Ph.D. in any area of Molecular biology or reproductive physiology for undertaking the research activity and supervising the work in the field along with the scientists.

4. Senior Research Fellows (3 posts)

Researchers having M.Sc. in any area of biology or life sciences with two years experience in research would be required to help senior scientists in carrying out the project work. One person would be required for the genetic studies, one for the assisted reproduction work and one for cryobanking and ultrasound monitoring.

5 Junior Research Fellows (3 posts)

Candidates with an M.Sc degree in any area of biology with interest in research would be required to assist the scientists and research associates with respect to all activities of the project including preparation of reagents, preparation of the animals, recording data both in the lab and the field etc. One person would be required for

the genetic studies, one for the assisted reproduction work and one for cryobanking and ultrasound monitoring.

B2. Consumables and feed

The budget estimates for consumables have been worked out taking into account the requirements for the genetic studies and the assisted reproduction studies. A number of imported chemicals and molecular biology kits would be needed for , the project. Feed of good quality and sufficient quantities would be required for the animals.

B3. Travel and Miscellaneous expenses

Travel would include travel to various zoos to collect samples etc. and to attend meetings.

Miscellaneous expenses would include stationary, postage and other contingent expenses.

8. PROJECT IMPLEMENTATION

8.1 Organisation of Work Elements

Year 1

1. Recruitment of staff.
2. Ordering for equipment and consumables.
3. Collection and processing of biological material for genetic polymorphism.
4. Development of non-invasive methodologies to study genetic variation in wild animals.
5. Collection of fecal samples for evaluating ovarian cyclicity and fertility status.
6. Continuation of studies on induction of ovulation and transcervical artificial insemination in big cats and ungulates.
7. Semen collection and standardization of AI in big cats, deers and vultures.
8. Collection of feather samples birds

Year 2

1. Procurement of consumables, etc.
2. Development of species-specific molecular probes for studies of wild animals.
3. Continuation of sequencing and characterization of mitochondrial genome of big cats.
4. Genotyping of wild animals using molecular markers.
5. Continuation of work on assisted reproduction of big cats with respect to faecal steroid analysis and artificial insemination.
6. Standardization of cryopreservation of semen and tissue in endangered animals.

Year 3 to Year 5

1. Procurement of consumables, etc.
2. Characterisation and completion of species-specific molecular probes for leopard, deer and other animals suggested by CZA
3. Completion of sequencing and characterization of mitochondrial genome of big cats and other animals suggested by CZA.
4. Genotyping of leopards and deers and other animals suggested by CZA. using molecular markers.
5. Completion of work on assisted reproduction of big cats and other animals suggested by CZA with respect to faecal steroid analysis and artificial insemination.
6. Completion of standardization of cryopreservation of semen and tissue in endangered animals.

8.2 Suggested plan of action for utilization of research outcome expected from the project

1. Studies on molecular biology of wild animals will have application in the *in situ* conservation strategies, population ecology, phylogenetics and also in translocation of wild animals for population management.
2. These studies will help to determine occurrence of inbreeding depression in zoo animals.
3. Studies on basic reproductive biology will help in identification of the most suitable individuals for *ex situ* conservation.
4. Development of assisted reproductive techniques would be used to facilitate breeding in endangered animals.
5. Human resource development in wildlife reproduction, molecular ecology and wildlife forensic.
6. Organisation of training programmes for zoo personnel.

8.3 Time schedule of activities giving milestones

S.No.	Name of Milestone	Expected date	
		Initiation	Completion
A.	**Equipment purchase**	Immediately after sanction	Before 31st March, 2008
B.	**Recruitment of staff**	Immediately after sanction	Within two months of sanction
C.	**Research Activity**		
	Collection of blood from individuals of different species and isolation of DNA	Immediately after sanction	Would continue till the end of project
2.	Analysis of various individual DNA samples using microsatellites and mitochondrial D-loop sequencing	Three months after sanction	By the end of the project
3.	Sequencing and characterization of complete mitochondrial genome of big cats and other animals suggested by CZA.	Three months after sanction	By the end of the project
4.	Collection of semen, semen analysis and cryopreservation of semen of big cats, deer and vulture and other animals suggested by CZA.	Three months after sanction	By the end of the project
5.	Estimation of steroid hormones/hormonal metabolites in serum/faecal samples	Three months after sanction	By the end of the project
6.	Induction of ovulation and transcervical AI in big cats and vultures and other animals suggested by CZA.	Three months after sanction	By the end of the project
7.	Cryopreservation of oocytes and fibroblasts in big cats and vultures and other animals suggested by CZA.	Three months after sanction	By the end of the project

