CONSERVATION OF ENDANGERED ANIMALS

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PROJECT COMPLETION REPORT

(APRIL, 2007 TO MARCH, 2012)

Submitted to :

Central Zoo Authority of India

Ministry of Environment and Forests, New Delhi

Laboratory for Conservation of Endangered Species (LaCONES)

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BACKGROUND, INTRODUCTION AND DEFINITION OF THE PROBLEM

Habitat destruction and poaching are known to fragment habitats and populations thus facilitating inbreeding and genetic homogenization, which has negative effects such as poor reproductive performance, low fecundity, increased juvenile mortality and susceptibility to diseases. The need for conservation is therefore immense. Habitat preservation and captive breeding are the best ways to conserve biodiversity. However, the reproduction process may be impaired in captivity due to space restriction, inadequate diet, health and husbandry problems, modified sexual behaviour or pair incompatibility, etc. so the only alternative is to develop new captive breeding strategies to improve the fertility status and the reproductive performance with the help of biotechnological approaches, which are better referred to as assisted reproduction (AR). It involves application of techniques such as : semen collection, gamete and embryo cryopreservation, oestrus induction and artificial insemination (AI) and more complex methods such as oocyte pick up (OPU), *in vitro* Fertilization (IVF), *in vitro* production of embryos (IVP), intra-cytoplasmic sperm injection (ICSI), embryo transfer (ET) and cloning. Hand in hand with AR, molecular markers based on the genetic make up of the animals needs to be developed and applied to ascertain the extent of genetic polymorphism in the surviving wildlife populations. This would help in planning captive breeding programmes, which would further facilitate maintenance of genetic heterozygosity and prevent genetic homogenization, which leads to extinction. The ultimate aim of this project would be to evaluate the genetic and fertility status of the endangered animals and develop technologies to boost their numbers

Objectives

The three main objectives are :

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

I. DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNOLOGIES FOR THE CONSERVATION OF ENDANGERED SPECIES

1. Non-surgical artificial insemination in the Indian Leopard (*Panthera pardus*)

As per the advise of the Central Zoo authority, artificial insemination in Leopards was attempted using leopards which were shifted from the Nehru zoological park, Hyderabad, to the animal holding facility in LaCONES (Fig. 1). Three males and two female Leopards were shifted to LaCONES animal holding facility and maintained in cages constructed in the LaCONES premises. Animlas were allowed to acclimatise to the environment of LaCONES cages for a few months prior to artificial insemination. Artificial insemination (AI) is the most widely used reproductive technology for conservation and genetic management of endangered species. Felids are induced ovulators, and for AI to be successful, a precise protocol for induction of oestrus and ovulation needs to be developed before insemination. Superovulation was induced in 2 females using PMSG followed by LH injection after 48 h to induce ovulation. Such animals were inseminated 96 h after LH injection.

Fig. 1. A male leopard (a) and a leopard playing in the exercise area of the cage at LaCONES (b).

Synchronized females showed oestrus discharge and ovarian follicular response to the hormone treatment as monitored by ultrasonography (Fig. 2). On the day of insemination, ultrasonographic scanning revealed that successful ovulation had occurred. The female leopards were inseminated non-surgically with freshly collected electroejaculated semen under anaesthesia and semen was deposited deep into the cervix using an

insemination catheter which was guided with a rigid endoscope and a light source. No pregnancy resulted in any of the animals. Further studies are underway to refine the protocol and insemination timing to achieve pregnancy and live birth.

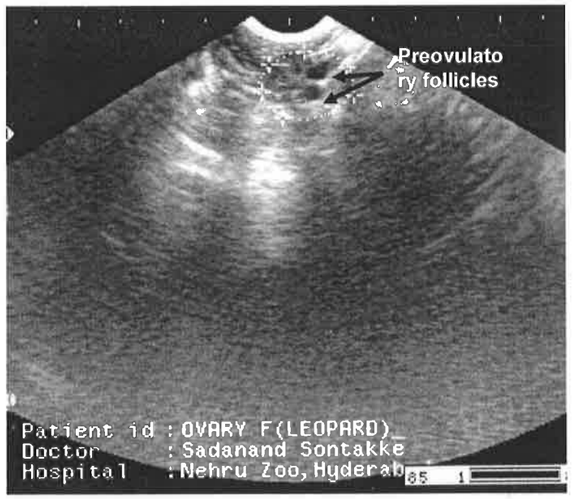

Fig. 2. Ultrasonographic detection of preovulatory follicle of leopard just prior to insemination

2. Short-term preservation of semen of endangered Indian Leopard for effective captive breeding program

Spermatozoa of wild felids are very sensitive to cold shock because of the high content of polyunsaturated fatty acids in the sperm membrane and are therefore susceptible to peroxidative damage resulting in subsequent loss of sperm motility and eventually affecting the sperm function. The maximum post-thaw sperm recovery (cryopreserved semen) so far reported in wild felids is < 40%. In such instances, fertilizing efficiency of frozen semen remains questionable for successful artificial insemination.

So ideally, in addition to the long-term semen cryopreservation, short-term storage methods would be useful especially to exchange genetic pool between zoos. However, so far, there have been no attempts of short-term semen storage in any of the wild felids. In the present study, attempts were made to assess the feasibility of such a short-term storage of semen of wild felids using Leopard (*Panthera pardus*) as a model for other wild felids in India.

