CONSERVATION OF ENDANGERED ANIMALS BASED ON GENETIC POLYMORPHISM STUDIES AND ASSISTED REPRODUCTION

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1. Title : CONSERVATION OF ENDANGERED ANIMALS BASED ON GENETIC POLYMORPHISM STUDIES AND ASSISTED REPRODUCTION

2. Background, introduction and definition of the problem

Human activities like habitat destruction and fragmentation, over exploitation, pollution and introduction of species into new locations, are directly or indirectly causing a rapid depletion of all biological diversity in the world. The rate of species loss is of such a great magnitude that it resembles a period of mass extinction, and species are lost at rates far greater than the origin of new ones. An unknown but definitely large number of species are already extinct, and several others are of such small population sizes that they are at a risk of extinction. These species are particularly vulnerable to accidental natural events like environmental fluctuations and catastrophes. Genetic stochasticity includes the harmful effects of inbreeding, loss of genetic diversity and accumulation of dangerous mutations. Inbreeding generally reduces birth rates and increases the death rates (inbreeding depression) in the inbred offspring, while loss of genetic diversity reduces the ability of the individuals to adapt to changing environments via natural selection. This vicious circle of reduced population size, loss of genetic diversity and inbreeding spins out of control in many cases resulting in an extinction vortex.

Such vulnerable species, in the near future may be incapable of surviving in their natural habitats, predominantly due to various human impacts, necessitating *ex-situ* conservation. Endangered species in captivity have to be systematically managed to maximize retention of genetic diversity over long periods, usually by minimizing kinship. These captive populations may provide individuals for reintroductions, whose success depends on off-setting inbreeding depression, loss of genetic diversity and genetic adaptation to captivity. With advances in conservation genetics it is now possible to clearly demarcate founder members and estimate numbers required to maintain a viable founding population in captivity. Later as the population approaches its target size, genetic issues in management like minimizing inbreeding and consequent inbreeding depression, and retaining genetic diversity can be addressed. Accumulation of deleterious mutations and genetic adaptation to captivity can be avoided so as to improve the success rate of reintroduction programs of the captive bred species into the wild.

In addition to genetic studies advances in reproductive biology have made several modern techniques available as aids in *ex-situ* conservation of endangered species. These techniques are especially suitable to overcome logistic problems or when the animals are

not conducive to mating. Although numerous assisted reproduction techniques are available today, the ones most commonly used are artificial insemination with fresh or frozen/ thawed sperm, combined with extensive non-invasive monitoring of urinary or faecal hormones. Embryo technologies are less frequently used mostly due to the lack of basic knowledge about embryology in these rare species and also non-availability of surrogate mothers. Often these applied studies should be accompanied with systematic basic research studies like understanding sperm quality and its cryosensitivity in various species to eventually allow artificial insemination of thawed spermatozoa. Assisted reproduction techniques will play a significant role in both understanding the biology and saving endangered animal species. However, progress will take many years, as the successful use of assisted reproduction techniques in one animal species cannot easily be applied to another, because the way that animals reproduce is as different as their genetic diversity and physical appearances.

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 was 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.

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3. OBJECTIVES

The main objectives are :

1. GENETIC POLYMORPHISM STUDIES IN ENDANGERED SPECIES FOR CONSERVATION BREEDING

i. Evolutionary and genetic polymorphism studies in the Indian deer.
ii. Genetic studies in Pheasants.
iii. Genetic studies in primates.
iv. Genetic studies and population management in captive Clouded leopards.
v.. Genetic polymorphism in Rhino.
vi Genetic studies in Pygmy hog.

2. DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNOLOGIES FOR THE CONSER VATION OF ENDANGERED SPECIES

i. Assisted reproduction in Rhino, Musk deer, Red panda, Snow leopard and Lion-tailed macacque
ii. *Ex situ* conservation of pheasants and vultures by artificial insemination
iii. Non-invasive monitoring of reproductive status in Rhino, Musk deer, Brow-antelered deer, Red panda, Snow leopard and Lion-tailed macacque based on fecal steroid analysis

3. DNA BASED DIAGNOSIS OF WILDLIFE DISEASES

i. Development of DNA based detection of viral and bacterial diseases
ii. Development of multiplex PCR for simultaneous detection of major feline and canine diseases

4. CRYOBANKING OF GENES, TISSUES, GAMETES AND EMBRYOS OF ENDANGERED ANIMALS

i. Cryobanking of genomes.
ii. Cryobanking of gametes and embryos.

1. GENETIC POLYMORPHISM STUDIES IN ENDANGERED SPECIES

i. Evolutionary and genetic polymorphism studies in the Indian deer (Manipur brow-antlered deer and Mouse deer),

Of the 40 existing deer species in the world, nine occur in India. The Manipur brow-antlered deer (*Cervus eldi eldi*) and Mouse deer (*Tragulus memmina*), are two of the endangered deer in India. The present population of the Manipuri deer is approximately 150 individuals restricted to Keibul Lamjao National Park in Manipur. The Indian mouse deer is known to have existed for more than 25 million years. However, it has not evolved much as compared to the extant deer species. Therefore it is known as a 'living fossil'. The anatomy and morphology of this species has been extensively studied, but evidences related to phylogenetics and evolution are still lacking. Because of their status, we would like to continue studies to develop non-invasive methods for genetic characterization of these deer species and to study their genetic relationship with other related species/ subspecies using modern molecular genetic methods.

ii. Genetic studies in Pheasants (Western tragopan, Himalayan monal, Cheer pheasant and Blyth's tragopan)

The pheasants represent an important taxon in the order Galliformes. The pheasants are relatively large birds with most species exhibiting extreme sexual dichromatism. Typically, male pheasants are brightly colored and have well developed ornamental traits such as elongated tails, crests, and specialized fleshy structures. Although the order Galliformes is well defined, taxonomic relationships are less clear within the group, due to the low variability in anatomical and osteological traits. We would study genetic variation in endangered pheasant populations in India and conduct phylogenetic analyses based upon mitochondrial DNA sequencing from representatives of endangered pheasant species such as western tragopan (state bird of Himachal Pradesh), Himalayan monal (state bird of Uttarakhand), Cheer pheasant, Blyth's tragopan etc. of India, currently facing major threats like hunting, habitat destructionand population fragmentation.

iii. Genetic studies in primates (Lion-tailed macaque, Golden langur and Hoolock gibbon)

The genetics of primates is a fascinating area of research for many biologists due to their close proximity to humans. The lion-tailed macaque is an endemic, highly endangered primate facing various threats in its last rainforest habitat patches. These macaques, restricted to the evergreen forests of the Western Ghats in

Karnataka, Tamil Nadu and Kerala, have been classified as endangered because of their selective feeding habits, limited range of occupancy, delayed sexual maturity, long interbirth intervals, low population turnover and small remaining populations in the wild. Population surveys have suggested that there are less than 4000 individuals in the wild. Similarly, Golden langur is an old world monkey found in a small region of western Assam, India. It is currently endangered, with a total Indian population of about 1000 individuals. Of these, approximately 60% are adults indicating a relative lack of infants and juveniles. The relative dearth of infants and juveniles indicate a declining population and with the habitat being degraded by human activity. Further, another important species, the hoolock gibbon, is a rare primate of the Indian sub-continent. It is protected under Schedule I of Wild life (Protection) Act and is listed as 'Endangered' by IUCN. Destruction of forest by felling of trees, encroachment for agriculture including tea plantation and settlement, oil mining and exploration and open cast coal mining are major threats.

Our studies will provide a deeper molecular insight into the earlier phylogenetic studies and present genetic diversity status of these endangered primates. Further, this would be a more authentic study since it will be based on nuclear and organellar genes combined together, in contrast to earlier studies that were based on Morphology/Anatomy/Nuclear genes/mitochondrial genes taken separately.

iv. Genetic studies and population management in captive Clouded leopards

The clouded leopard is a medium-sized wild cat found in the forests of Asia. Their natural habitat stretches through southern China, the eastern Himalayas, northeast India, and Southeast Asia. It is believed that this species has become extinct in Taiwan. In addition to habitat disturbance, the number of clouded leopards is also continuously dwindling as they are increasingly becoming targets of international trade for their skins, bones, and meat in lieu of tigers and leopards. This may lead to loss of genetic diversity in the existing population in captivity as well as in the wild. Therefore, it is very important to monitor the genetic health of the species and assess the extent of genetic polymorphism. This will provide better understanding of the inter- and intra-species relationships and species status, significant for long term conservation and population management.

v. Genetic polymorphism in Rhino

The population of Indian one horned rhinoceros (*Rhinoceros unicornis*) was severely threatened in the last century. Population in the Brahmaputra Valley, Assam, reached a low

of 20 individuals in 1908. In Chitwan Valley in Nepal, a strong population persisted until about 1950; then, poaching and land clearing reduced the number to approximately 60–80 survivors in 1962. Since then the species has been brought back from the brink of extinction by strict protection and a sustained conservation effort, and numbers have increased from under 200 in the 1950s to around 2,600 today. However, there is no room for complacency, and the small population is still very vulnerable. The species is inherently at risk because over 70% of its population occurs at a single site, Kaziranga National Park. This area is subject to incessant poaching and human-wildlife conflicts. In fact, any single catastrophic event such as disease, poaching, habitat loss, etc., would wipe out the existing population. Further, the maintenance of a genetically viable and demographically stable captive population is still an important component for the recovery of the species. Species with small and declining populations are faced in with the loss of genetic variability due to the limited mating choices available and the smaller original gene pool. It therefore, becomes necessary to maintain a genetically healthy captive population of this threatened species. This will expand extant knowledge of the conservation status and genetics and will enlighten conservationists for selecting best management option to maximize the recovery potential of this species.

vi. Genetic studies in Pygmy hog

Pygmy hog (*Porcula salvania*) is an endangered species of small wild pig, previously spread across India, Nepal, and Bhutan but now only found in Assam. The current world population is about 150 individuals or fewer. Its population has been decimated by habitat destruction due to the expansion of human and cattle populations, uncontrolled thatch burning in the region and the development of commercial plantations. However, recent conservation measures have improved the prospects of survival in the wild of this critically endangered species. The species was first described as the only member of the genus *Porcula* , but was then regarded as the closest relative of the Eurasian pig *Sus scrofa* and named *Sus salvanius*. New genetic analysis of a large section of mitochondrial DNA supports the original classification of the pygmy hog as a unique genus. Being the sole representative of *Porcula*, the conservation of this critically endangered species is even more important as its extinction would result in the loss of a unique evolutionary branch of pigs. It is highly necessary and all efforts are required to maintain a genetically healthy founder population.

2. DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNOLOGIIES FOR THE CONSERVATION OF ENDANGERED SPECIES

i. Assisted reproduction in Rhino, Musk deer, Red panda, Snow leopard and lion-tailed macaque

In situ conservation strategies require live populations of animals to be maintained in their adaptive environments. However, in some cases these efforts are not sufficient for the propagation of small populations and maintaining adequate genetic diversity in endangered wild animals. Hence, *ex situ* conservation strategies like Artificial Insemination (AI), in-*vitro* fertilization (IVF) and embryo transfer (ET) need to be developed to help the conservation efforts of endangered animals. These strategies are commonly referred to as assisted reproduction and have been used very effectively in conservation of wild animals at national and international levels. AI has been one of the widely used technique for conservation and has led to successful birth of young ones of wild animals like the tiger (Donoghue et al., 1993), cheetah (Howard et al., 1992), puma (Barone et al., 1994), snow leopard (Roth et al., 1997), white Rhino (Hildebrant et al., 2007), spotted deer (Umapathy et al., 2007) and blackbuck (Sontakke et al., 2009).

Rhinocerous

Indian Rhinoceros (*Rhinoceros unicornis*) is the second largest animal, after the elephant. This animal is found in only two places in the world, Assam (India) and Nepal, inhabiting the tall grasslands and forests in the foothills of the Himalayas. Indian rhinos found a mention in the list of the endangered species. Their population had been reduced to less than 100 in the early 20th century. Due to conservation efforts their population has increased. However they are still under threat of extinction due to poaching, habitat loss and fragmentation.

Musk deer

The musk deer belongs to the family *Moschidae* and lives in India, Pakistan, Tibet, China, Siberia and Mongolia. Musk deer is the most endangered of all the species of deer. One of the main reasons for this is that they are highly poached for their musk. As part of conservation breeding program for endangered species, this deer needs intensive captive breeding effort using assisted reproductive techniques.