8.4 Project implementing Agency/Agencies

Name of Agency	Address of Agency	Proposed Research Aspects
Lacones an Annexe of Centre for Cellular and Molecular Biology	CCMB, Uppal Road, Hyderabad 500 007	Scientific research

9. EXPECTED BENEFITS

Anticipated Products & Processes of Practical / Technological utility / Socio economic relevance expected to be evolved by pursuing the project

The anticipated gains from the above project are that the results of the various studies on DNA of the endangered animals would highlight the degree of biological relatedness, the level of inbreeding, if any, and the presence of chromosomal abnormalities. Simultaneously, the basic studies on reproduction would reveal the fertility status of the animals and facilitate redesigning of the breeding programmes by selecting suitable animals so as to achieve improved breeding performance of the endangered animals. Standardization of assisted reproductive technologies would help in human intervention to improve the fertility status and numbers of the endangered animals. Furthermore the effort would generate scientific manpower with skills in assisted reproduction of endangered animals which is an absolute necessity to conserve our mega wild animals.

The anticipated benefits of the project :

1. It would establish relatedness and the degree of inbreeding in endangered animals, which will help in designing future breeding programmes by selecting unrelated mating partners.
2. It would establish the fertility status of the animals, which will help in captive breeding programmes.
3. It would lead to standardization of protocols for ovulation induction and artificial insemination in endangered animals.
4. It would lead to the setting up of gene resource bank.
5. It would lead to human resource development in the field of wildlife molecular ecology and reproductive biology.
7. It would help to train zoo personnel and wildlife Managers for effective management of wildlife.

PART V : EXISTING FACILITIES

20. Available equipment and accessories at LaCONES to be utilized for the project:

S. No.	Name of equipment/accessories
1.	Vehicle for mobile lab
3.	Refrigerator
5.	CO_2 incubator
6.	Laminar flow hood
7.	Electronic balance
8.	Microfuge
9.	Field camera
10.	Generator portable
11.	UPS portable
12. & 13.	Dart gun, pistol and related equipment for anaesthising animals
14.	Electroejaculator plus probes
15.	Phase contrast microscope with camera
16.	Micropipettes
17.	Cryogenic containers
18.	Ultrasound scanner
19.	Suction pump
20.	Oocyte aspiration set
21.	Catheters
23.	Motility analyzer
24.	Incubator (ordinary)
25.	Refrigerated microfuge
26.	Personal centrifuge
27.	Computation facility
28.	Power pack
29.	Microwave oven
30.	Trangamete portable incubator
31.	Inverted microscope
32.	Millipore filtration units with pump
33.	Animal holding facility and cages for big cats

1.	Thermal cycler
2.	Spectrophotometer
3.	-70oC freezer
4.	Ultracentrifuge
5.	Laminar flow
6.	GC machine
9.	Precision balance
10.	Hot air oven
11.	Autoclave
12.	Fluorescent microscope
13.	Phase contrast microscope with photomicrography
14.	Bacteriological incubator
15.	Shaker
16.	Incubator shaker
19.	CO_2 incubator
21.	Electrophoretic apparatus with blot, elution, vertical, horizontal, scanner and computer
22.	UV transilluminator
23.	Ice flaker

Facilities available for use from CCMB

All other equipment required for the project such as lyophiliser, DNA sequencer, HPLC, GC-MS, scintillation counter etc would be utilized at CCMB. In addition all the other facilities as and when required would also be available for use at CCMB.