Semen samples were diluted with Tris-yolk buffer (TYB) containing different concentrations of egg yolk (5, 10 and 20%) and were then placed in the refrigerator (at ~ 5°C) in a cryocontainer for 2-3 days. Sperm variables such as motility, plasma membrane

integrity and acrosomal integrity declined over a period of storage. Of the three media, after 24 h storage, TYB containing 5% and 10% egg yolk were comparable and found better than in medium containing 20% egg yolk. However, at 48 h, motility up to 45% was maintained in an extender containing 5% egg yolk as compared to only 10-20% in other concentrations of efgg yolk (Fig. 3 a-c).

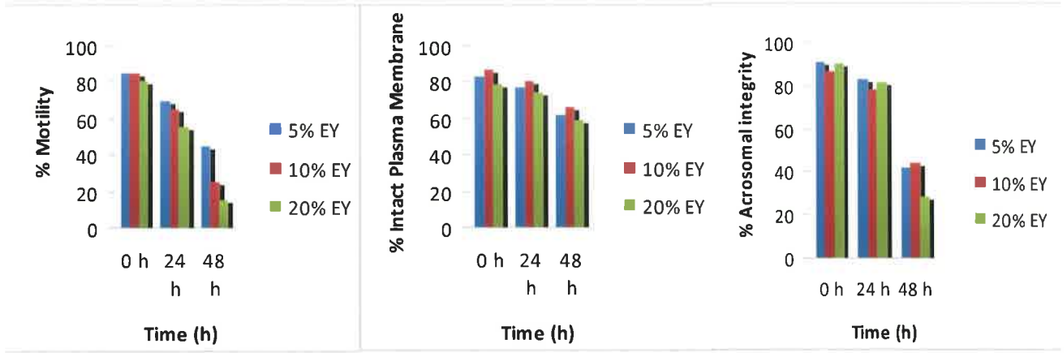

Fig. 3. Effect of short-term cold storage of Leopard semen stored in Tris buffer containing 5, 10 & 20% of egg yolk on (a) sperm motility, (b) plasma membrane integrity and (c) acrosomal integrity

3. Standardization of a protocol for induction of superovulation in spotted deer

Oocyte aspiration from live wild animals is mandatory for developing assisted reproductive techniques like *in-vitro* fertilization and nuclear transfer (cloning). The first step in this direction would be to develop a suitable and a reproducible protocol for superovulation so as to induce optimal number of oocytes and optimal size (diameter) of the follicle. Six protocols have so far been tested using either porcine FSH (Folltropin V) or ovine FSH (Ovagen). Prior to superovulation animals were oestrus synchronized using two injections of Prostaglandin $F_2\alpha$ (15 mg each on day 1 and day 10). Subsequently, porcine or ovine FSH was administered 96 h after the second Prostaglandin $F_2\alpha$ injection. All injections were scheduled at morning 6 am and evening 6 pm (12 h interval). Ultrasongraphic evaluation was then done after anaesthetising the animals with ketamine and xylazine combination 56 h after the first FSH injection for treatments 1-4 and after 92 h in the case of treatments 5 and 6.

The results revealed that use of either pFSH or oFSH for three days produces optimum size (0.5 cm) of the follicle with the required number of follicles (7 to 8) (Table 1). A large follicle is advantageous since it could be easily pierced with the ultrasound guided needle and the oocytes could also be retrieved easily. Single injection protocols did not

yield satisfactory results with respect to follicular size. In conclusion, FSH of either ovine or porcine origin could be used successfully for inducing superovulation in the spotted deer.

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Table 1. Number of follicles and follicular size in spotted deer following induction of superovulation using either porcine or ovine FSH

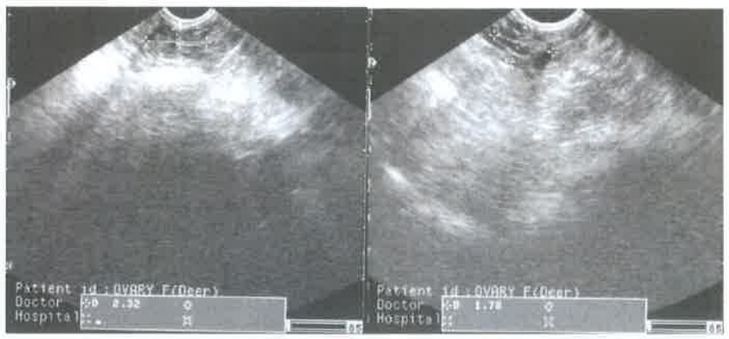
Treatment group	Treatment	Mean number of follicles	Mean follicle size (cm)
1	pFSH 100 mg (intramuscular injection of 25 mg every 12 h for 2 days)	7.00 ± 0.6	0.25 ± 0.01
2	pFSH 140 mg (intramuscular injection of 35 mg every 12 h for 2 days)	11.83 ± 1.20	0.34 ± 0.03
3	oFSH 7 mg (intramuscular injection of 1.75 mg every 12 h for 2 days)	9.33 ± 0.56	0.31 ± 0.01
4	pFSH 200 mg (intramuscular injection of 200 mg as a single injection)	8.33 ± 0.5	0.39 ± 0.02
5	oFSH 10 mg (intramuscular injection of 1.7 every 12 h for 3 days)	7.83 ± 1.25	0.50 ± 0.03
6	pFSH 180 mg (intramuscular injection of 30 mg every 12 h for 3 days)	7.66 ± 1.12	0.52 ± 0.03

pFSH – Porcine Follicle stimulating hormone (Folltropin – V)

oFSH – Ovine Follicle stimulating hormone (Ovagen)