Red Panda

The main threat to the survival of red pandas (*Ailurus fulgens fulgens)* is loss and fragmentation of habitat. Additional threats are continued poaching for skins and for pet trade. The species shows a declining population trend across its range. It is therefore listed in Schedule I of the Wildlife Protection Act (1972) of India and is listed as Endangered in the IUCN Red List. The range of *Ailurus fulgens fulgens* extends from Nepal through

northeastern India (West Bengal, Sikkim, Arunachal Pradesh), Bhutan and into China *(Bahuguna et al. 1998)*. The red panda range is being increasingly fragmented and the population continues to decline sharply. In, India Padmaja Naidu Himalayan zoological park under the Conservation breeding programme funded by CZA had undertaken *insitu* conservation of red panda. Along with the *insitu* conservation strategies we need to develop *exsitu* strategies like artificial insemination and other assisted reproductive techniques. Initially we would like to standardize the procedure of artificial insemination in this species.

Snow leopard (*Uncia uncia*)

Due to the threats, especially human animal conflict and the declining population trends the species has been listed in Schedule I of the Wildlife Protection Act (1972) of India and is listed as endangered in the IUCN Red List of Threatened Species. Only around 20 individuals are available in captivity in four different zoos out of which around 17 individuals are present in the Pt. Govind Ballabh Pant High Altitude Zoo, Nainital as per the record of stud book. Artificial Insemination would help in increasing the number of this species.

Lion-tailed macaque

The lion-tailed macaque is an endangered primate, endemic to the tropical rain forest of the Western Ghats in south India. Most of the remaining populations live in fragmented rain forest habitats. In contiguous forests, it lives in groups of 8 to 40 animals, with a mean of 18 animals. Compared to other macaques, this species has a high age at first birth (6.6 yr), low birth rate (0.31 infants/female/year), and low mortality rate (0.045/animal/year). Apart from *insitu* conservation efforts, *exsitu* conservation efforts using various assited reproductive techniques need to be undertaken in Chennai or Mysore zoo as part of Conservation breeding program

The specific tasks under this objective would be :

1. Collection of semen from Rhino, Musk deer, Red panda, snow leopard and lion-tailed macaque.
2. Cryopreservation of semen from Rhino, Musk deer, Red panda, snow leopard and lion-tailed macaque.
3. Artificial Insemination in the above species.

ii. *Ex situ* conservation of vultures and pheasants by artificial insemination

Gyps vultures in the Indian subcontinent and South-East Asia have declined catastrophically during the last decade, and current populations are estimated to be <5% of the original. The major reason for this decline appears to be the use of the veterinary drug diclofenac for treating cattle (Oaks et al. 2004), Therefore, an immediate need exists to conserve the species either by captive breeding or by assisted reproduction. Captive breeding in birds of prey is not always successful because of stress-related behavioral

changes. Therefore, assisted reproduction may be the method of choice. The present study would be carried out in one of the vuture captive breeding centres in India.

Pheasants, one of the most beautiful group of birds on earth, are threatened due to habitat loss, hunting and live bird trade (IUCN 2008). In India, many pheasants [Cheer pheasant (*Catreus wallichi)*, Reeves's pheasant (*Syrmaticus reevesii),* Western tragopan (*Tragopan melanocephalus),* Blyth's tragopan (*Tragopan blythii),* Sclater's monal (*Lophophorus sclateri*) and Grey jungle fowl (*Gallus sonneratii)]* are endangered and some of them are critically endangered (IUCN 2004). The proposed study would be carried out on some of the commonly available pheasants such as Silver and Ring-necked pheasant as model species of other critically endangered species such as Western tragopan, Monal pheasant and Cheer pheasant.. The specific tasks would be to standardize semen collection , semen cryopreservation, artificial insemination and artificial incubation of eggs, so as to facilitate breeding and increase in the number of birds. Thus this study would be useful for the conservation breeding programs of critically endangered pheasant species and thus enhance their population and to strengthen *ex situ* conservation measures in India.

iii. Non-invasive monitoring of reproductive status in Rhino, Manipur–brow antlered deer, musk deer, red panda, snow leopard, lion-tailed macaque etc based on fecal steroid analysis

Monitoring of reproductive cycles in wild and endangered animals is extremely difficult since it would involve collection of blood for which purpose the animal would have to be anaesthetized. It is not advisable to anaesthetize the animal on a regular basis to monitor the reproductive cycle and detect the day of ovulation, since regular exposure to anaesthesia would put the animal under stress. Under such circumstances, it would be advisable to carry out non-invasive monitoring of the reproductive cycle by using faeces as the source of the hormonal steroids. Fecal steroid metabolites would help to understand reproductive cycle and assessment of fertility status in captive animals for intensive breeding programs of endangered animals. In the present study it is proposed to assess the reproductive cycle and fertility status of Rhinos, elephants, Manipur–brow antlered deer and Hangul using fecal samples collected at regular intervals from these animals.

3. DNA-BASED DIAGNOSIS OF WILDLIFE

Many infectious diseases (bacterial, viral and parasitic) are common in both captive and free-ranging wild animals and some of these diseases have been responsible for the large-scale decline in endangered animals and possibly extinction. Thus from the point of conservation of wild animals there is a need to have in place methods for prognosis, diagnosis and therapy for major diseases. The first step in this direction would be to identify the most prevalent diseases in wild animals in India, develop rapid and accurate diagnostic

methods for the disease and propose therapy for the same. Data collected from different zoos, wild life sanctuaries and reserve forests have indicated that the most prevalent diseases in wild animals include viral, bacterial and parasitic diseases (Table 1).

In India, disease diagnosis of wild animals follows the conventional approach of culturing of bacteria and virus, staining for identifying the bacteria, histopathology, and post-mortem analysis. This approach is time consuming and not always accurate. Compared to the above approach, molecular approach employing PCR-based diagnosis of infectious diseases of wild animals would be more rapid and accurate. Further, molecular characterization of infectious agents would help to establish whether the causative agent in wild and domestic animals is identical and also establish whether the infectious agent is identical across geographical boundaries. In addition to conventional PCR where a single disease is detected one could also think of developing strategies for detecting multiple infectious agents in a single reaction by using Multiplex Polymerase Chain Reaction (PCR).

Table 1. A survey of disease prevalence in captive wild animals

Infectious disease	Prevalence (%)	Species affected
Tuberculosis (T.B.)	50-60%	Most species in the wild
Feline Parvo Virus (FPV)	50	Wild cats (lion, leopard and tiger)
Foot and Mouth Disease (FMD)	35	Cloven-hoofed animals
Haemoprotozoan Diseases (*Babesia, Theileria, Hepatozoon*)	50	Most species in the wild
Infectious Canine Hepatitis (ICH)	40	Indian sloth bear
Feline Leukemia Virus (FeLV)	Data not available	Wild cats (lion, leopard and tiger)
Feline immunodeficiency Virus (FIV)	Data not available	Wild cats (lion, leopard and tiger)
Avianpox Virus (APV)	40	Wild birds

With this in view, the specific objectives would be :

i. Development of DNA-based detection of viral, bacterial and parasitic diseases

ii. Development of multiplex PCR for simultaneous detection of major feline and canine diseases

4. CRYOBANKING OF GENES, TISSUES, GAMETES AND EMBRYOS OF ENDANGERED ANIMALS

i. Cryobanking of genomes

The systematic banking of genome resources using cryopreserved germ plasm offers the opportunity to further conservation strategies of endangered species by assisting in the effective genetic management of captive populations. Cryopreserved germplasm will allow indefinite preservation of the presently available gene diversity represented in either captive or wild populations. If properly utilized, Genome Banks have the potential to decelerate the loss of gene and allelic diversity in captive populations.

At LaCONES, we are maintaining a Genome Bank of properly quantified and catalogued DNA samles of different species of Mammals (34), Birds (63), Reptiles (06) and Corals (25). A unique barcode ID has been provided to all the samples. This material has practical use for propagating endangered species and for assessing health and cause of diseases. We will continue to procure more samples for the Genome Bank from various sources.

ii. Cryobanking of gametes

The conventional method is to collect semen and cryopreserve the sperm. But, to collect oocytes or embryos one needs to use ultrasound guided recovery which needs to be standardized. The other alternative is to recover oocytes, semen and embryos from wild/endangered animals from natural/ accidental deaths and preserve them. Such gametes would help to establish base line data on the physiology of the species and to develop species-specific protocols for production of competent oocytes for *in vitro* fertilization and for somatic cell nuclear transfer technology (SCNT) to conserve endangered animals.

iii. Cryobanking of embryos

An alternative approach with potential for conservation involves exploiting gonadal tissue to preserve the genetic potential of individuals. This is a critical advantage for species that suffer from high rates of neonatal/juvenile mortality. Ovary could also be used for preservation of the reproductive potential of individual animals that receive known insult (e.g. the administration of a chemotherapeutic agent). Therefore, preservation of ovaries could be one of the most powerful tools for the conservation of germplasm of threatened/endangered mammalian species. In this context the following tasks need to be undertaken

1. To establish species - specific protocols for cryopreservation of ovaries and testes from dead wild/endangered animals.

2. To establish species-specific protocols for *in vitro* production of embryos in wild/ endangered animals using *in vitro* maturation and fertilization of oocytes and sperm collected from dead wild/endangered animals.
3. To analyse the viability and differentiation ability of preserved ovarian tissues.

3.1 REVIEW OF STATUS (NATIONAL AND INTERNATIONAL) OF RESEARCH AND DEVELOPMENT UNDER EACH OBJECTIVE AND JUSTIFICATION

1. GENETIC POLYMORPHISM STUDIES IN ENDANGERED SPECIES

Greater the genetic diversity greater is the survival fitness of a species. Normally, if Nature is left unperturbed by natural forces or calamities and more importantly free from anthropogenic influences such as habitat destruction and poaching wild populations of both flora and fauna would flourish. But, this is not to be in a world dominated by human beings and thus necessitating methods to monitor and evaluate genetic variation among populations, relationship among individuals within a population and to select those genotypes which show higher degree of genetic diversity to ensure that prevalent genetic diversity is maintained in these species (Deyoung and Honeycutt, 2005; Hall et al., 2007; Primmer, 2009). DNA fingerprinting is a reliable method for monitoring genetic variation and this could be accomplished by using molecular genetic markers such as polymorphic nuclear microsatellites and mitochondrial markers (Sunnucks, 2000). But, the accuracy and reliability of these markers is dependent on the development of species-specific probes. Several nuclear microsatellite and mitochondrial markers have been developed and used extensively in big cats, elephants, ungulates, reptiles and birds to ascertain the genetic variability (Chang et al., 2008; Nguyen et al., 2007; Pearse et al., 2006; Vidya et al. 2009; Wu et al., 2007; Zhang et al., 2005).

In India, species-specific microsatellite markers have been developed for Asiatic lion (Singh et al., 2002; 2003; Sachdeva et al., 2005; Gaur et al., 2006), Bengal tiger (Bhagavatula and Singh, 2006; Reddy et al. 2010), chital (Gaur et al., 2003), Eld's deer (Balakrishnan et al., 2003), Olive Ridley turtles (Shanker et al., 2004), langurs (Karanth et al., 2004) etc. and were used to establish that the genetic diversity in these animals was high enough and did not warrant any intervention in their in-situ conservation. Hand in hand with these developments Indian scientists have developed DNA isolation from scat and hair samples (Bhagavatula and Singh, 2006; Reddy et al. 2010) and thus paved the way to the use of non-invasive methods for studying the degree of genetic polymorphism in endangered animals. The ability to source DNA from scats and genotype such DNA has also become the

accepted mode for enumeration of lions and tigers in the wild (Bhagavatula and Singh, 2006; Reddy et al. 2010; Mondol et al., 2009)Thus this approach is proposed to study genetic variation in the Rhino, Swamp deer, Hangul, Sangai and captive Clouded leopards which are highly endangered .