PART VI : DECLARATION / CERTIFICATION

It is certified that

(a) the research work proposed in the scheme/project does not in any way duplicate the work already done or being carried out elsewhere on the subject

(b) the same project has not been submitted to any other agency/agencies for financial support

(c) the emoluments for the manpower proposed are those admissible to persons of corresponding status employed in the institute/university or as per the Ministry of Science & Technology guidelines (Annexure-III)

(d) necessary provision for the scheme/project will be made in the Institute/University/State budget in anticipation of the sanction of the scheme/project

(e) if the project involves the utilization of genetically engineered organism, it is agreed that we will ensure that an application will be submitted through our Institutional Biosafety Committee and we will declare that while conducting experiments, the Biosafety Guidelines of the Department of Biotechnology would be followed *in toto*

(f) if the project involves field trials/experiments/exchange of specimens, etc. we will ensure that ethical clearances would be taken from concerned ethical Committees/Competent authorities and the same would be conveyed to the Department of Biotechnology before implementing the project

(g) it is agreed that any research outcome or intellectual property right(s) on the invention(s) arising out of the project shall be taken in accordance with the instructions issued with the approval of the Ministry of Finance, Department of Expenditure, as contained in Annexure-V

(h) we agree to accept the terms and conditions as enclosed in Annexure-IV. The same is signed and enclosed

(i) the institute/university agrees that the equipment, other basic facilities and such other administrative facilities as per terms and conditions of the grant will be extended to investigator(s) throughout the duration of the project

(j) the Institute assumes to undertake the financial and other management responsibilities of the project

L. Singh

Signature of Executive Authority of Institute/University with seal
Dr Lalji Singh
Director
Centre for Cellular and Molecular Biology
Uppal Road, Hyderabad 500 007

Dr. Lalji Singh
Director
Centre for Cellular and Molecular Biology
Uppal Road, Hyderabad - 500 007, India

Shivaji

Signature of Principal Investigator (1)
Name : Lalji Singh
Date : 29-12-2006

Signature of Principal Investigator (2)
Name : S. Shivaji
Date : 29-12-2006

PART VII : PROFORMA FOR BIODATA OF PRINCIPAL INVESTIGATOR 1

Name :**Lalji Singh** Designation : **Director**

Department/Institute/University : **Centre for Cellular and Molecular Biology**

Date of Birth : **05-07-1947** Sex (M/F) : **M** SC/ST : **No**

Education *(post-graduation onwards & professional career)*

S. No.	Institution Place	Degree Awarded	Year
1.	Banaras Hindu University, Varanasi	M.Sc.	1966
2.	Banaras Hindu University, Varanasi	Ph.D.	1971

Research Experience in various institutions (*if necessary, attach separate sheets)*

Designation	Organization & Place	From	To	Mode of Appointment	Nature of duties
Jr. Research Fellow	Dept. of Zoology, BHU	1966	1970	U.G.C. Fellowship	Research & Training
Sr. Research Fellow	Dept. of Zoology, BHU	1970	1972	CSIR Fellowship	Research
Research Associate	Dept.of Zoology Calcutta Univ.	1972	1974	Permanent Post	Research & Training
Pool Officer	Dept.of Zoology Calcutta Univ.	April 1974	Sept 1974	CSIR Appointment	Research
Postdoctoral Research Fellow	Edinburgh Univ. U.K.	1974	1976	Commonwealth Fellowship	Research
Guest Scientist	Dept.of Zoology Calcutta Univ.	Oct 1976	April 1977	On leave from U.K.	Research
Research Associate	Edinburgh Univ. U.K.	1977	1979	Medical Research Council Fellowship	Research
Visiting Fellow	Austr. National Univ., Canberra	July 1979	Sept 1979	Welcome Foundation	Research
Research Associate	Edinburgh Univ. U.K.	Oct 1979	May 1987	Medical Research Council Fellowship	Research

Scientist EII	CCMB Hyderabad	June 1987	May 1990	CSIR Appointment	Research
Scientist F	CCMB Hyderabad	June 1990	June 1995	CSIR Appointment	Research
Scientist G	CCMB Hyderabad	June 1995	July 1998	CSIR Appointment	Research
Officer on Special Duty (OSD)	CDFD Hyderabad	Oct 1995	Feb 1999	DBT Appointment	Setting up of Centre for DNA Fingerprinting & Diagnostics
Director	CCMB Hyderabad	July 1998-to-date		CSIR Appointment	Research