4. Ultrasound guided Oocyte aspiration in spotted deer

This work was initiated towards the goal of recovering oocytes non-surgically from the spotted deer, when there is limited chance of adopting surgical procedures like laparotomy or laparoscopy. Superovulation is an important step towards this goal, since appropriate follicular diameter should be achieved without creating dominant follicle so that the developmental potential can be maintained during *in vitro* procedures. Appropriate hormonal preparation and dose rate have been standardized to achieve good superovulatory response (Fig. 4) for the oocyte aspiration procedure. We are trying to standardize the procedure of ultrasound guided transvaginal oocyte recovery, since the problem as anticipated are smaller size of the animal which will not permit transrectal manipulation of ovaries and also anatomical make up of cervix (in this species cervix is a muscular organ) makes this procedure complicated but continuous attempt may produce fruitful results.



Left ovary Right ovary

Fig. 4. Detection of ovaries in spotted deer by ultrasonography

5. Consistent production of live births of endangered Blackbucks by artificial insemination technology

A successful application of AI technology to wildlife conservation depends upon the development of a successful oestrous synchronization protocol, and determining the exact timing of insemination to obtain live births consistently. Further, the ability to tightly regulate ovulation allows insemination of females at a pre-determined time, thereby maximizing labour efficiency and avoiding handling and anaesthetic stress to females. Last year, we had produced a live birth of blackbuck "named *Blacky*" following synchronization of oestrus and non-surgical trans-cervical artificial insemination. Further, to evaluate the repeatability and the efficiency of the technique, this year 6 females were artificially inseminated using the same procedure as used for the production of *Blacky*. In short, oestrus was synchronized using two injections of $PGF_{2\alpha}$ given 11-days apart and females were inseminated transcervically with freshly collected electroejaculated semen. Four of the 6 (67 %) remained pregnant as evident by ultrasonography on 58–63 days of AI (Fig. 5). Unfortunately, only two females carried to term and delivered live offsprings after 177 to 180 days of gestation (Fig. 5). This demonstrates the potential application of artificial insemination technology in the conservation and genetic breeding of endangered species.

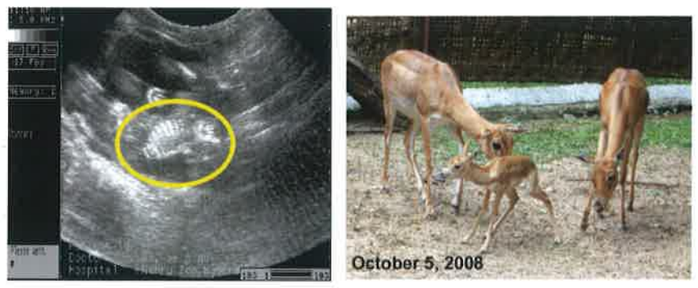


Fig. 5. Ultrasonographic picture of a 63-days old foetus of Blackbuck and birth of a live female fawn "Blacky" at LaCONES on 23rd August, 2007 following artificial insemination

6. Short-term cold storage of Blackbuck semen

Genetic resource banks offer new solutions to facilitate the genetic management of endangered species and also help to insure the catastrophic losses of a given species almost indefinitely. However, many a times, the efficiency/fertilizing efficiency of frozen sperm remains the major obstacle for a successful artificial insemination. So ideally, in addition to the long-term semen storage, effective methods for short-term storage over short periods would be useful. Transport of liquid semen between the zoos of the country is generally possible within 24 – 72 hours by road or air. The present study was undertaken to address the development of methods for such short-term storage in Blackbuck. Semen samples were diluted with either Tes-*n*-tris buffer (TEST) or TEST supplemented with trehalose (TEST-Trehalose) buffers. Sperm aliquots were placed in a 1.8-ml polypropylene cryotubes, which were then kept in the refrigerator (at ~ 5°C) for 7 days. Semen assessments such as sperm motility, PMI and acrosomal integrity were performed at 0 hour and after every 24 hours for up to 7 days of cold storage. The results indicate that both buffers were effective in maintaining cold storage of Blackbuck spermatozoa at 5°C up to 7 days and TEST–trehalose buffer was found to be comparatively superior to TEST alone. TEST–trehalose buffer maintained more than 50% of viable spermatozoa up to 7 days of cold storage (Fig. 6).

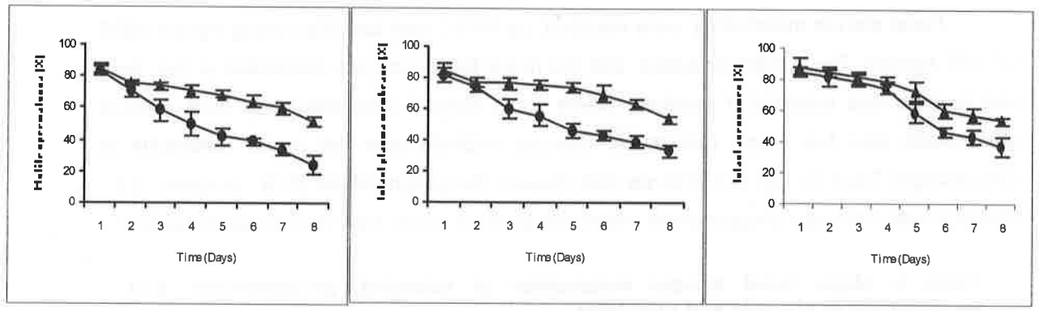


Fig. 6. Effect of short-term cold storage of Black buck semen stored in TEST (●) and TEST-trehalose (▲) on (a) sperm motility, (b) plasma membrane integrity and (c) acrosomal integrity