Genetic variation could also be studied using other molecular markers. The MHC loci of Asiatic lions was studied and abundant polymorphism in MHC loci was observed not only between different individuals but also amongst clones from the same individual in spite of the population bottlenecks these wild cats were being subjected to (Sachdeva et al., 2005). However, when control region sequences of mitochondrial DNA (mt DNA) of Sangai deer were analysed population genetic parameters, including nucleotide diversity and haplotype diversity indicated lack of genetic variation among the Sangai deer probably due to inbreeding and would thus benefit from the incorporation of new genetic material (Balakrishnan et al., 2003).

Justification for genetic polymorphism studies

LaCONES has developed methods and capacity for the study of genetics of endangered species like big cats, ungulates, pheasants and primates. However, this area of study is very vast and the molecular tools required for such study are not sufficiently available. Such study requires species-specific molecular markers for population, evolutionary and phylogenetic analysis of the endangered species. Development of species-specific molecular markers is a tedious process. Also, not much work has been done on the genetics of above-mentioned group of animals of which many are highly endangered. Therefore, it will be worth studying the same.and the study would provide immediate solution and support for their conservation requirements and subsequent management. This study would also lead to the development of novel and efficient usage of already developed species-specific DNA markers for genetic polymorphism studies and phylogenetic analysis and thus help in conservation efforts.

2. DEVELOPMENT OF ASSISSTED REPRODUCTIVE TECHNOLOGIES FOR THE CONSER VATION OF ENDANGERED SPECIES

i. Assisted reproduction in Rhino, musk deer, red panda, snow leopard and lion-tailed macaque

Incompatibility between males and females, non-availability of a particular sex and male factor involvement could be few of the reasons responsible for infertility and low fecundity. AI could thus be used under such circumstances to overcome the hinderances to fertility. AI, has not been commonly applied to wild animals and, success has been reported in some 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), Eld's deer, white Rhino

(Hildebrant et al., 2007), spotted deer (Umapathy et al., 2007) and blackbuck (Sontakke et al., 2009). In the present proposal AI would be attempted in Rhino, Swamp deer, Hangul and Sangai deer which are all very endangered.

Justification for AI in Rhino, , Sangai deer, musk deer, red panda, snow leopard and lion-tailed macaque

Rhino, Swamp deer, Sangai deer, musk deer, red panda, snow leopard and lion-tailed macaque are considered very endangered and antropogenic factors such as habitat destruction and poaching have devasted populations of these animals. Therefore there is a need to develop protocols such as AI to supplement the numbers by ex-situ conservation. The specific technologies or protocols that need to be standardised would include collection of semen by electroejaculation, cryopreservation of semen, induction of super ovulation in females and AI.

ii. *Ex situ* conservation of vultures and pheasants by artificial insemination

In situ conservation strategies require live populations of animals to be maintained in their adaptive environments. However, in some cases these efforts are not sufficient for the propagation of small populations and maintaining adequate genetic diversity. Hence, *ex situ* conservation strategies like artificial insemination (AI), in-*vitro* fertilization (IVF) and embryo transfer (ET) would need to be developed for the purpose of conserving the endangered animals. These strategies commonly referred to as assisted reproduction have been used very effectively in conservation of wild animals. AI has been one of the widely used technique for conservation and has led to successful birth of young ones of wild animals like tiger (Donoghue et al., 1993), puma (Barone et al., 1994), snow leopard (Roth et al., 1997), black buck (Sontakke et al., 2009), spotted deer (Umapathy et al., 2007), blue rock pigeon (Sontakke et al., 2004), Northern pintail duck and Mallard duck (Stunden et al., 1998; Penfold et al., 2001), pheasants (Jalme et al., 2003) etc. These studies clearly indicated that AI could be used as a method for *ex situ* conservation but also revealed that methods developed for one species normally are not applicable to another species thus necessitating species specific development of protocols for successful AI. With this in view it is proposed to undertake AI in pheasants .

Justification for AI in vultures and pheasants

AI would be standardised in the commonly available species such as Silver pheasant and Ring-necked pheasant. These methods could then be applied in captive breeding programs of critically endangered pheasant species such as, Western Tragopan, Monal pheasant and Cheer pheasant in the future, to enhance their population and to strengthen *ex situ* conservation in India.

iii. Non-invasive monitoring of reproductive status in Rhinos, , Manipur–brow-antlered deer, musk deer, red panda, snow leopard and lion-tailed macaque , etc. based on fecal steroid analysis

Monitoring of fertility status is essential for assessing the reproductive potential of the animal. It is in this context that it is important to assess the endocrine levels throughout the year so as to determine optimal time of mating, reproductive status, fertility of captive animals, to understand the ovulation, implantation, pregnancy and parturition (Larson et al., 2003). Normally this is accomplished by monitoring steroid hormone levels in the blood. But, repeated blood sampling is not advised or encouraged in wild animals due to stress caused due to anesthesia. Therefore, non-invasive fecal hormone analysis could be used as an alternative method for hormonal analysis in wild animals.

Fecal hormone analysis has recently become a regular method to assess the reproductive physiology of non-human primates (Wasser et al., 1994; Ziegler et al., 1996; Mohle et al., 2002; Lynch et al., 2003), jaguar, sea otters (Larsen et al., 2003), brown bear, sika deer (Takahashi et al., 2002), small carnivores (Morai et al., 2002), great hornbill (Crofoot et al., 2003) and other birds (Dehnhard et al., 2003). This technique has also been used to assess the adrenal activity to judge the stress in captive and wild animals due to handling and environmental disturbance in mice (Touma et al., 2004), carnivores and sea lions (Hunt et al., 2004). Thus, fecal steroid analysis has enormous potential for evaluating changes in the hormonal profile during ovarian cycling or during pregnancy (Wasser et al., 1994; Larson et al., 2003; Hunt et al., 2004).

Justification for non-invasive monitoring of reproductive status in Rhinos, , Manipur–brow-antlered deer, musk deer, red panda, snow leopard and lion-tailed macaque , etc.

At LaCONES many ELISA kits for non-invasive monitoring of steroids such as estradiol, progesterone, 5-α-pregnane-3-α-ol 20-one, testosterone and cortisol to assess the reproductive function and stress in many Indian animals including big cats, ungulates, primates and elephants has been developed. Sukumar and his group have extensively studied the population dynamics, behavioral ecology and genetic polymorphism of Asian elephants (Vidya and Sukumar, 2005a; Vidya and Sukumar, 2005b). The present proposal to monitor the reproductive status in Rhinos, , Manipur–brow antlered deer, musk deer, Red panda, snow leopard, lion-tailed mcaque based on fecal steroid analysis would help to assess the reproductive potential of endangered animals both in captivity and wild. The data accumulated over a period of a year or more would be important to assess the endocrine levels, to determine optimal time of mating, reproductive status, fertility, ovulation status, implantation, pregnancy and parturition (Larson et al., 2003). Normally this is accomplished

by monitoring steroid hormone levels in the blood. But, repeated blood sampling is not advised or encouraged in wild animals due to stress caused due to anesthesia. Therefore, non-invasive fecal hormone analysis could be used as an alternative method for hormonal analysis in wild animals.

3. DNA-BASED DIAGNOSIS OF WILDLIFE DISEASES

i. Development of DNA-based detection protocols of viral, bacterial and parasitic diseases in wild animals

Feline leukemia virus, Feline immunodeficiency virus, Feline Panleukemia virus, Infectious Canine Hepatitis virus, Canine Parvovirus, Foot and mouth disease and Tuberculosis are among the most prevalent viruses and bacteria causing disease and death in animals. In addition, parasitic protozoa such as *Hepatozoon, Babesia* and *Theileria* live in mammalian blood cells, sometimes causing severe disease and even death of infected animals . These hematozoans have wide geographic distributions. The causative agent, the wild animal affected and the disease caused are summarised in Table 2.

Tuberculosis (TB) is a current and re-emerging disease of both captive and free ranging wild animals in India and has been reported to affect deer, antelopes, gazelles, nilgai, giraffe, chital, wild pigs, barking deer, etc. (Singh et al., 1981; Rao et al., 1982; Chakraborty et al., 1993; Arora, 1994). It is also regarded as a serious disease in primates such as rhesus monkeys, golden langurs, common langurs, pig-tailed monkeys, bonnet monkeys etc. (Singh et al., 1951; Rahman et al., 1981). Carnivores are relatively resistant to TB but a few cases have been reported in tigers, leopards and lions from different Indian zoos (Rathore and Khera, 1983; Arora, 1994). Further, all species of birds are susceptible including ducks, geese, swans, peafowl, pigeons, turkeys, parrots, canaries, wild fowl, pheasants, sparrow, crows, etc. (Joshi et al., 1991, Dvorska et al., 2007).

Feline panleucopenia, commonly known as feline distemper, is characterized by fever, enteritis and bone marrow changes and is caused by feline parvovirus, a highly contagious DNA virus infecting the cat family (Siegl et al., 1985; Parrish et al., 1988; Kariatsumari et al., 1991). This disease causes high morbidity and mortality in wild animals in various zoological parks or gardens in the country, and killed or modified live vaccines have been used for prophylactic immunization (Rathore and Khera, 1981; Singh and Gupta, 1988). The Zoological Parks in New Delhi and Chandigarh have experienced outbreaks of FPL which resulted in the death of pumas, snow leopard, golden cat, and tiger cubs and disease symptoms were observed in tigers and panther cubs (Singh et al., 1983; Singh and Gupta, 1988). A recent study by Ramanathan et al. (2007) showed that FPV is sero-prevalent in Asiatic lion (*Panthera leo persica*). It was suggested that these sero-positive animals may pose a risk of infection to other sero-negative animals. Hence, it is imperative

to carefully consider any movement, translocation, or reintroduction of these animals to new regions. Research on TB and FPL in Indian wild animals is lacking.

Table 2. Viral, bacterial and parasitic diseases of wild animals to be studied

Causative agent	Type of agent	Target gene	Affected animal	Disease symptoms
Feline Leukemia Virus	Family: *Retroviridae*, (ssRNA-RT)	*gag*	Domestic and wild cats	Immunosupression anemia, and neoplasms.
Feline immune-deficiency Virus	Family: *Retroviridae*, (ssRNA-RT)	*Pol*, reverse transcriptase	Domestic and wild cats	Immunodeficiency
Feline Panleukemia virus	Family: *Parvoviridae* (ssDNA)	*VP2*, structural protein	Domestic and wild cats	Fever, enteritis and bone marrow changes
Infectious Canine Hepatitis	Family: *Adenoviridae* (dsDNA)	*pVII* , major core protein	Dogs, wolves coyotes, bears, foxes	Virus infects blood, liver and kidneys.
Foot and mouth disease	Family: *Picornaviridae* (+)ssRNA)	*VP1*, polyprotein ; RNA polymerase	Cloven-hoofed wildlife and livestock	High fever, blisters inside the mouth and on the feet
M.tuberculosis, M. bovis, M. africanum, M. micriti, and M. pinnipedii	Genus: *Mycobacterium* (DNA)	*IS6110* , 16s rRNA	Mammals, birds, reptiles, amphibians and fish	Coughing blood, chest pain, shortness of breath
Hepatozoon, Babesia and *Theileria*	Blood Protozoans	18s rRNA	Domestic and wild ruminants, dogs, cats and even to humans.	Fever, anaemia and haemoglubinuria

Justification for DNA based detection of viral, bacterial and parasitic diseases in wild animals

Despite the urgency of the situation in India, an exclusive laboratory for diseases diagnosis of wild animals is lacking. Preliminary studies carried out at LaCONES based on data collected from different zoos, wild life sanctuaries, and field veterinarian suggested that tuberculosis (T.B.) and infectious feline enteritis (FPV) are most prevalent in India and thus there is a need to develop a DNA based diagnostic method for accurate and rapid detection of TB and FPV.

ii. Development of multiplex PCR for simultaneous detection of major feline and canine diseases

Multiplex polymerase chain reaction (PCR) is defined as the simultaneous amplification of multiple regions of DNA templates using multiple primers specific to more

than one gene (Henegariu et al., 1997). Multiplex PCR is a rapid and essentially cost-saving technique for large-scale genotyping with significant scientific, clinical, and commercial applications including diagnosis of infectious diseases, gene expression, forensic analysis, and paternity testing. Attempts would be made to develop Multiplex PCR for the simultaneous detection of several diseases like Feline Leukemia Virus, Feline immunodeficiency Virus, Feline Panleukemia virus, Infectious Canine Hepatitis, Canine parvo virus and Tuberculosis.