Publications *(Numbers only)* : **100**

Books : **-** Research Papers, Reports : **92** General articles : **6**

Patents : **2** Others *(Please specify)* : **-**

List of important publications relevant to the proposed area of work

1. Shankaranarayanan, P., Banerjee, M., Kacker, R.K., Aggarwal, R.K. and Singh, L. (1997) Genetic variation in Asiatic lions and Indian tigers. *Electrophoresis*, 18: 1693-1700.
2. Shivaji, S., Jayaprakash, D. and Patil, S.B. (1998) Assessment of inbreeding depression in big cats: Testosterone levels and semen analysis. *Current Science*, 75 : 923-930.
3. Shankaranarayanan, P. and Singh, L. (1998) A rapid and simplified procedure for isolating DNA from scat samples. *Current Science*, 75 : 883–884.
4. Shankaranarayanan, P. and Singh, L. (1998) Mitochondrial DNA sequence divergence among big cats and their hybrids. *Current Science*, 75 : 919–923.
5. Singh, A., Shailaja, K., Gaur, A. and Singh, L. (2002) Development and characterization of novel microsatellite markers in Asiatic lion (*Panthera leo persica*). *Molecular Ecology Notes*, 2 : 542 – 543.
6. Singh, A., Gaur, A., Shailaja, K., Satyarebala, B. and Singh, L. (2003) A novel microsatellite (STR) marker for forensic identification of big cats. *Forensic Science International*, 141 : 123-127.
7. Gaur, A., Singh, A., Arunabala, V., Umapathy, G., Shailaja, K. and Singh, L. (2003) Development and charecterisation of ten novel microsatellite markers from Chital deer (*Cervus axis*) and their cross-amplification in other related species. *Molecular Ecology Notes*, 3 : 607–609.

8. Balakrishnan, C.N., Monfort, S.L., Gaur, A., Singh, L. and Sorenson, M.D. (2003) Phylogeography and conservation genetics of Eld's Deer (*Cervus eldi*). *Molecular Ecology*, 12 : 1–10.

9. Shivaji S., Kholkute, S.D., Verma, S.K., Gaur, A., Umapathy, G., Singh, A., Sontakke, S., Shailaja, K., Reddy, A., Monika, S., Sivaram, V., Jyotsna, Bala, S., Ahmed, S., Bala, A., Chandrashekar, B.V.N., Gupta, S., Surya Prakash and Singh, L. (2003) Conservation of wild animals by assisted reproduction and Molecular marker technology- A review. *Indian Journal of Experimental Biology*, 41 : 710 – 723.

10. Verma, S.K. and Singh, L. (2003) Novel universal primers establish identity of enormous number of animal species for forensic application. *Molecular Ecology Notes*, 3 : 28-31.

11. Aggarwal, R.K., Ramadevi, J. and Singh, L. (2003) Ancient origin and evolution of the Indian wolf : Evidence from mitochondrial DNA typing of wolves from Trans-Himalayan region and Peninsular India. Genome Biol. 4 : P6.

12. Verma, S.K., Prasad, K., Nagesh, N., Sultana, M. and Singh, L. (2003) Was elusive carnivore a panther? DNA typing of faeces reveals the mystery. *Forensic Science International,* 137 : 16-20.

13. Aggarwal, R.K., Velavan, T.P., Udaykumar, D., Hendre, P.S., Kartik Shanker, Choudhury, B.C. and Singh, L. (2004) Development and characterization of novel microsatellite markers from the olive ridley sea turtle (*Lepidochelys olivacea*). Mol. Ecol. Notes, 4 : 477-479.

14. Verma, S.K., Sinha, R.K. and Singh, L. (2004) Phylogenetic position of *Platanista gangetica* : insights from the mitochondrial cytochrome b and nuclear interphotoreceptor retinoid-binding protein gene sequences. Molecular Phylogenetic & Evolution, 33 : 280-288.