7. Development of fecal steroid analysis as a non- invasive method to monitor reproductive function in Indian endangered animals

Understanding the basic reproductive physiology of a given species is fundamental for successful captive breeding programs using assisted reproductive techniques such as artificial insemination, IVF, embryo transfer, cloning, etc. During artificial insemination, the exact timing of ovulation is important for successful conception. Investigations directed towards monitoring reproductive hormones (estrogen and progesterone in female and testosterone in male) in blood are crucial for artificial insemination since the data helps to assess gonadal function on the basis of sex, age, and seasonality; the timing of

spermatogenesis and ovulation; the type of ovulation and ways to overcome infertility. But repeated blood sampling, which is possible in domesticated animals and human beings, is not permissible in wild animal. Further sporadic blood sampling in wild animals suggested that hormonal patterns in wildlife are different from domesticated animals due to stress of anesthesia. Thus, there is a need to develop non-invasive methods for fecal / urine hormone analysis which appears to be species specific. The objectives of the present study are to identify major fecal steroid metabolites of progesterone, estradiol, and testosterone in felids (lion, leopard and tiger) and ungulates (black buck and spotted deer), and to develop and validate immunoassays (RIA/ ELISA) for monitoring of ovarian function and pregnancy detection of Indian wild animals.

For the purpose of identification and monitoring of steroid hormone metabolites, fecal samples of felids (Lion, tiger, leopards) and ungulates (Black buck, spotted deer), were collected from the Nehru Zoological Park, Hyderabad, and Indira Gandhi Zoological Park, Visakapatnam. Overall 3,988 fecal samples were collected from 53 individuals of nine species over a period of one year. Further, these fecal samples were extracted and preserved in -30^0 C for future processing and analysis.

Fecal steroid metabolites were resolved by HPLC and identified using Agilent 6900 GC-MS system. The results indicated that the major fecal estrogen metabolite of lion, tiger and leopard was estradiol 3 sulphate, where as in spotted deer and black buck estrone glucuronide was the major metabolite. Estrone sulphate was the major metabolite in Choushinga (Table 2). GC-MS analysis also showed that pregnanediol (5- β - pregnan- 3 β - 20α-diol) was the major progesterone metabolite present in lion, tiger and leopard (Table 2).

Table 2. Major fecal steroid metabolites of estradiol, progesterone, and testosterone in big cats and ungulates

Species	Major metabolite		
	Estradiol	Progesterone	Testosterone
Lion	Estradiol-3-sulphate	5- β - pregnan- 3 β -20α-diol	Incomplete#
Tiger	Estradiol-3-sulphate	5- β - pregnan- 3 β -20α-diol	Incomplete#
Leopard	Estradiol-3-sulphate	5- β - pregnan- 3 β -20α-diol	Incomplete#
Spotted deer	Estrone glucuronide (E1G)	Pregnanediol (5β pregnan- 3α-20α-diol)	Incomplete#
Thamin deer	Estrone glucuronide	Incomplete#	Incomplete#
Chousingha	Estrone sulphate (E1-3-sulphate)	Incomplete#	Incomplete#
Mouse deer	Incomplete#	Dihydroprogesterone (Pregnan –3, 20-dione)	Androst-16-en-3-ol (3 β, 5α); Dihydro testosterone
Black buck	Estrone glucuronide (E1G)	17 α- hydroxy-progesterone	Incomplete

8. Non-invasive method of detecting pregnancy in Indian wild animals

i. Analysis of total fecal progesterone metabolites for pregnancy detection in big cats using commercial RIA kits and ELISA developed by LaCONES

Health care and management of wild animals in captivity is most important for a successful captive breeding program. The recent development of non-invasive method to monitor reproductive hormone using fecal sample is advantage for better management practices in captive animals. Detection of pregnancy at earlier stage is most essential for successful breeding program of endangered species especially using non-invasive method. Therefore, the present study was undertaken to standardize pregnancy detection using fecal steroid metabolites in big cats and ungulates. For this purpose, fecal samples from known pregnant animals and non-pregnant animals were collected from tiger, lion, leopard, spotted deer, black buck, four-horned antelope and mouse deer. The fecal samples were extracted for progesterone metabolites and stored in -20 ° C until the analysis. The fecal progesterone metabolites were analyzed using commercial RIA kits and also by ELISA developed by LaCONES. The preliminary results show that the pregnancy in big cats can be detected as early as 18 days and in ungulates as early as 25 days. The progesterone metabolites concentration was highly significant between pregnant and non pregnant animals as early as two weeks of pregnancy till the delivery (Fig. 7).

ii. Detection of pregnancy in lion, tiger, jaguar, black buck, etc. using EIA developed against one of the major progesterone metabolite in the feces of big cats and ungulates

In continuation of the above work an EIA was developed against one of the major progesterone metabolite in the feces of big cats and ungulates The present EIA showed a high sensitivity, low intra and inter assay (6.2 % and 10.2% respectively), higher recovery rate (98.28%) and low cross reactivity with other steroid metabolites and a significant correlation with commercially available RIA based on progesterone antibodies (Fig. 8). The sensitivity of the present EIA (6 pg/ well) is comparable to that reported in previous studies for tigers (15 pg/ well, Graham et al 2001; 30 pg/ml Brown et al., 1999). Cross reactivity of the present antibody to estrogens was below 1% and this antibody could react with only progesterone metabolites in the fecal sample. Due to the high sensitivity of the developed assay, increase in fecal progesterone metabolite concentration was detectable within 3 weeks in the big cats after successful mating (Fig. 9). Therefore, the present assay is more reliable and efficient in measuring fecal progestogens and for detection of pregnancy in a wide range of animals like lion, tiger, jaguar, black buck, etc. (Fig. 9).