Justification for the Development of multiplex PCR for simultaneous detection of major feline and canine diseases

Multiplex PCR is rapid, cost-saving technique for large-scale detection and simultaneous detection of several diseases like Feline Leukemia Virus, Feline immunodeficiency Virus, Feline Panleukemia virus, Infectious Canine Hepatitis, Canine parvo virus and Tuberculosis.

4. CRYOBANKING OF GENES, TISSUES, GAMETES AND EMBRYOS OF ENDANGERED ANIMALS

i. Cryobanking of genes and tissues

Cryobanking of total genomic DNA of wild animals is very easily achieved since DNA from a biological sample could be purified and stored at -70^{0}C. Such DNA could be used as a reference sample to compare gene diversity of existing populations with past populations. Cryobanking of tissues could also be achieved using standard protocols to set up fibroblast cultures using fine needle aspirates from a live animal or tissue derived as early as possible from a dead animal. These tissues could also serve the purpose as indicated for DNA. More importantly somatic cells could also be used to resurrect an endangered animal by somatic cell nuclear transfer by either using a surviving animal or a closely related species.

ii. Cryobanking of gametes and embryos

Cryopreservation of semen of endangered species is an ongoing activity in LaCONES and cryopreservation protocols have been optimized for semen from lions, tigers, (Patil et al., 1998), leopards (Jayaprakash et al., 2000), spotted deer (Umapathy et al., 2007) and black buck, blue rock pigeon (Sontakke et al., 2004) and white-backed (Umapathy et al., 2005). These studies indicated that the protocol for cryopreservation is species specific and needs to be standardized for and each every species. The cryopreserved semen samples could be used for AI under circumstances when a male factor is involved in normal mating, could be used to produce embryos by *in vitro* fertilization and in addition the DNA could also be used for purposes indicated above.

In contrast to semen, non-availability of oocytes of wild animals posses a major challenge in cryopreservation of the female gamete. Though electroejaculation is permitted for semen collection laproscopic intervention is not permitted for oocyte collection. In most animals this is the best method. An alternative approach would be to recover oocytes from the ovaries at postmortem activate them, mature them and store them. A few attempts have been made to explore the possibility of recovery, *in vitro* maturation (IVM) and IVF of oocytes collected from the ovaries of various wild/ endangered species either from supervised culls or after their death as in felids (Johnston et al., 1991) and antelope species (Lukstoff et al., 2001). In India, LaCONES has carried out pioneering studies and has succeeded in oocyte recovery, cryopreservation, IVM and IVF of oocytes collected post-mortem from the ovaries of black buck, nilgai and chousingha at the Nehru Zoological Park (NZP), Hyderabad (Rao et al., 2010; 2011; Umamahesh et al., 2011).

iii. Cryobanking of ovaries and testes

In addition attempts could be made to establish species-specific protocols for cryopreservation of ovaries and testes from dead wild/ endangered animals. Sheep ovarian tissues have been cryopreserved and cryopreserved ovaries could be revived and cultured *in vivo* as xenografts or *in vitro* as explant cultures. Snow et al. (2002) produced live offspring from the in-vitro matured and fertilized oocytes collected from xenografted mice ovary in rat host. Similar outcomes in endangered species would have tremendous implications in conservation biology. Ovarian xenotransplantation in various wild species such as elephant (Gunasena et al., 1998), wallaby (Candy et al., 1995), wombat (Wolvekamp et al., 2001) and in domestic species such as sheep (Gosden et al., 1994), cow (Hernandez-Fonseca et al., 2005) and pig (Kagawa et al., 2005) have been demonstrated with variable success. In human, ovarian xenograft in mice host with development of primordial follicles to antral follicles have also been shown (Weissman et al., 1999). In carnivores, there has been some progress in embryo freezing (Pope, 2000), and healthy offspring have been produced following the same freeze-thaw protocol for embryos in three non-domestic felid species (ocelot, African wild cat and cercal; Pope, 2000; Swanson, 2001; Swanson and Brown, 2004).

Justification for cryobanking of genes, tissues, gametes, ovaries, testes and embryos

Cryobanking of genes, tissues, gametes, ovaries, testes and embryos will thus provide an holistic approach towards preservation of the germplasm from endangered animals. These cryopreserved bioresources could over the years be used for comparing genetic polymporphism with the existing populations and also used for somatic cell nuclear transfer or inter-species nuclear transfer, thus satisfying the long term goal of LaCONES in wildlife conservation.

Justification for cryobanking of genes, tissues, gametes, ovaries, testes and embryos

Developing cryobanking of genes, tissues, gametes, ovaries, testes and embryos will thus provide an holistic approach towards preservation of the germplasm from endangered animals. These cryopreserved bioresources could over the years be used for comparing genetic polymporphism with the existing populations and also used for somatic cell nuclear transfer or inter-species nuclear transfer, thus satisfying the long term goal of LaCONES in wildlife conservation.

3.2 WORK ACCOMPLISHED AT LACONES (CCMB)

1. DNA fingerprinting, chromosomal analysis, RAPD analysis, microsatellite DNA analysis and mitochondrial DNA sequencing studies were carried out with blood samples to ascertain the genetic variability and phylogenetic position of endangered animals like lions, tigers, leopards, wolves, deers etc. (Shankaranarayanan et al., 1997).
2. 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 animals in all zoos.
3. Scat samples of tigers were used as a source for DNA and for arriving at population numbers of tigers and lions in the wild (Bhagavatula and Singh, 2006; Reddy et al., 2010).
4. Sexing of birds based on a rapid non-invasive PCR- based method has been accomplished (Reddy et al., 2006).
5. Developed an universal primer for forensic use to identify animal species (Verma and Singh, 2003).
6. Identified the Himalayan wolf as a new species *Canis himalayensis* (Aggarwal et al., 2003; 2006).
7. Demonstrated that the Olive Ridleys' are ancestral to all the other known Ridleys (Aggarwal et al., 2004).
8. Based on molecular marker studies endangered star tortoise confiscated from smugglers have been rehabilitated successfully (Gaur et al., 2006).
10. A number of cases related to wild life forensics have also been resolved (Verma et al., 2003; Gupta et al., 2006).
11. Methods have been 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).

12. Semen of big cats and ungulates have been cryopreserved and evaluated their fertilizing ability (Patil et al., 1998; Sontakke et al., 2004; Umapathy et al., 2005).
13. Protocols have been developed for non-invasive analysis of fecal sex steroids in Asiatic lion and ungulates (Umapathy, et al., 2007).
14. Artificial insemination leading to pregnancy and live birth has been achieved in spotted deer and black buck (Umapathy et al., 2007; Sontakke, et al., 2009a).
15. For the first time in the world, a method was standardized for the collection of semen from the white backed vulture (Umapathy et al., 2005).
16. Established that vulture populations also decline due to avian malaria (Reddy et al., 2009).
17. Reversal of anaesthesia in captive Indian wild felids, spotted deer and black buck by administration of yohimbine hydrochloride or Tolazoline (Sontakke et al., 2007; 2009a; 2009b).

All the above studies were carried out in collaboration with Nehru Zoological Park, Hyderabad; Sakkarbaug Zoo, Junagadh; Nandankanan Biological Park, Bhubaneshwar; Darjeeling Zoo, Darjeeling; Indira Gandhi Zoological Park, Visakhapatnam; S V Zoological Park, Tirupathi; Arignar Anna zoological park, Chennai; Delhi zoo, Delhi; Sepphajala zoo, Tripura etc.

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1. Pandit, M.W., Shivaji, S. and Lalji Singh (2007) You Deserve, We Conserve : A biotechnological approach to wildlife conservation. I.K. International, New Delhi.

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).

Award received

1. S.K. Verma and Lalji Singh received the CSIR Technology Award for the year 2008 for the development of Universal primers for wildlife identification.

4. DETAILED WORK PLAN

4.1 METHODOLOGY FOR GENETIC POLYMORPHISM STUDIES

1. Collection of samples

Fecal, hair and blood (wherever possible) samples of focal species would be collected from various zoos under sterile conditions. The samples will be properly preserved with suitable additives and transported to LaCONES for genetic analyses.

2. DNA Isolation

DNA would be isolated from various biological samples such as from blood, tissue, sputum, hair or nasal swab samples 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; Sachdeva et al., 2005) or by using standard proteinase K/ phenol: chloroform protocol. DNA would be dissolved in TE (10 mM Tris-HCL, 1.0 mM EDTA, pH 8.0) buffer and stored at -20^{0}C until use.

3. Identification of species-specific/ cross-specific molecular markers

Molecular markers serve as invaluable tools for genetic individualization, parentage assessment and gene mapping, and as population monitors of genetic diversity, especially in the area of molecular ecology and breeding programmes. Attempts will be made to identify nuclear and mitochondrial markers already reported in the public domain/database from the same species or related species to conduct the genetic analyses. In case, no useful markers are found, we will develop species-specific markers in the lab. The markers would serve as invaluable tool for genetic individualization, parentage assessment and gene mapping, and as population monitors of genetic diversity, especially in the area of molecular ecology and breeding programmes.

4. Microsatellite typing

All the PCRs will be performed in a laminar flow hood to avoid any contamination. Fluorescent-tagged amplification products will be size fractionated and visualized on ABI 377 DNA sequencer (Applied Biosystems Inc, USA), and allele size will be determined using HD400 [ROX-0350] or [Tamara-350] size standard and the GENESCAN 3.1 software [Applied Biosystems, USA]. All samples will be amplified twice. The loci that are heterologus in both replicates will be scored as reliable and the genotype will be recorded.

5. Mitochondrial DNA sequencing

An aliquot of the PCR product will be treated with Shrimp Alkaline Phosphatase (SAP) and exonuclease-I to degrade the single stranded primers. Cycle sequencing reactions containing exonuclease-SAP treated PCR product and either of forward or reverse primers, will be carried out with a fluorescent Big Dye Terminator cycle sequencing kit (Applied Biosystems, USA). Sequences will be resolved on an ABI 3700 automated DNA sequencer (Applied Biosystems, USA).

6. Data Analysis

Genetic diversity will be quantified as the total number of alleles at each microsatellite locus and over all loci as observed heterozygosity (H_o), and expected heterozygosity (H_E) at each locus using ARLEQUIN 1.1. CERVUS version 2.0 will be used to estimate the value of the Polymorphic Information Content (PIC),

Mitochondrial DNA sequences will be edited and aligned using Autoassembler software (Applied Biosystem, USA). The edited sequences will be aligned using CLUSTALX and visually checked. Initial comparison, measures of variability and phylogenetic analysis of the identified sequences will be performed using different approaches in various softwares like MEGA,. Measures of population genetics parameters such as gene diversity and nucleotide diversity will be estimated from the mtDNA dataset using ARLEQUIN 1.1.

4.2 METHODOLOGY FOR ASSISTED REPRODUCTIVE TECHNOLOGIES

1. Anaesthesia / Restraining

Standardisation of anaesthetic procedure in Rhino and other ungulate species is a critical part in the development of assisted reproductive techniques. In Rhino, opioid drugs like Etorphine HCl (Hildebrant et al., 2007), Carfentanil, etc. could be used to sedate and naltrexone would be used to overcome the anaesthesia induced by Ethorphine HCl. In ungulate species, initial attempts will be made with a combination of xylazine HCl and ketamine HCl. If the desirable anaesthetic effect is not achieved then opioid drugs would be used in these proposed ungulate species.

Restraining chute would be used in the case of Rhino, to avoid any accidents to the handling personnel.

2. Collection and evaluation of semen from Rhino, Swamp deer, Hangul and Sangai deer

Semen would be collected by electroejaculation from Rhino, Swamp deer, Hangul and Sangai deer. The procedure requires standardization with respect to the positioning of electroejaculator probe in the rectum, such that the prostrate is stimulated to achieve successful erection and ejaculation.

The semen collected would be 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). Morphology of spermatozoa would be assessed by phase contrast microscopy 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 cryopreservation.

3. **Collection and evaluation of semen from Silver and Ring-necked pheasants and White-backed vultures**

Semen from the Silver and Ring-necked pheasants would be collected using the massage method (Umapathy et al., 2005). Before semen collection, the bird would be captured using a sweep-net, manually restrained, and the head and beak covered with a cotton sock that could be easily slipped over the head and bill and fastened around the back of the head. A team of 2-3 people would be required for the collection of semen so as to hold the limbs, head, and wings respectively. The bird's back would be massaged gently from midabdomen to ent for 1 or 2 min. Following the massage, the bird may respond by everting its cloaca which would then be grasped and the semen collected using a glass funnel.