15. Shanker, K., Ramadevi, J., Choudhury, B.C., Singh, L. and Aggarwal, R.K. (2004) Phylogeography of olive ridley turtles (*Lepidochelys olivacea*) on the east coast of India : Implications for conservation theory. Mol. Ecol. 13 : 1899-1909.

16. Sachdev, M., Sankaranarayanan, R., Reddanna, P., Thangaraj, K. and Singh, L. (2005) Major histocompatibility complex Class I polymorphism in Asiatic lions. Tissue Antigens, 66 : 918.

17. Gupta, S.K., Verma, S.K. and Singh, L. (2005) Molecular insight into a wildlife crime : A case of peafowl slaughter. Forensic Sci. Int. 154 : 214-217.

18. Ajay Gaur, K. Shailaja, Anju Singh, V. Arunabala, B. Satyarebala and Lalji Singh 2006. Twenty polymorphic microsatellite markers in the Asiatic lion (*Panthera leo persica*). Conservation Genetics 7 (6):1005-1008.

19. Bhagavatula J, Singh L. 2006. Genotyping faecal samples of Bengal tiger *Panthera tigris tigris* for population estimation: a pilot study. BMC Genet. 2006 Oct 17;7:48.

20. Aggarwal, R.K., Kivisild, T., Ramadevi, J. and Singh, L. (2006) Mitochondrial DNA coding region sequences support the phylogenetic distinction of two Indian wold species. J. Zool. Syst. Evol. Res. In press.

Project(s) being pursued / carried out by Investigator as on December 2006

Sl No	Title of the Project	Funding Agency	Duration From	To	No. of Scientists working under the project	Total Approved Cost of Project (in Rs.)	Status
1	Molecular Characterization of Silk Worm Races and Sex Determining Genes in Silk Worms	CSB	1994	1997	Lalji Singh J Nagaraju, R K Aggarwal	57.630	Completed
2	Molecular Markers in Cattle & Buffaloes	NDRI	1994	1995	Lalji Singh	3.00	Completed
3	Sex related disorders and reproductive failures	BMMRC	1994	1995	Lalji Singh	1.50	Completed
4	Molecular Characterisation of Wild Animals	CZA	1994	1997	Lalji Singh	41.54	Completed
5	Characterization, Sequencing & Molecular Organization of the YACs containing Testis Organising (TO) Gene in Human	DBT	1996	1998	Lalji Singh	75.00	Completed
6	Conservation of endangered wild animals	CZA	1999	2002	Lalji Singh S Shivaji	446.66	Completed
7	"X-Chromosome: functional genomics approach	CNRS	2000	2003	Satish Kumar Lalji Singh	--	Carried out
8	Study on genetic diversity in tribal populations of Andaman & Nicobar islands and creation of immortalised cell lines of vanishing tribes.	ICMR	2000	2003	Lalji Singh S C Sehgal	40.67	Completed
9	Advanced Molecular/chromosomal diagnostics for genetic disorders and cancers, stem cells	BE	2000	2002	Lalji Singh, J Dhawan, A Singh, G R Chandhak, G Pande	132.74	Completed
10	Characterization of a sex and species specific heterochromatic DNA transcripts(s) expressed during spermatogenesis in man	DBT	2001	2004	A J Rachel Lalji Singh	28.47	Completed
11	Genetic causes of male infertility in India	ICMR	2001	2004	K Thangaraj L Singh, B N Chakravarty	21.61	Completed
12	Chromosomal and Molecular Genetic Evaluation of Infertile Men	DST	2002	2005	Lakshmi Rao Lalji Singh, M. Deendayal	15.00	Completed
13	Conservation of Genetics of marine turtle on the mainland coast of India and offshore islands, using mitochrondrial DNA sequencing analysis and microsatellite analysis to assess the phylogeography and population structure at various roorkeries on the coast	WII	2001	2004	Ramesh Kumar Agarwal, Lalji Singh, Karthik Shankar	18.00	Completed
14	Genetics of Reproductive Dysfunction in Women	ICMR	2002	2005	Lakshmi Rao Lalji Singh, M. Deendayal	48.99	Completed
15	Molecular basis of sex determination and infertility in humans	CSIR	2001	2004	Lalji Singh, Nalini J Gupta K Thangaraj	22.30	Completed
16	Clinical and molecular manifestations of anophthalmia	ICMR	2002	2005	S Hari Naryana Rao, Lalji Singh, Sai Baba Goud	11.80	Completed
17	Genetic analysis of pancreatitis in Indian population	ICMR	2002	2005	G R Chandak Lalji Singh, D Nageshwar Reddy	31.71	Completed
18	Genome diversity in the tribal population of Kumaon region	CSIR	2005	2007	S P Singh, Lalji Singh, Veena Pandey, Thangaraj	8.35	Ongoing