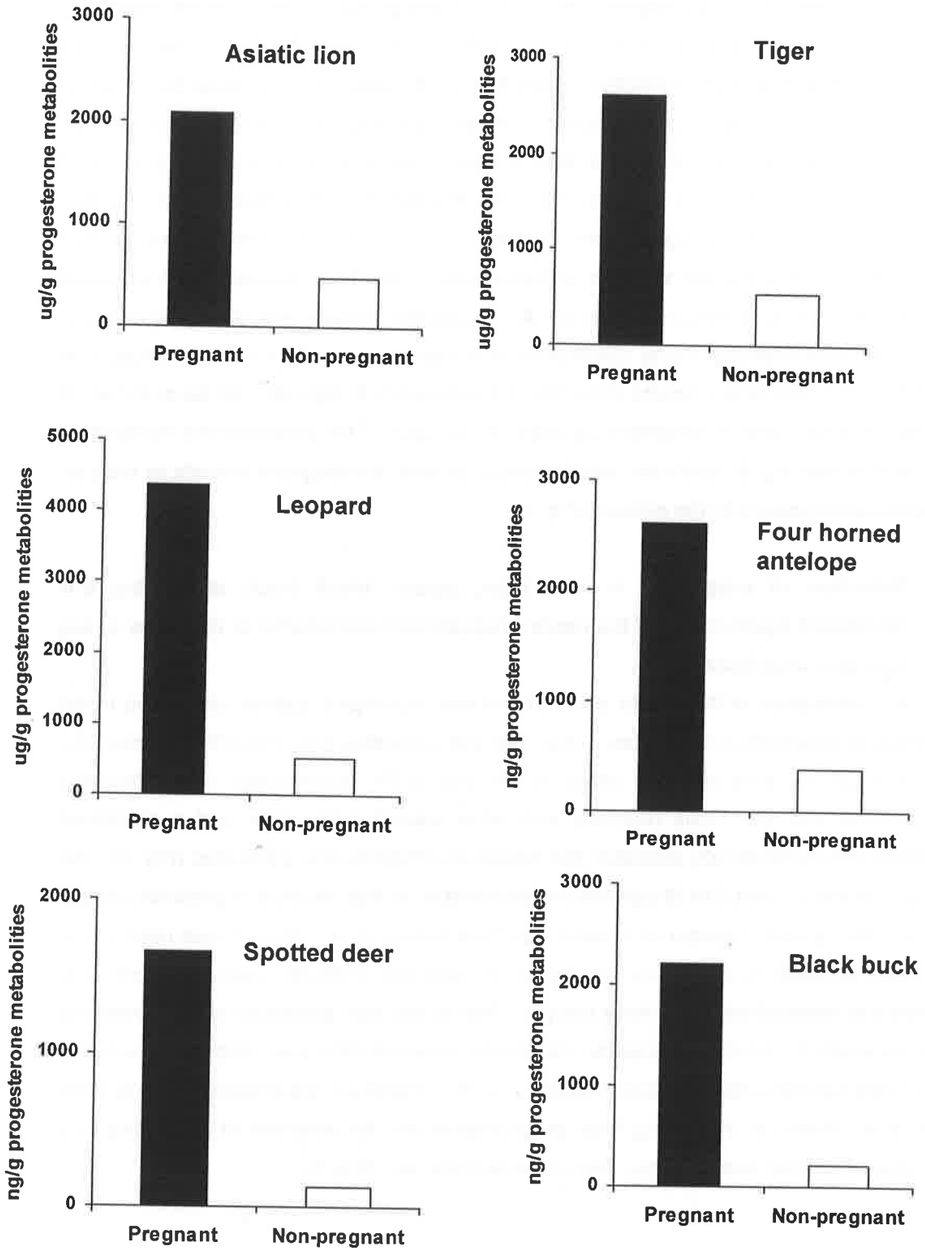


Fig. 7. Average progesterone metabolite concentration (n=5) between pregnant and non-pregnant wild animals in captivity

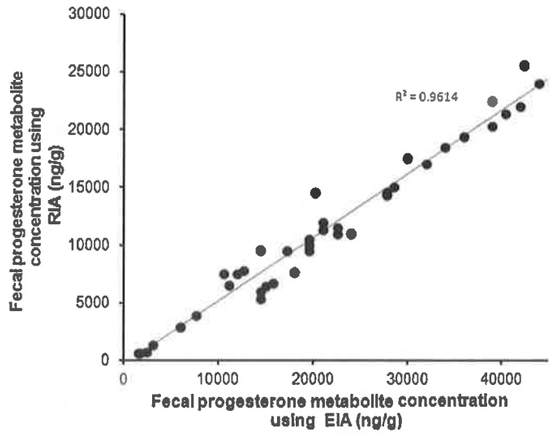


Fig. 8. Comparison of fecal progesterone values using EIA developed at LaCONES with commercially available RIA kit based on progesterone antibodies.