Immediately after semen collection, the neat ejaculate would be evaluated for semen volume, pH, sperm concentration, percentage motile spermatozoa, and percentage normal/ abnormal spermatozoa. Ejaculate volume would be estimated by aspirating the semen into a calibrated positive-displacement pipette, pH by using pH indicator strips (Qualigens Fine Chemicals, Glaxo India Ltd., Mumbai, India), and concentration of sperm using a Makler chamber as described by Sontakke et al. (2004). The percentage motile spermatozoa would be determined using a phase-contrast microscope at 400 magnification, and a minimum of four separate fields would be examined. For sperm morphology studies, 2 ml of neat semen would be fixed in 100 ml of 0.5% gluteraldehyde, smeared on a glass slide, and observed under the microscope (400 X). Approximately 100 spermatozoa per ejaculate would be analyzed for sperm pleiomorphism.

4. Selection of an optimum buffer for semen analysis

Various buffers such as Tyrodes buffer, modified Tyrodes buffer, 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.

5. Computerized analysis of the motility parameters of spermatozoa

Computer aided motility analysis of spermatozoa facilitates rapid analysis of various motility parameters which were hitherto not possible or were 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).

6. Induction of ovulation in Rhino, musk deer, red panda, snow leopard and lion-tailed macaque

A commercially available kit, CRESTAR (Intervet, Boxmeer, The Netherlands) 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).

Induction of ovulation in Rhino could be achieved by using Rhino OvuPlant (subcutaneous application). This requires a small surgical incision caudo-ventral to the ear to penetrate the rhinoceros dermis. Due to the extreme thickness of the dermis in rhinoceros and associated difficulties to access the subcutis the applied implants may not be removed.

7. **Ultrasonographic monitoring of ovarian follicular response to various hormone regimen**

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 hormonal regimens would be evaluated. Based on the effectiveness of the protocols the best hormone-treatment protocol would be used for estrus induction in the endangered wild animals.

8. **Artificial insemination in Rhino, musk deer, red panda, snow leopard and lion-tailed macaque**

Based on the ovarian status and ovarian follicular response to hormone treatment as monitored by ultrasonography, 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.

9. **Artificial insemination in Silver and Ring-necked pheasants and White-backed vulture**

Each female would be restrained manually and inseminated intracloacally with freshly collected semen diluted in TALP such that the sperm number is approximately 250–300 million spermatozoa with 70–80% motility per insemination. In these experiments at least five females would be inseminated and the semen would be pooled from males to achieve the concentration and volume required for insemination. A human insemination catheter (Gynetics Medical Products, N.V. Hamont-Achel, Belgium) attached to a Gilson-P100 pipette or a 1 ml syringe would be used for transferring the semen in to the cloaca. Before insemination, the females

would be stimulated by cloacal massage to facilitate opening of the cloaca. After the eggs are laid, they would be allowed to be incubated by the respective female till hatching. After 12–15 days of incubation, eggs would also be candled to ascertain fertilization and embryo development (Penfold et al., 2001).

10. Analysis of fecal steroids to monitor cyclicity of the animals

Fecal samples would be collected on alternate days from animals from various Indian zoos subjected to gonadotropin-induced ovulation and naturally ovulating animals. The fecal samples would be collected in morning hours in vials containing 95% methanol and would be stored at -20^0C until further analysis. Samples from at least four individuals from each species and in each sex would be collected for two-year period. Fecal samples would be dried in oven at 60^0C. 90% ethanol would be added to about 0.2 gm of dried fecal sample. . Samples would be vortexed and boiled in a water bath for 15- 20 min. After centrifugation at 1000 rpm for 10-15 minutes, supernatant would be recovered and the pellet would be resuspended in 3 ml of 90% ethanol, vortexed for 1 min and centrifuged. Both ethanol supernatants would be combined, dried completely and reconstituted in 1 ml of absolute methanol. Methanol extract would be vortexed (1 min), sonicated (30 sec) and revortxed prior to decanting into a plastic tube for storage at -30^0C. The efficiency of steroid extraction from feces of each species would be evaluated by adding radio labeled hormones to a subset of fecal samples prior to boiling the extracts. The mean recoveries would be estimated to find efficiency of fecal extraction and loss during the process. ELISA developed at LaCONES would be used for assaying fecal progesterone, estradiol and testosterone.

Estradiol and progesterone (metabolites) would also be measured in duplicates 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 is 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.

4.3 METHODOLOGY FOR DNA BASED DIAGNOSIS TECHNOLOGIES

1. Extraction of RNA and cDNA synthesis

Total RNA would be extracted from blood cells, tissue, nasal swab using TRIzol (Invitrogen, USA) and cDNA would be synthesized using the High capacity cDNA archive kit (Applied Biosystems Inc., USA) following manufacturer's instructions.

2. **Multiplex PCR**

Multiplex PCR would be established using different sets of primers in same reaction tube. This mPCR assay will target conserved genes of infectious agents. This assay will help in detecting infectious agents which would produce different product size in agarose gel. Validation of sensitivity and specificity of mPCR assays would be carried out.

3. **Real time–PCR**

Real time PCR assay would be standardised using SYBR green master mix (Applied Biosystems Inc., USA). This study will help in understanding of virus/bacterial load in infected samples.

4. **Detection of hematozoan infection through microscope analysis (Giemsa stain)**

Hematozoan infection like (*Hepatozoon, Babesia* and *Theileria*) would be detected in blood smear using Giemsa stain. Blood smears would be air dried and then fixation of smears would be done using methanol. Staining of blood smear would be carried out with diluted Giemsa stain (1:20, vol/vol) for 20 min and observed under microscope.

4.4 METHODOLOGY FOR CRYOBANKING

1. **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. 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 37^0C. 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: 24^0C to 4^0C @ 1^0C /min and subsequently from 4^0C to -80^0C @ 6^0C /min. Finally, the straws would be plunged and stored in liquid nitrogen. Two to three days later, the straws would be thawed in a 37^0C water bath for one minute and evaluated for post thaw sperm motility.

2. **Cryopreservation of Cumulus oocyte complexes (COCs)**

Open pulled straws (OPS) would be prepared as described earlier in our laboratory (Rao et al., 2011). Briefly, French mini straws slightly melted over a flame would be

hand pulled to achieve a diameter that was half of its original diameter. The straws would then be held in air for a few seconds prior to cutting at the narrowest point of the pulled portion. During the last 10 s of exposure to vitrification solution–II (VS-II), open end of the pulled straw would be placed on the surface of the third droplet. The COCs enter the straw by capillary action.

3. **Warming of vitrified immature oocytes**

A four-well tissue culture plate containing 1.2 ml of warming solutions -I and -II (WS-I and WS-II) in first two wells and the holding medium in the other two wells would be used to remove the cryoprotectives from thawed COCs. For warming, OPS with COCs would be taken out of LN_2 and the open end of the OPS immersed vertically in WS-I solution. The vitrification medium liquefied within 2–4 s and released into the WS-I. After one minute, they were transferred into WS-II solution for 5 min. Finally they were washed twice in holding medium for 5 min each.

4. **Source of ovaries and oocyte collection**

The methods used for collection of testicles, ovaries, sperm and oocytes, and *in vitro* maturation and fertilization and cryopreservation of oocytes would be followed as established earlier in our laboratory (Rao et al., 2010; 2011; Uma Mahesh et al., 2011). Ovaries/testicles would be collected from the wild/endangered animals at postmortem as early as possible to avoid postmortem changes in the ovaries/testicles. The ovaries/testicles would be transported to the laboratory in prewarmed (35°C) Dulbecco's phosphate buffered saline supplemented with antibiotics. After reaching the laboratory, each ovary would be examined for the presence of visible follicles and corpora lutea for cyclicity of the animal. Cumulus-oocyte complexes (COCs) would be recovered usinig slicing method. COCs with more than three layeres of cumulus cells would be considered as culture grade COCs and selected for cryopreservation.

5. **IVM of cumulus oocyte complexes**

Culture grade COCs would be washed extensively in fresh droplets of handling medium followed by IVM medium and finally transferred into a 4-well plate containing 500 µl of pre-equilibrated IVM medium. The medium would be overlaid with equilibrated mineral oil and cultured in an incubator with 5% CO_2 under humidified air at 38.5°C for 24 h to 30 h as per the species.

6. **Evaluation of oocytes following IVM**

After IVM oocytes would be examined for cumulus cell expansion and those oocytes exhibiting cumulus cell expansion would be used for either in vitro fertilization or

assessment of nuclear maturation. Subsequently, the oocytes will be denuded of cumulus cells by treating with hyaluronidase (100 IU/ml) for 15 min and repeatedly passing through a fire polished narrow bore glass pipette and examined for extrusion of the 1st polar body. Denuded oocytes will be stained with 5 µg/ml HOECHST 33342 for 20 min and permeabilized in 3.7% paraformaldehyde and 1% Triton-X100, and then placed in 0.3% polyvinylpyrrolidone for 15 min at room temperature. The oocytes will subsequently be incubated overnight at 4°C in D-PBS supplemented with 90% glycerol. oocytes will then be examined using a fluorescence microscope for nuclear stages. Nuclear maturation is defined as the number of oocytes that matured to the MII stage and those oocytes arrested at the GVBD stage or only progressed to MI will be considered as immature.

7. IVF and culture of embryos

Oocytes collected from ovaries of dead animals would be denuded, allowed to mature and subjected to IVF with spermatozoa collected from either cauda epididymis of a dead animal or from the electroejaculate obtained from a live animal. The motile fraction of spermatozoa would be collected by swim-up method and resuspended in fertilization medium. Concentration of spermatozoa will be then adjusted to 2 x 10^6/ml by diluting in fertilization medium. Five microlitres of the sperm suspension (equivalent to 10^4 spermatozoa) would then be transferred into each of the 75 µl fertilization droplets (containing 10 partially denuded *in vitro* matured oocytes), over laid with mineral oil in 35 mm tissue culture dishes and incubated for 12 h at 38.5°C in a 5% CO_2 humidified atmosphere. Following oocyte-sperm co-incubation, the oocytes with sperm attached to zona pellucida will be washed with modified synthetic oviductal fluid and cultured for another 7 days at 38.5°C in 5% CO_2 under humidified air. Development of the embryos will be assessed every 24 h.

8. Cryobanking of fibroblast cultures of endangered animals

Fibroblast cultures from endangered animals such as 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 genetics and resurrection of the species.

9. Genome banking

A genome bank consisting of DNA isolated from endangered species would be maintained at LaCONES in duplicates at -70^0C. LaCONES already has a bank of almost hundred different endangered animals.

Animal requirement and source

Animal	Males required	Females required	Source Zoo
Rhino	3	6	Guwahati or Patna Zoo (CZA is requested to coordinate)
Red panda	3	6	Darjeeling and Sikkim zoos (CZA is requested to coordinate)
Snow leopard	3	6	Darjeeling and Sikkim zoos (CZA is requested to coordinate)
Lion-tailed macaque	3	6	Mysore and Chennai zoos (CZA is requested to coordinate)
Musk deer	3	6	Uttarakhand zoo (CZA is requested to coordinate)

5. JUSTIFICATION / OUTCOME / EXPECTED BENEFITS

The anticipated results of the project would help conservation of wild and endangered animals as follows:

1. Genetic polymorphism studies in the endangered musk deer, Manipur–brow antlered deer, tragopans, monal, lion-tailed macaque, golden langur, clouded leopards, Rhino, pygmy hog etc. would help in ascertaining the degree of polymorphism in designing breeding programmes.
2. Evolutionary, molecular systematic and phylogenetic studies would help to establish the evolutionary relationships in the closely related animals.
3. Assisted reproduction would help in *ex situ* breeding of pheasants, vultures, Rhino, , musk deer, snow leopard, red panda and lion-tailed mcaque.
4. Assisted reproduction would also help in creating a healthy captive population and reintroduction to the native habitat.
5. Non-invasive monitoring of reproductive status in Rhinos, Manipur–brow antlered deer, musk deer, snow leopard, red panda, lion-tailed macaque, etc. based on fecal steroid analysis would be of great help to establish the fertility status, ovarian status and pregnancy of captive and wild animals and in planned breeding programmes.
6. DNA based diagnostic methods would be available for the diagnosis and therapy of various bacterial, viral and protozoan diseaseses in wild animals.
7. Cryobanking of genes, tissues, gametes, ovaries, testes and embryos would thus provide an holistic approach towards preservation of the germplasm of endangered animals.
8. The cryopreserved bioresources could be used in the future for comparing genetic polymporphism with the existing populations and also used for somatic cell nuclear transfer or inter-species nuclear transfer thus satisfying the long term goal of LaCONES in wildlife conservation.
9. Manpower development in colleges, universities and institutes and NGOs for undertaking research related to conservation of endangered animals through workshops, lecture series and collaborative research activities.