19	Molecular epidemiology of alcoholism : The Kota Tribe in Nilgiri Hills – The unique opportunity to study total population	DBT	2004	2007	Lalji Singh, V R Rao, Thangaraj, J Partha-sarathi, K S Ashokan	36.410	Ongoing
20	Cytogenetic and molecular evaluation of infertile males	CSIR	2004	2007	P M Gopinath, Lalji Singh, K Satya-moorthy, K Thangaraj, K Sasi-dharan, G G Laxman Prabhu	7.55	Ongoing
21	Genome diversity in the selected tribal populations of Maharashtra	CSIR	2004	2007	Lalji Singh, K Thangaraj, S R Walimbe	8.2	Ongoing
22	Molecular genetic analysis of Archacological human specimens: Taxonomic and health perspectives	DBT	2003	2006	Lalji Singh Walimbe, Subhash R.	38.54	Ongoing
23	Genomic diversity in the caste, tribal and religious populations of Andhra Pradesh and its border areas in the broader context of the peopling of India	CSIR	2003	2--6	Lalji Singh, B Mohan Reddy, Thangaraj K	23.22	Ongoing
24	Genome diversity in the caste and tribal populations of Himalayan region	CSIR	2003	2006	Asha Chandola Saklani, Lalji Singh K Thangaraj	28.98	Ongoing
25	Conservation of Endangered Big cats in India	CZA	2006	2009	Lalji Singh, Shivaji	70.94	On going
26	Setting up a clinical research facility to develop Stem Cell Technologies and Regenerative Medicine	DST/ CSIR	2006	2008	Lalji Singh, Prasada Rao, NIMS	21.93	On going
27	National Facility for Real time Imaging of live samples	DST/ CSIR	2006	2010	Lalji Singh, Shashidhara	5.55	On going
28.	National Facility for Conservation of Endangered Species of Animals (NaFCONES)	CZA, DBT, Govt. of A.P., CSIR	2003	2008	Lalji Singh S. Shivaji	746.787	Ongoing

Place : Hyderabad

Date : 29-12-2006

L. Singh

Signature of Investigator(s)

Dr. Lalji Singh
Director
Centre for Cellular and Molecular Biology
Uppal Road, Hyderabad - 500 007, India

PART VII : PROFORMA FOR BIODATA OF PRINCIPAL INVESTIGATOR 2

Name :**S. Shivaji** Designation : **Scientist F**

Department/Institute/University : **Centre for Cellular and Molecular Biology**

Date of Birth : **17-6-1950** Sex (M/F) : **M** SC/ST : **No**

Education *(post-graduation onwards & professional career)*

S. No.	Institution Place	Degree Awarded	Year
1.	Birla Institute of Technology & Science, Pilani	M.Sc.	1971
2.	University of Delhi, Delhi	Ph.D.	1974
3.	University of Delhi, Delhi	Lecturer	1976 - 1978
4.	Indian Institute of Science, Bangalore	Research Associate	1978 - 1980
5.	Centre for Cellular and Molecular Biology, Hyderabad	Scientist	1980 - 1996
6.	Centre for Cellular and Molecular Biology, Hyderabad	Scientist F (Deputy Director)	1996 onwards

Research Experience in various institutions (*if necessary, attach separate sheets)*

1971 - 1973	M.Sc. thesis in Radiation Biology, Birla Institute of Technology and Science, Pilani.
1973 - 1978	Ph.D. thesis in Cell Biology, University of Delhi, Delhi.
1978 - 1980	Research Associate at Indian Institute of Science, Bangalore.
1980 onwards	Scientist at Centre for Cellular and Molecular Biology, Hyderabad.
1981 - 1982	Post-Doctoral Fellow at Max-Planck-Institut fur Biophysikalische Chemie, Gottingen, West Germany.
September, 1984 - November, 1984	Visiting Scientist at National Institute of Health and Family Welfare,New Delhi.
December, 1984 - March, 1985	Visited Antarctica as a Member of the Fourth Indian Scientific Expedition to Antarctica.