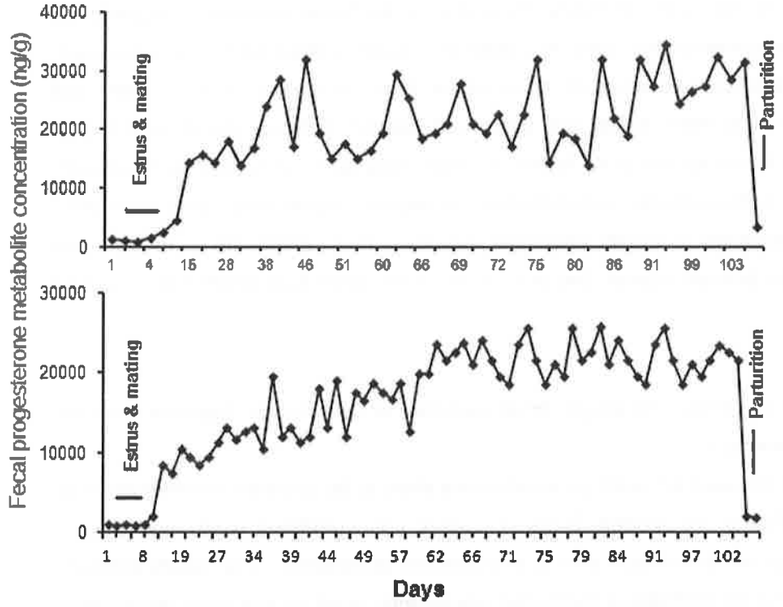


Fig. 9. Fecal progesterone metabolite concentration (ng/g) during estrus, mating, pregnancy and parturition in tiger (above, age-8 years) and jaguar (age 10 years).

9. Monitoring of 'musth' and stress in the Asian elephant using fecal hormone metabolites

The Asian elephant, one of the largest mammalian species, is under severe threat from habitat loss, poaching for ivory, bones, hair, etc. Though it is an endangered wild animal, it is also domesticated in certain parts of India. It is not uncommon to find elephants being used as beasts of burden and in temples for processions and other religious functions. Due to these activities, these elephants undergo stress and health related problems in captivity, resulting in inability to reproduce and musth-related physiological and endocrine problems. Monitoring of 'musth' and reproductive status of elephants would help for better management and conservation of captive elephants. The present study was an attempt to standardise non-invasive monitoring of musth and stress related steroid hormones in captive elephants using fecal and urine samples. Fecal and urine samples were collected from three bulls and two female elephants, in Nehru Zoological Park, Hyderabad, and examined for steroid metabolites and their concentration.

Preliminarily results showed that testosterone was detectable in both the fecal and urine samples of all the male elephants. HPLC/ELISA analyses revealed a significantly higher testosterone concentration was in males in 'musth' compared to the non-musth males. Further, the concentration of testosterone level increased during musth and decreased to the basal level during the non-musth period. Further, significantly higher concentrations of corticosterone were found in musth elephants compared to non-musth elephants and . corticosterone concentration increased significantly with increasing testosterone concentration in musth elephants indicating that elephants in musth were stressed. It is for this precise reason that animals in musth were kept isolated and chained during musth.

10. Monitoring of cortisol levels in fecal samples of the Asian elephant used for public procession

Elephant bulls used for public procession are likely to be stressed. In order to check whether such elephants are indeed stressed cortisol concentrations were monitored in elephants used for public procession prior to and after the procession. The results indicated that the fecal cortisol concentrations increased significantly three to four folds immediately after the public procession of elephants. But, the cortisol concentration decreased to the basal level after three days of public display. This finding suggests that the elephants are severely stressed during the public procession. The present study suggests that stress levels in captive Asian elephants can be monitored using non-invasive methods.

11. Impact of Habitat Fragmentation on the Demography of Lion-tailed Macaque (*Macaca silenus*) Populations in the Rainforests of Anamalai Hills, Western Ghats, India

Habitat fragmentation is considered the most serious threat to primate conservation in the tropics and understanding it effects on lion-tailed macaque is very important as most of the populations live in fragmented habitats. We examined demographic parameters of nine lion-tailed macaque groups in eight rain forest fragments with reference to fragment area, tree density, canopy cover, tree height and total basal area of food trees. Group size ranged 7 - 90 individuals but was not related to habitat variables (Table 3). Tree density, canopy cover and total basal of food tress all had strong positive correlations with fragment area. Birth rate ranged from 0.17 - 0.50 infants/female (Fig. 10) and these were observed in the smallest (Tata coffee) and the largest fragments (Varagaliar and Akkamalai) respectively. Groups in larger fragments had a mean of 0.42 infants/female, while smaller fragments had 0.31 infants/female, but this difference was not significant (M-W test U = 14.5, p = 0.41). Growth rate ranged from 0.06 in a small fragment to 0.30 in one of the largest fragments (Akkamalai). Groups in larger fragments had a growth rate of 0.16 while the smaller fragments had 0.08, but this difference was not significant (M-W test U = 17, p = 0.11). Growth rate correlated with tree density, but there were no other significant relationships between birth or growth rate and habitat variables. The percentage of immatures in the group was significantly positively associated with the total basal area of food trees, but not with any other habitat variable (Fig. 10 and Table 3).