Overall it is anticipated that the results of the various genetic studies of the endangered animals would highlight the degree of biological relatedness, the level of inbreeding, if any, and resolve phylogenetic issues, if any. 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.

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7. BUDGET ESTIMATES

A. NON-RECURRING

(Rs. in Lakhs)

S. No.	Item	Year 1 2012-2013	Year 2 2013-2014	Year 3 2014-2015	Year 4 2015 – 2016	Year 5 2016 - 2017	Total
1.	Minor equipment such as gel rocker, microwave oven, field microscope, pH meter, printer, vortex mixer, lap top computer, balance, magnetic stirrer, pipettes,refridgerator etc.	2.00	1.50	1.50	1.00	1.00	7.00
	Sub Total (A1)	**2.00**	**1.50**	**1.50**	**1.00**	**1.00**	**7.00**

Total of A = Rs. 7.00 Lakhs

B. RECURRING

B1. Manpower

(Rs. in Lakhs)

S. No	Position	No. of posts	Scale (Rs.)	Year 1 2012-2013	Year 2 2013-2014	Year 3 2014-2015	Year 4 2015 – 2016	Year 5 2016 - 2017	Total
1.	Helper	1	7,000*	0.84	0.84	0.84	0.84	0.84	4.20
2.	Keeper	1	7,000*	0.84	0.84	0.84	0.84	0.84	4.20
3.	Research Associate	2	22,000 + 30% HRA	6.86	6.86	6.86	7.18**	7.18**	34.94
4.	Senior Project Assistant	3	18,000*	6.48	6.48	6.48	6.48	6.48	32.40
5.	Junior Project Assistant	8	16,000*	15.36	15.36	15.36	15.36	15.36	76.80
6.	Laboratory / Field Assistant	2	10,000*	2.40	2.40	2.40	2.40	2.40	12.00
7	Consultant	1	50000*	6.00	6.00	6.00	6.00	6.00	30.00
	Total	17		38.78	38.78	38.78	39.10	39.10	194.54

Sub-Total (B1) = Rs. 194.54 Lakhs

* **Consolidated salary per month.**

** **Rs. 23,000 + 30% HRA per month.**

B2. Consumables

(Rs. in Lakhs)

S. No	Item	Year 1 2012-2013	Year 2 2013-2014	Year 3 2014-2015	Year 4 2015 – 2016	Year 5 2016 - 2017	Total
1	Molecular biologicals	12.00	12.00	12.00	12.00	12.00	60.00
2	Animal feed (5 leopards and 5 deers)	5.00	5.00	5.00	5.00	5.00	25.00
	Total	**17.00**	**17.00**	**17.00**	**17.00**	**17.00**	**85.00**

Sub-Total (B2) = Rs. 85.00 Lakhs

B3. Other Items

(Rs. in Lakhs)

Item	Year 1 2012-2013	Year 2 2013-2014	Year 3 2014-2015	Year 4 2015 – 2016	Year 5 2016 - 2017	Total
(i) Travel in India including TA/DA etc.	5.00	5.00	5.00	3.00	2.00	20.00
(II) Travel for conferences abroad	0	1.00	1.00	1.00	1.00	4.00
(III) In house Workshop/Conference /Lectures etc	2.00	2.00	2.00	2.00	2.00	10.00
(IV) Miscellaneous for stationary, postage, contingencies etc.	0.50	0.50	0.50	0.50	0.50	2.50
Total	**7.50**	**8.50**	**8.50**	**6.50**	**5.50**	**36.50**

Sub-Total (B3) = Rs. 36.50 Lakhs

Total B = (B1 to B3) = (Rs. 194.54 + 85.00 + 36.50) = Rs. 316.04

GRAND TOTAL (A + B) = (Rs. 7.00 + 316.04) = Rs. 323.04 LAKHS

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C. BUDGET AT A GLANCE (FOR FIVE YEARS)

(Rs. in Lakhs)

S. No	Item	Year 1 2012-2013	Year 2 2013-2014	Year 3 2014-2015	Year 4 2015–2016	Year 5 2016-2017	Total
	Non-Recurring						
A1	Equipment	2.00	1.50	1.50	1.00	1.00	**7.00**
	Recurring						
B1	Manpower	38.78	38.78	38.78	39.10	39.10	194.54
B2	Consumables	12.00	12.00	12.00	12.00	12.00	60.00
	Feed	5.00	5.00	5.00	5.00	5.00	25.00
B3	(I) Travel in India	5.00	5.00	5.00	3.00	2.00	**20.00**
	(II) Travel to conferences abroad	0	1.00	1.00	1.00	1.00	4.00
	(III) In house Workshop/Conference/Lectures etc	2.00	2.00	2.00	2.00	2.00	10.00
	(IV) Miscellaneous expenses	0.50	0.50	0.50	0.50	0.50	2.50
	TOTAL	**65.28**	**65.78**	**65.78**	**63.60**	**62.60**	**323.04**

GRAND TOTAL = Rs. 323.04 LAKHS

ROUNDED OFF TO = Rs. 323.00 LAKHS

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

A1. 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. Manpower

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 (2 posts)

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

Research Associate (2 positions)	Justification
One	One in the area of Molecular biology / Biotechnolgy for undertaking research activity and supervising the work in the field along with the scientists
One	One in the area of Reproductive biology/ Biotechnology for undertaking research activity and supervising the work in the field along with the scientists

4. Senior Project Assistants (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.

Senior Project Assistants (3 Positions)	Justification
One	For genetic polymorphism and evolutionary studies of the Indian deer, Pheasants, primates, Clouded leopards, Rhino, Pygmy hog.etc
One	For assisted reproduction in Rhino, Musk deer, Red panda, Snow leopard and Lion-tailed macacque
One	For testis/ovary tissue xenografting, hormone analysis of xenografted animals, molecular analysis, immune-histochemistry and histology.

5 Junior Project Assistants (8 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. The specific projects in which they would be involved are:

Junior Project Assistants (8 postions)	Justification
One	For genetic polymorphism and evolutionary studies of the Indian deer, Pheasants, primates, Clouded leopards, Rhino, Pygmy hog.etc
Two	For assisted reproduction in Rhino, Musk deer, Red panda, Snow leopard and Lion-tailed macacque including artificial insemination, embryo transfer, follicular dynamics by ultrasonography,
Two	Non-invasive monitoring of reproductive status in Rhino, Musk deer, Brow- antelered deer, Red panda, Snow leopard and Lion-tailed macacque based on fecal steroid analysis. (Some time would also be devoted to *in vitro* fertilization, artificial insemination, embryo transfer)
One	In vitro fertilization, Cryobanking of eggs and embryos
Two	Development of multiplex and real time PCR based detection of viral (Panleucopenia virus), bacterial (Tuberculosis) and protozoon diseases .

6. Laboratory / Field Assistant (2 posts)

Candidates with an M.Sc. degree in biology and with interest in field work and lab work, would be required to carry out routine lab activities and also assist all the staff in the lab and field. The two assistants would be absolutely essential during field work.

7. Consultant (1 post)

Candidates with a veterinary degree and minimum of ten years experience in the handling, upkeep and treatment of endangered animals would be absolutely essential for taking care of the animals at LaCONES, to attend to routine health check, feeding supervision and also assist the staff in field in tranquilisation.

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, Training Programmes and Miscellaneous expenses

1. Travel in India

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

2. Travel Abroad

To attend conferences/ workshops/ symposia etc. outside India by scientist from LaCONES. To meet TA, DA and other expenses.

3. Training Programmes / Workshops / Lectures

Training programmes for Zoo personnel, forest personnel, staff and students from colleges, universities and institutes and NGOs for undertaking research related to conservation of endangered animals through workshops, lecture series and collaborative research activities.

4. Miscellaneous for stationary, postage, contingencies etc

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

9. PROJECT IMPLEMENTATION

9.1 ORGANISATION OF WORK ELEMENTS

Year 1

1. Initiate studies on genetic polymorphism in musk deer, Manipur–brow antlered deer, tragopans, monal, lion-tailed macaque, golden langur, Rhino, pygmy hog etc..
2. Collection of fecal samples for evaluating ovarian cyclicity and fertility status Rhinos, Elephant, Manipur–brow antlered deer, Hangul, etc. based on fecal steroid analysisInitiate studies on induction of ovulation and artificial insemination in Rhino, Swamp deer, Hangul and Sangai deer.
3. Development of DNA based detection of viral and bacterial and diseases.
4. Initiate studies on cryobanking of gametes and embryos.
5. Training programmes for Zoo personnel, forest personnel, staff and students from colleges, universities and institutes and NGOs for undertaking research related to conservation of endangered animals through workshops, lecture series and collaborative research activities.

Year 2

1. Continue studies on genetic polymorphism in musk deer, Manipur–brow antlered deer, tragopans, monal, lion-tailed macaque, golden langur, Rhino, pygmy hog etc..
2. Initiate genetic polymorphism studies in clouded leopard.
3. Continue evaluating ovarian cyclicity and fertility status in Rhino, Elephant, Manipur–brow antlered deer, Hangul, etc. based on fecal steroid analysis and ultrasound.
4. Continue studies on induction of ovulation and artificial insemination in Rhino, Swamp deer, Hangul and Sangai deer.
5. Initiate studies on *ex-situ* conservation of silver and ring necked pheasants by artificial insemination.
6. Continue DNA based detection of viral and bacterial and diseases.
7. Continue studies on cryobanking of gametes and embryos and devlop fibroblast cultures of endangered animals.
8. Training programmes for Zoo personnel, forest personnel, staff and students from colleges, universities and institutes and NGOs for undertaking research related to conservation of endangered animals through workshops, lecture series and collaborative research activities.

Year 3

1. Continue studies on genetic polymorphism in musk deer, Manipur–brow antlered deer, tragopans, monal, lion-tailed macaque, golden langur, clouded leopards, Rhino, pygmy hog etc..
2. Complete evaluating ovarian cyclicity and fertility status in Rhinos, Elephant, Manipur–brow antlered deer, Hangul, etc. based on fecal steroid analysis
3. Continue studies on induction of ovulation and artificial insemination in Rhino, Swamp deer, Hangul and Sangai deer .
4. Continue studies on *ex-situ* conservation of Indian silver and ring necked pheasants by artificial insemination.
5. Complete DNA based detection of either viral or bacterial disease.
6 Training programmes for Zoo personnel, forest personnel, staff and students from colleges, universities and institutes and NGOs for undertaking research related to conservation of endangered animals through workshops, lecture series and collaborative research activities.

Years 4 and 5

1. Complete studies on genetic polymorphism in musk deer, Manipur–brow antlered deer, tragopans, monal, lion-tailed macaque, golden langur, clouded leopards, Rhino, pygmy hog etc.
2. Complete s tudies on induction of ovulation and artificial insemination in Rhino, Swamp deer, Hangul and Sangai deer.
3. Complete studies on on *ex-situ* conservation of silver and ring necked pheasants by artificial insemination.
4. Complete DNA based detection of either viral or bacterial disease.
5. Complete development of multiplex PCR for simultaneous diagnosis of multiple diseases.
6. Training programmes for Zoo personnel, forest personnel, staff and students from colleges, universities and institutes and NGOs for undertaking research related to conservation of endangered animals through workshops, lecture series and collaborative research activities.

9.2 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. Training programmes for Zoo personnel, forest personnel, staff and students from colleges, universities and institutes and NGOs for undertaking research related to conservation of endangered animals through workshops, lecture series and collaborative research activities.