April, 1997 - July, 1997	Visiting Scientist at National Institute of Basic Biology, Okazaki, Japan.
October, 1997	Indo-French Programme of Cooperation in the Field of Polar Research and Technology
September, 1998 - October, 1998	Visiting Scientist at Institute for Medicine, Muenster, Germany.
January 2000 - March 2000	Visiting Scientist at National Institute of Basic Biology, Okazaki, Japan
December 2000 - February 2001	Visiting Scientist at National Institute of Basic Biology, Okazaki, Japan.
November 19, 2001 - January, 20, 2002	Visiting Scientist at National Institute of Basic Biology, Okazaki, Japan.

Publications *(Numbers only)* : 165

Books : **1** Research Papers, Reports : **159** General articles : **5**

Patents : - Others *(Please specify)* : -

List of important publications relevant to the proposed area of work

1. Shivaji, S., Jayaprakash, D. and Patil, S.B. (1998) Assessment of inbreeding depression in big cats: Testosterone levels and semen analysis. *Current Science*, 75 : 923-930.
2. Patil, S.B., Jayaprakash, D. and Shivaji, S. (1998) Cryopreservation of semen of tigers and lions : Computerized analysis of the motility parameters of spermatozoa. *Current Science*, 75 : 930-935.
3. Jayaprakash, D., Patil, S., Navin Kumar, M., Muzumdar, K.C. and Shivaji, S. (2001) Semen characteristics of the captive Indian leopard. *Journal of Andrology*, 22 : 25-33.
4. Shivaji S., Kholkute, S.D., Verma, S.K., Gaur, A., Umapathy, G., Singh, A., Sontakke, S., Shailaja, K., Reddy, A., Monika, S., Sivaram, V., Jyotsna, Bala, S., Ahmed, S., Bala, A., Chandrashekar, B.V.N., Gupta, S., Surya Prakash and Singh, L. (2003) Conservation of wild animals by assisted reproduction and Molecular marker technology- A review. *Indian Journal of Experimental Biology*, 41 : 710 – 723.
5. Sontakke, S.D., Umapathy, G., Sivaram, V., Kholkute, S.D., Shivaji, S. (2004) Semen characteristics, cryopreservation, andsuccessful artificial insemination in the Blue rock pigeon (*Columba livia*) *Theriogenology*, 62 : 139-153.

6. Gaur, A., Reddy, A., Annapoorni, S., Satyarebala, B. and Shivaji, S. (2005) The origin of Indian Star tortoises (*Geochelone elegans*) based on nuclear and mitochondrial DNA analysis: a story of rescue and repatriation. Conservation Genetics, In press.

7. Umapathy, G., Sontakke, S., Reddy, A., Ahmed, S. and Shivaji, S. (2005) Semen Characteristics of the Captive Indian White-Backed Vulture (*Gyps bengalensis*). Biology of Reproduction, 73(5) : 1039-45.

8. Sontakke, S.D., Reddy, A., Umapathy, G., Shivaji, S. (2006) Anesthesia induced by administration of xylazine hydrochloride alone or in combination with ketamine hydrochloride and reversal by administration of yohimbine hydrochloride in captive Axis deer (*Axis axis*). Amer. J. Vet. Res. In press.

9. Reddy, A., Prakash, V. and Shivaji, S. (2006) A rapid, non-invasive, PCR-based method for the identification of the sex of the endangered Old World Vultures (white-backed and long-billed vultures) - Implications for captive breeding programs. Curr. Sci. In press.

10. Umapathy, G., Sontakke, S.D., Srinivasu, K., Kiran, T., Kholkute, S.D. and Shivaji, S. (2006) Estrus behaviour and fecal steroid profiles in the Asiatic lion (*Panthera leo persica*) during natural and gonadotropin-induced estrus. Anim. Reprod. Sci. In press.