Comparison of our data with data available for the same population in 1996 indicated a slight decline in birth rate but an increase in total number of individuals from 154 to 242. Of the five small fragment groups, three have increased in size since 1996 while the other two groups have remained the same size. Based on this study we advocate that to manage the fragile lion-tailed macaque groups following steps need to be taken: (a) create dispersal corridors between the fragments using fruit trees to facilitate male dispersal, (b) construct canopy bridges across the prevailing roads, (c) protect the fragments from further degradation and (d) periodically monitor these populations for long term conservation.

Table 3. Demographic parameters of nine lion-tailed macaque groups in eight rainforest fragments in Anamalai Hills

Fragment	Area (ha)	Number of adult males	Adult females* (%)	Immature animals* (%)	Number of adult females per male	Group size	Birth rate
Varagaliyar	2500	1	4 (40.0)	5 (50.0)	4.0	10	0.50
Akkamalai	2000	1	4 (30.7)	8 (61.5)	4.0	13	0.50
Monompoly	500	2	6 (37.5)	8 (50.0)	3.0	16	0.33
Andiparai	185	4	10 (31.2)	18 (56.2)	3.5	32	0.30
Puthuthottam- I	65	7	36 (40.0)	47 (52.2)	5.1	90	0.44
Puthuthottam -II	65	2	14 (43.7)	16 (50.0)	7.0	32	0.29
Pannimedu	50	1	3 (42.9)	3 (42.9)	3.0	7	0.33
Korangumudi	35	3	11 (36.7)	16 (53.3)	5.5	30	0.36
Tata Coffee	24	1	6 (50.0)	5 (41.7)	6.0	12	0.17

*- Percentage is given in parenthesis.

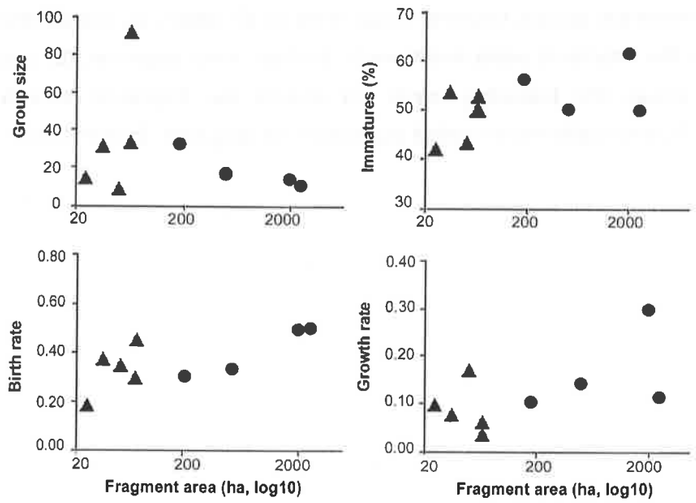


Fig. 10. Relationship between fragment area and demographic parameters of lion-tailed macaques in Anamalai Hills (triangles, fragments <100 ha; solid circles, fragments >100 ha.

12. DNA based sex identification of birds

As in mammals, avian gender is determined chromosomally. In mammals, the male is heterogametic (XY) and the female is homogametic (XX), but the reverse is true in the birds, where females are heterogametic (ZW) and males homogametic (ZZ). In 1996, Griffiths *et al*., developed a PCR-based approach that can be used in a wide variety of avian taxa for sexing of birds. This was a major achievement as birds are difficult to sex. Nestlings rarely show sex-linked morphology. Adult females appear identical to males in over 50% of the world's bird species. DNA-based sex identification using the CHD gene provides a solution. This gene is highly conserved and is linked to the W and Z chromosomes in birds. In birds, the CHD-W gene is located on the W chromosome and is therefore unique to females. The other gene, CHD-Z, is found on the Z chromosome and occurs in both sexes (female, ZW; male, ZZ). The test employs a single set of primers, which amplifies homologous sections of both genes and incorporates introns whose lengths usually differ. When examined on a gel there is a single CHD-Z band in males but females have a second, distinct CHD-W band.

In the present study DNA was extracted from different sources like blood and feathers using the phenol-chloroform method. After quantification, the DNA obtained was subjected to PCR amplification using the procedure of Griffiths *et al*. (1998) to determine the sexes. The gel picture (Fig. 11) shows the banding patterns of CHD-Z and CHD-W in a male and female sample respectively.

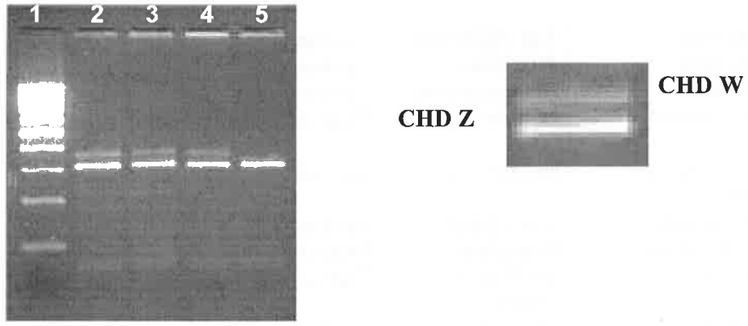


Fig. 11. Sexing of birds based based on the CHD gene. The females have both a CHD-Z and CHD-W band (lanes 3-5) whereas the male shows a single CHD-Z band (lane 5). Lane 2 is a positive control for female and in lane 1 the 100 bp ladder was loaded.