9.3 TIME SCHEDULE OF ACTIVITIES GIVING MILESTONES

S.No.	Name of Milestone	Expected date	
		Initiation	Completion
A.	**Equipment purchase**	Immediately after sanction	Before 31st March, 2013
B.	**Recruitment of staff**	Immediately after sanction	Within two months of sanction
C.	**Research Activity**		
1	Initiate studies on genetic polymorphism in Rhino, Swamp deer, Hangul and Sangai deer	Immediately after sanction	By the end of project
2.	Collection and processing of biological material for genetic polymorphism in the clouded leopard	One year after sanction	By the end of 3 years
3.	Collection of fecal samples for evaluating ovarian cyclicity and fertility status in Rhinos, Elephant, Manipur–brow antlered deer, Hangul, etc. based on fecal steroid analysis	Three months after sanction	By the end of 3 years
4.	Initiate studies on induction of ovulation and artificial insemination in Rhino, Swamp deer, Hangul and Sangai deer	Three months after sanction	By the end of the project
5.	Development of DNA based detection of viral and bacterial and diseases	Three months after sanction	By the end of the project
6.	Initiate studies on cryobanking of gametes and embryos	Three months after sanction	By the end of 3 years
7.	Initiate studies on *ex-situ* conservation of silver and ring necked pheasants by artificial insemination	Initiate in year 2 or 3	By the end of the project
	Complete development of multiplex PCR for simultaneous diagnosis of multiple diseases	Start in year 5	By the end of the project

9.4 PROJECT IMPLEMENTING AGENCY

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

10. OUTCOME / EXPECTED BENEFITS

The anticipated results of the project would help conservation of wild and endangered animals as follows:

1. Gnetic polymorphism studies in clouded leopards, Rhino, Swamp deer, Hangul and Sangai deer would help in ascertaini ng the degree of polymorphism in designing breeding programmes.
2. Evolutionary, molecular systematic and phylogenetic studies would help to establish the evolutionary relationships in the closely related animals.
3. Assisted reproduction would help in *ex situ* breeding of pheasants, Rhino, Swamp deer, Hangul and Sangai deer.
4. Assisted reproduction would also help in creating a healthy captive population and reintroduction to the native habitat.
5. Non-invasive monitoring of reproductive status in Rhinos, Elephant, Manipur–brow antlered deer, Hangul, etc. based on fecal steroid analysis would be of great help to establish the fertility status, ovarian status and pregnancy of captive and wild animals and in planned breeding programmes.
6. DNA based diagnostic methods would be available for the diagnosis and therapy of various bacterial, viral and protozoan diseaseses in wild animals.
7. Cryobanking of genes, tissues, gametes, ovaries, testes and embryos would thus provide an holistic approach towards preservation of the germplasm of endangered animals.
8. The cryopreserved bioresources could be used in the future for comparing genetic polymporphism with the existing populations and also used for somatic cell nuclear transfer or inter-species nuclear transfer thus satisfying the long term goal of LaCONES in wildlife conservation.
9. Manpower development in colleges, universities and institutes and NGOs for undertaking research related to conservation of endangered animals through workshops, lecture series and collaborative research activities.

Overall it is anticipated that the results of the various genetic studies of the endangered animals would highlight the degree of biological relatedness, the level of inbreeding, if any, and resolve phylogenetic issues, if any. 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.

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
2	Refrigerator
3	CO_2 incubator
4	Laminar flow hood
5	Electronic balance
6	Microfuge
7	Field camera
8	Generator portable
9	UPS portable
10	Dart gun, pistol and related equipment for anaesthising animals
11	Electroejaculator plus probes
12	Phase contrast microscope with camera
13	Micropipettes
14	Cryogenic containers
15	Ultrasound scanner
16	Suction pump
17	Oocyte aspiration set
18	Catheters
19	Motility analyzer
20	Incubator (ordinary)
21	Refrigerated microfuge
22	Personal centrifuge
23	Computation facility
24	Power pack
25	Microwave oven
26	Trangamete portable incubator
27	Inverted microscope
28	Millipore filtration units with pump
29	Animal holding facility and cages for big cats

30	Thermal cycler
31	Spectrophotometer
32	-70oC freezer
33	Ultracentrifuge
34	Laminar flow
35	GC machine
36	Precision balance
37	Hot air oven
38	Autoclave
39	Fluorescent microscope
40	Phase cóntrast microscope with photomicrography
41	Bacteriological incubator
42	Shaking water bath
43	Incubator shaker
44	CO_2 incubator
45	Electrophoretic apparatus with blot, elution, vertical, horizontal, scanner and computer
46	UV transilluminator
47	Ice flaker
48	DNA sequencer
49	HPLC
50	Scintillation counter

Facilities available for use from CCMB

All other equipment required for the project such as lyophiliser liquid nitrogen facility, cell imaging, proteomics, real time PCR would be utilized 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 necessary provision for the scheme/project will be made in the Institute/University/State budget in anticipation of the sanction of the scheme/project

(d) 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

(e) 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

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

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

Dr. Ch. Mohan Rao
DIRECTOR
CENTRE FOR CELLULAR AND MOLECULAR BIOLOGY
UPPAL ROAD, HYDERABAD - 500 007

Signature of Principal Investigator (1)
Name : Ch Mohan Rao
Date : January 16, 2012

Signature of Principal Investigator (2)
Name : S. Shivaji
Date : January 16, 2012

PART VII : PROFORMA FOR BIODATA OF PRINCIPAL INVESTIGATOR 1

Name : **Ch Mohan Rao** Designation : **Director**

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

Date of Birth : 19 January, 1954 Sex (M/F) : **M** SC/ST : **No**

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

S. No.	Institution Place	Degree Awarded	Year
1.	Kakatiya University, Warangal, India	M.Sc. (Chemistry)	1977
2.	University of Hyderabad, Hyderabad	Ph.D.	1984

Research Experience in various institutions

1. 1984-1988, Scientist-C, Centre for Cellular and Molecular Biology, Hyderabad
2. 1988- 1991, Scientist-E -I, Centre for Cellular and Molecular Biology, Hyderabad
3. 1990-1992, Visiting Associate, National Eye Institute, NIH, Bethesda, MD, USA
4. 1991- 1996, Scientist E -II (Asst. Director) Centre for Cellular and Molecular Biology, Hyderabad
5. 1996, Visiting Professor, Science University of Tokyo, Noda, Tokyo, Japan
6. 1996 -2001, Scientist F (Deputy Director) Centre for Cellular and Molecular Biology, Hyderabad
7. 2001 – 2009, Scientist G (Director-Grade Scientist), Centre for Cellular and Molecular Biology, Hyderabad
8. 2001, Visiting Scientist, University of Texas Medical Branch, Galveston, USA
9. 2002, Visiting Professor, Institute for Protein Research, Osaka University, Osaka, Japan

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

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

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

List of important publications

1. Sankaralingam Prabhu, Volety Srinivas, Tangirala Ramakrishna, Bakthisaran Raman and Ch. Mohan Rao: Inhibition of Cu^{2+}-mediated generation of reactive oxygen species by the small heat shock protein, αB-crystallin *Free Radical Biology and Medicine,* doi.org/10.1016/j.freeradbiomed. 2011.05.021
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Ongoing Research Projects

Sl No.	Title of Project	Funding Agency	Amount	Date of sanction and Duration
1.	Devpt of novel sensitizers based on NIR dyes for photo dynamic therapy (multi-institutional- NIIST, IISc., RGCB & CCMB)	DST	1500.00 lakhs	March 2008 – five years
2.	Devpt of DNA macrochips for simultaneous detection of pathogens causing AES septicaemia and antibiotic resistance	NMITLI-CSIR	676.47 lakhs 470.47 lakhs (addl. Fund – Mar'11)	March 2008 – three years
3	Nanotechnology for biomedical applications	DST	1173.00 lakhs	March 2009
4	Joint R&D nanomedicine & cellular engineering (CCMB and Purdue University)	Indo-US Science & Technology Forum	50.6 lakhs	Jan 2010

Completed Research Projects

Sl No.	Title of Project	Funding Agency	Amount	Date of completion
1.	NMITLI - Eye and Vision (multi-institutional)	CSIR	300.00 lakhs	2009
2.	DNA based diagnostics using capacitance monitoring	DST	30.00 lakhs	2010
3	Prion-protein	IFCPAR (Indo-French)	45.00 lakhs	2007

Place : Hyderabad

Date : January 16, 2012

Signature of Principal Investigator 1

(Ch Mohan Rao)

Dr. Ch. Mohan Rao

DIRECTOR

CENTRE FOR CELLULAR AND MOLECULAR BIOLOGY

UPPAL ROAD, HYDERABAD - 500 007

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PART VII : PROFORMA FOR BIODATA OF PRINCIPAL INVESTIGATOR 2

Name : **S. Shivaji** Designation : **Scientist G (Director-grade Scientist)**

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

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.
October, 1989	Visiting Scientist at Max-Planck-Institut fur Biophysikalische Chemie, Gottinggen, West Germany.
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)* : 247

Books : **2** Research Papers, Reports : 232 General articles : **5**

List of important publications relevant to the proposed area of work

1. Rao, B.S., Uma Mahesh, Y., Venu Charan, K., Suman, K., Sekhar, N. and Shivaji, S. (2012) Effect of vitrification on meiotic maturation and expression of genes in immature goat cumulus oocyte complexes. Cryobiology, In press.
2. Sontakke, S.D., Patil, M.S., Lakshmikantan, U. and Shivaji, S. (2011) Ultrasonographic characterization of ovarian follicular development in the Indian blackbuck antelope (*Antilope cervicapra*). Small Rumi. Res. In press.
3. Reddy, P.A., Gour, D.S., Bhavanishankar, M., Jaggi, K., Hussain, S.M., Harika, K. and Shivaji, S. (2011) Genetic evidence of tiger population structure and migration within an isolated and fragmented landscape in Northwest India. Plos One. In press.
4. Uma Mahesh, Y., Rao, S.B., Venucharan, K., Suman, K., Devassy, C., Lakshmikantan, U., Pawar, R.M. and Shivaji, S. (2011) Cell cycle synchronization of *in vitro* cultured bison (*Bos gaurus*) ear fibroblasts. Reprod. Domes. Anim. In press.

5. Pawar, R.M., Poornachandar, Srinivas, P., Rao, K.R., Lakshmikantan, U. and Shivaji, S. (2011) Molecular characterization of *Hepatozoon* spp. Infection in endangered Indian wild felids and canids. Vet. Parasitol. In press.

6. Shivaji, S. (2011) Conserve biodiversity and save the earth. Geography and You, 11 : 20-21.

7. Pawar, R.M., Poornachander, A., Arun, A.S., Manikandan, S. and Shivaji, S. (2011) Molecular prevalence and characterization of Hepatozoon ursi infection in Indian sloth bears (*Melursus ursinus*). Vet. Parasitol. In press.

8. Umapathy, G., Hussain, Sk. and Shivaji, S. (2011) Impacts of habitat fragmentation on the demography of lion-tailed macaque (*Macaca silenus*) populations in the rain forests of Anamalai Hills, Western Ghats, India. Int. J. Primato. 32 : 889-900.

9. Senthil Kumar, S., Gaur, A. and Shivaji, S. (2011) Phylogenetic studies in Indian Scleractinian corals based on mitochondrial cytochrome b gene sequences. Curr. Sci. 101 : 669-676.

10. Pawar, R.M., Sasi Bhushan, S., Poornachandar, A., Lakshmikantan, U. and Shivaji, S. (2010) Avian pox infection in different wild birds in India. Eur. J. Wildlife Res. 57 : 785-793.

11. Rao, B.S., Uma Mahesh, Y., Suman, K., Venu Charan, K., Lakshmikantan, U., Gibence, H.R.W. and Shivaji, S. (2010) Meiotic maturation of vitrified immature chousingha (*Tetracerus quadricorni*) oocytes recovered postmortem. Cryobiology, 62 : 47-52.

12. Uma Mahesh, Y., Rao, B.S., Suman, K., Lakshmikantan, U., Venu Charan, K., Gibence, H.R.W. and Shivaji, S. (2010) *In vitro* maturation and fertilization in the nilgai (*Boselaphus tragocamelus*) using oocytes and spermatozoa recovered postmortem. Reprod. Domes. Anim. 10.1111/j.1439-0531.2011.01751.x

13. Reddy, P.A., Kumaraguru, A., Yadav, P.R., Ramyashree, A., Bhagavatula, J. and Shivaji, S. (2010) Studies to determine presence or absence of the Indian tiger (*P. tigris tigris*) in Kawal Wildlife Sanctuary, India. Eur. J. Wildlife Res. 57 : 517-522.