Project(s) submitted / being pursued / carried out by Investigator

S. No.	Title of the Project	Funding Agency	Duration From	To	No. of Scientists working under the project	Total Approved Cost of the Project (in Rs.)	Status
1	Biodegradation of human refuse by psychrotrophic microbes at low temperatures: Identification and strain improvement	DRDO	1994	1996	S Shivaji M K Ray	20.70	Completed
2	Semen Analysis and Cryopreservation of Spermatozoa of Indian Wild Animals	CZA	1994	1997	S Shivaji, K C Majumdar	39.22	Completed
3	Ribotyping of Plague Bacillii	DGHS	1995	1995	S Shivaji	2.50	Completed
4	Targets for male contraception : proteins involved	Volks-wagen	1997	2000	S Shivaji T G Cooper	17.25	Completed
5	Polyunsaturated fatty acids and acyl-lipid desaturases of psychrotrophic cyanobacteria from Anatartica	DST, JSPS	1999	2001	S Shivaji P Mohanty, Norio Murata, Hidetoshi Okuyama	15.11	Completed
6	Microbial Biodiversity of psychrophiles and psychrotrophs from cold habitats	DBT	1999	2004	S Shivaji M K Ray	29.79	Completed
7	Cryosampler (Baloon) Experiment	IUCAA	2000	2001	S Shivaji	2.00	Completed
8	Biodiversity and function of bacteria from Antarctica: a study of pack ice and sea water bacteria	IFCPAR	2000	2003	S Shivaji Daniel Delille	24.67	Completed
9	Genome-wide screen in two hundred sister-pairs with endometriosis	Wellcom e Trust	2000	2005	S Shivaji S Kennedy	90.17	Completed
10	IT solutions to Biotech and Pharmaceutical companies	PereNea	2001	2004	S Shivaji	0.50	Completed
11	Inventory on Microbial Resources of India	DBT	2002	2003	T. Chakrabarti S Shivaji, K V B Tilak, B N Ganguli, A K Paul, S Nautyal	1.95	Completed

12	Technical advise on research & production of recombinant proteins	Virchow Consultancy	2002	2002	S Shivaji	0.60	Completed
13	Molecular typing of Lactobacillus sporogenes	Unique Biotech	2002	2002	S Shivaji	1.00	Completed
14	Conservation of endangered wild animals	CZA	1999	2002	Lalji Singh S Shivaji	446.66	Completed
15.	Special Grant from CSIR for Antarctic Research	CSIR	2005	2005	S Shivaji	1.00	Completed
16.	Novel expression systems	NMITLI	2005	2007	S. Shivaji	65.00	Ongoing
17.	Bacteria from stratosphere – Cryosampler Balloon Experiment	ISRO IUCAA	2005	2006	S Shivaji	2.00	Ongoing
18.	Molecular basis of cold adaptation : Antarctic cyanobacteria and bacteria as model systems	DST, JSPS	2003	2006	S. Shivaji	10.92	Completed
19.	National Facility for Conservation of Endangered Species of Animals (NaFCONES)	CZA, DBT, Govt. of A.P.,CSIR	2003	2008	Lalji Singh S. Shivaji	746.787	Ongoing
20.	Bacterial biodiversity of Antarctica polyphasic and a rRNA approach	NCAOR, DOD	2005	2008	S. Shivaji	10.93	Ongoing
21.	Microbial biodiversity, phylogency and bioprospecting of East Antarctica	NCAOR	2006	2009	S. Shivaji	21.10	Ongoing
22.	Bacterial diversity and bioprospecting of bacteria from Himalayan glaciers : A culture-dependent and a culture-independent approach	DBT	2006	2009	S. Shivaji	32.343	Ongoing
23.	Molecular mechanisms of cold acclimation in a cyanobacterium *Synechocystis* sp. PCC 6803 : Role of molecular chaperonins during cold acclimation	DST	2006	2009	S. Shivaji	3.6	Ongoing

Place : Hyderabad

Date : 29-12-2006

Signature of Investigator(s)