14. Umapathy, G. and Singh, M. 2010. Long-tailed macaques (Crab-eating macaque, *Macaca fascicularis umbrosa*) in Nicobar Islands, India. In: *Recent Trends in Biodiversity in Andaman and Nicobar Islands*. Ramakrishna, Raghunathan and Sivaperuman (Eds), Zoological Survey of India, Kolkatta 2010.

15. Umapathy, G., Hussain, Sk. and Shivaji, S. (2009) Status and distribution of vultures in Andhra Pradesh, India. Forktail, 25 : 164-166.

16. Sontakke, S.D., Patil, M.S., Umapathy, G., Rao, K.R. and Shivaji, S. (2009) Ejaculate characteristics, short-term semen storage and successful artificial insemination following synchronisation of oestrus in the Indian blackbuck antelope (*Antilope cervicapra*). Reprod. Fertil. Dev. 21 : 749-756.

17. Rao, B.S., Uma Mahesh, Y., Lakshmikantan, U., Suman, K., Venu Charan, K. and Shivaji, S. (2010) Developmental competence of oocytes recovered from post-mortem ovaries of the endangered Indian blackbuck (*Antilope cervicapra*). J. Reprod. Dev. 56 : 623-629.

18. Sontakke, S.D., Umapathy, G. and Shivaji, S. (2009) Yohimbine antagonism of ketamine-xylazine in captive Indian wild felids. Vet. Anaes. Analg. 36 : 34-41.

19. Poharkar, A., Reddy, P.A., Gadge, V.A., Kolte, S., Kurkure, N. and Shivaji, S. (2009) Is malaria the cause for the decline of a wild population of the Indian White-backed vulture (*Gyps bengalensis*). Curr. Sci. 96 : 553-557.

20. Sontakke, S.D., Patil, M.S., Umapathy, G., Ramachandra Rao, K. and Shivaji, S. (2009) Ejaculate characteristics, short term semen storage and successful artificial insemination following synchronization of oestrus in an Indian Antelope, the Blackbuck (*Antilope cervicapra*). Reprod. Fert. Dev. 21 : 749-756.

21. Sontakke, S.D., Umapathy, G. and Shivaji, S. (2009) Yohimbine antagonizes the anesthetic effect of ketamine-xylazine in captive Indian wild felids (Asitic lions, tigers and leopards). Vet. Anaes. Analg. 36 : 34-41.

22. Sontakke, S.D., Umapathy, G., Patil, M.S. and Shivaji, S. (2009) Tolazoline antagonizes ketamine-xylazine anaesthesia in an endangered Black buck (*Antilope cervicapra*). Eur. J. Wildlife Dis. 55 : 357-361.

23. Shivaji, S. (2007) From Dolly, the sheep, to cloning of an endangered animal. In : You Deserve, We Conserve : A biotechnological approach to wildlife conservation. pp. 63-72.

24. Sontakke, S.D., Reddy, A., Umapathy, G. and Shivaji, S. (2007) 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. 68 : 20-24.

25. Umpathy, G., Sontakke, S.D., Reddy, A. and Shivaji, S. (2007) Seasonal variations in semen characteristics, semen cryopreservation, estrus synchronization and successful artificial insemination in the spotted deer (*Axis axis*). Theriogenology, 67 : 1371-1378.

26. Umapathy, G., Sontakke, S.D., Srinivasu, K., Kiran, T., Kholkute, S.D. and Shivaji, S. (2007) Estrus behaviour and fecal steroid profiles in the Asiatic lion (*Panthera leo persica*) during natural and gonadotropin-induced estrus. Anim. Reprod. Sci. 101 : 313-325.

27. 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. 92 : 659-662.

28. Gaur, A., Reddy, A., Annapoorni, S., Satyarebala, B. and Shivaji, S. (2006) The origin of Indian Star tortoises (*Geochelone elegans*) based on nuclear and mitochondrial DNA analysis: a story of rescue and repatriation. Cons. Gen. DI:10-1007/6 10592-005-9002-z.

29. 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.

30. 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.

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32. Jayaprakash, D., Patil, S.B., Majumdar, K.C., Navin Kumar, M. and Shivaji, S. (2000) Semen characteristics of the captive Indian leopards, *Panthera pardus*. J. Androl. 22 : 25-33.

33. Shivaji, S., Jayaprakash, D. and Patil, S.B. (2000) Biotechnology for management of captive and free ranging endangered animals. Indian Zoo Year Book, Vol. III, pp. 20-31.

34. Patil, Suresh B., Jayaprakash, D. and Shivaji, S. (1998) Cryopreservation of semen of tigers and lions : Computerized analysis of the motility parameters of the spermatozoa. Curr. Sci. 75 : 930-935.

35. Shivaji, S., Jayaprakash, D. and Suresh B. Patil (1998) Assessment of inbreeding depression in big cats : testosterone levels and semen analysis. Curr. Sci. 75 : 923-930.

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

INTERNATIONAL PROJECTS

S.No	Title of the Project	Funding Agency	Duration From	Total Approved Cost of the Project (in Rs.)	Status
1	Polyunsaturated fatty acids and acyl-lipid desaturases of psychrotrophic cyanobacteria from Anatartica	Department of Science and Technology and Japanese Society for Promotion of Science	1999 - 2001	15.11	Completed
2	Targets for male contraception : proteins involved	Volkswagen Stiftung, Germany	1997 - 2000	17.25	Completed
3	Biodiversity and function of bacteria from Antarctica: a study of pack ice and sea water bacteria	Indo-French Centre for Promotion of Scientific Research, New Delhi	2000 - 2003	24.67	Completed
4	Genome-wide screen in two hundred sister-pairs with endometriosis	Wellcome Trust, UK	2000 - 2005	90.17	Completed
5	Molecular basis of cold adaptation : Antarctic cyanobacteria and bacteria as model systems	Department of Science and Technology and Japanese Society for Promotion of Science	2003 - 2006	10.92	Completed
6	Identification of molecular mechani for signal perception by a multi-stress sensor Hik33 in *Synechocystis* sp. PCC 6803	Department of Science and Technology	2008 – 2010	2.86	Completed
7.	Development of specific microbial consortia for the improvement of renewable bio-energy products in an Anglo-Indian partnership'	BBSRC, UK	2007-2010	7.40	Completed

NATIONAL PROJECTS

S.No	Title of the Project	Funding Agency	Duration From	Total Approved Cost of the Project (in Rs.)	Status
1	Biodegradation of human refuse by psychrotrophic microbes at low temperatures: Identification and strain improvement	Defence Research and Development Organization, Gwalior	1994 - 1996	20.70	Completed
2	Semen Analysis and Cryopreservation of Spermatozoa of Indian Wild Animals	Central Zoo Authority, New Delhi	1994 - 1997	39.22	Completed
3	Ribotyping of Plague *Bacillii*	Department of General Health Scheme (DGHS), Hyderabad	1995	2.50	Completed
4	Microbial Biodiversity of psychrophiles and psychrotrophs from cold habitats	Department of Biotechnology, New Delhi	1999 - 2004	29.79	Completed
5	Bacteria from stratosphere – Cryosampler Balloon Experiment	Inter University Centre for Astronomy and Astrophysics, Pune	2000 - 2001	2.00	Completed
6	Inventory on Microbial Resources of India	Department of Biotechnology, New Delhi	2002 - 2003	1.95	Completed
7	Conservation of endangered wild animals	Central Zoo Authority, New Delhi	1999 - 2002	446.66	Completed
8	Special Grant from CSIR for Antarctic Research	Council of Scientific and Industrial Research, New Delhi	2005	1.00	Completed
9	Bacteria from stratosphere – Cryosampler Balloon Experiment	Inter University Centre for Astronomy and Astrophysics, Pune	2004 – 2005	5.00	Completed
10	Molecular basis of cold adaptation : Antarctic cyanobacteria and bacteria as model systems	Department of Science and Technology and Japanese Society for Promotion of Science	2003 - 2006	10.92	Completed
11	National Facility for Conservation of Endangered Species of Animals (NaFCONES)	Central Zoo Authority, Department of Biotechnology, Govt. of A.P. and Council of Scientific and Industrial Research	2003 – 2008	746.787	Completed
12	Conservation of endangered wild animals	Department of Biotechnology, New Delhi	2003 – 2008	244.760	Completed
13	Bacterial biodiversity of Antarctica : a polyphasic and a rRNA approach	National Centre for Antarctic and Ocean Research, Goa	2005 - 2008	10.93	Completed
14	Bacterial diversity and bioprospecting of bacteria in Himalayan glaciers : A culture-dependent and culture-independent approach	Department of Biotechnology, New Delhi	2006 – 2009	32.343	Completed

15	Microbial biodiversity, phylogency and bioprospecting of East Antarctica	National Centre for Antarctic and Ocean Research, Goa (MOU)	2006 – 2009	21.10	Completed
16	Development of novel expression systems	NMITLI	2005 - 2008	65.00	Completed
17	Molecular mechanisms of cold acclimation in a cyanobacterium *Synechocystis* sp. PCC 6803 : Role of molecular chaperonins during cold acclimation	Department of Science and Technology (UOH)	2006 – 2009	3.6	Completed
18	Use of biotechnological interventions for conservation of endangered species of animals in the country	Central Zoo Authority	2007 – 2012	100.34	Ongoing
19	Atmosphere carbon dioxide sequestration through fertilization of a high-nutrients-low chlorophyll (HNLC) oceanic region with iron	CSIR Network Project (NIO – Nodal agency)	2007 - 2012	59.00	Ongoing
20	Exploitation of India's rich microbial diversity	CSIR Network Project (IMTECH – Nodal agency)	2008 - 2012	145.20	Ongoing
21	Project on conservation of endangered species	CSIR Network Project	2008 – 2012	132.65	Ongoing
22	Estimating the number of wild tigers in tiger reserves in India by DNA profiling of fecal samples	Department of Biotechnology, New Delhi	2008 – 2011	88.87	Completed
23.	Biological effects of Microgravity	National Microgravity Research Programme, ISRO	2008 – 2011	41.86	Completed
24.	Genetic studies on the phylogeography and population diversification in Himalayan finches	Department of Science and Technology, New Delhi	2008-2011	18.22	Completed
25.	Identification of molecular mechani for signal perception by a multi-stre sensor Hik33 in *Synechocystis* sp. 6803	Department of Science and Technology, New Delhi	2008 – 2010	3.18	Completed
26	DNA Barcoding of birds in India	Department of Biotechnology, New Delhi	2008-2011	70.00	Completed
27	Arctic bacteria as workhorses of biotechnology : Biodiversity of bact and bioprospecting for biomolecule	National Centre for Antarctic and Ocean Research, Goa	2009-2012	27.33	Ongoing
16.	Nanobiotechnology	CSIR Network Project	2008-2012	12.00	Ongoing
17..	Diversity of Arctic cyanobacteria	Department of Science and Technology, New Delhi	2009-2012	32.91	Ongoing

Place : Hyderabad
Date : January 16, 2012

Signature of Principal Investigator 2
(S. Shivaji)

PART VIII : LACONES SCIENTISTS WHO WOULD BE INVOLVED IN THE PROJECT

Sl.No.	Name of the scientist	Designation	Activity
1.	Dr Ch Mohan Rao	Director, CCMB	Principal Investigator 1
2.	Dr S Shivaji	Scientist-Incharge, LaCONES	Principal Investigator 2
3.	Dr Ajay Gaur	Scientist	Genetic polymorphism
4.	Dr P Anuradha Reddy	Scientist	Genetic polymorphism
5.	Dr U Lakshmikanthan	Scientist	Assisted reproduction
6.	Dr M Rahul Pawar	Scientist	Wildlife diseases
7.	Dr Sadanand D Sontakke	Scientist	Assisted reproduction
8.	Dr B Sambasiva Rao	Scientist	Cryobanking
9.	Dr Sandeep Goel	Scientist	Cryobanking
10.	Dr G Umapathy	Scientist	Assisted reproduction
11.	Dr Y Uma Mahesh	Scientist	Assisted reproduction