A total of 84 samples of different species of birds from Vandalur Zoo, Chennai, Rajiv Gandhi Zoological Park & Wildlife Research Centre, Pune, Nehru Zoological Park, Hyderabad, Mysore Zoo, Mysore, Thiruvananthapuram Zoo, Thiruvananthapuram, M C Zoo, Punjab, and Sepahijala Zoological Park, Tripura were sexed (Table 4) and the reports were submitted to the respective zoos.

Table 4. DNA based sexing of birds using feather and blood samples of birds from four different zoos

Sl. No.	Sample name	Markings	Source	Male / Female
Vandalur Zoo, Chennai				
1	Demoiselle crane	Big	Feather and Blood	Male
2	Demoiselle crane	Small	Feather and Blood	Female
3	Demoiselle crane	Not mentioned	Feather and Blood	Female
4	White stork	Not mentioned	Feather and Blood	Female
5	Sarus crane	Big	Feather and Blood	Male
6	Sarus crane	Small	Feather and Blood	Male
7	Sarus crane	Not mentioned	Feather and Blood	Female
8	Lesser adjutant	Not mentioned	Feather and Blood	Female
9	Greater adjutant	Not mentioned	Feather and Blood	Female
10	White bellied sea eagle	Not mentioned	Feather and Blood	Male
11	Sea eagle	Ringed (Guindy-vandalur)	Feather	Female
12	White backed vulture	Left leg ringed	Feather	Male
13	White backed vulture	Right leg ringed	Feather and Blood	Male
14	White backed vulture	Not ringed	Feather and Blood	Male
15	Red whiskered bulbul	A248925	Feather	No DNA
16	White browed bulbul	Ab157623	Feather	Male
17	Grey patridge	Not mentioned	Feather	No DNA
18	Emarald dove	Bald head	Feather	No DNA
19	Emarald dove	Not mentioned	Feather	No DNA
20	Blossom headed parakeet	Not mentioned	Feather	Female
21	Blossom headed parakeet	Not mentioned	Feather	Male
22	Malabar parakeet	Black billed	Feather	Male
23	Malabar parakeet	Red billed	Feather	Female
24	Rosy pelican	Black double ringed	Feather and Blood	Male
25	Rosy pelican	Green and Black double ringed	Feather and Blood	Female
26	Rosy pelican	Red and Black double ringed	Feather and Blood	Female
27	Rosy pelican	Blue and Black double ringed	Feather and Blood	Male
28	Rosy pelican	Black and White ringed	Feather and Blood	Female
29	Rosy pelican	Left leg green ringed	Feather and Blood	Female
30	Rosy pelican	Left leg black ringed	Feather and Blood	Male
31	Rosy pelican	Left leg blue ringed	Feather and Blood	Female
32	Spot billed pelican	Left leg ringed	Feather and Blood	Female
33	Spot billed pelican	Right leg ringed	Feather and Blood	Male
34	Little egret	Left ringed	Feather	Female
35	Little egret	Right leg ringed	Feather	Female

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Sl. No.	Sample name	Markings	Source	Male / Female
36	White ibis	Left leg white ringed	Feather	Male
37	White ibis	Right leg white ringed	Feather	Female
38	Bar headed goose	Left leg ringed	Feather and Blood	Female
39	Bar headed goose	Right leg ringed	Feather and Blood	Female
40	Painted stork	Left leg ringed	Feather	Female
41	Painted stork	Right leg ringed	Feather	Female
42	Night heron	Left leg ringed	Feather and Blood	Male
43	Comb duck	Left leg ringed	Feather and Blood	Male
44	Brahminy duck	Left leg ringed	Feather and Blood	Female
45	Brahminy duck	Right leg ringed	Feather and Blood	Male
46	Great horned owl	Not mentioned	Feather and Blood	Male
47	Red billed blue magpie	Not mentioned	Feather	Female
48	Great horned hornbill	Not mentioned	Feather	No DNA
Rajiv Gandhi Zoological Park & Wildlife Research Centre, Pune				
49	Black winged kite	Not mentioned	Blood stain	Male
50	Shikra	Not mentioned	Blood stain	Male
51	Common kestrel	Not mentioned	Blood stain	Female
52	Emerald dove	Not mentioned	Blood stain	Female
53	Large parakeet	Not mentioned	Blood stain	Female
54	Rose ringed parakeet	Not mentioned	Blood stain	Female
55	Malabar pied hornbill	Not mentioned	Blood stain	Male
56	Barn owl	Not mentioned	Blood stain	Female
57	Brahminy kite	Not mentioned	Blood	Male
58	Brahminy kite	Not mentioned	Blood	Female
59	Brown fish owl	Not mentioned	Blood	Female
60	Laggar falcon	Not mentioned	Blood	Male
61	Horned owl	Not mentioned	Blood	Female
62	Short toed snake eagle	Not mentioned	Blood	Female
63	Steepe eagle	Not mentioned	Blood	Male
64	Serpent eagle	Not mentioned	Blood	Female
65	Tawny eagle	Not mentioned	Blood	Male
66	Cinerous vulture	Not mentioned	Blood stain	Male
67	Long billed vulture	Not mentioned	Blood	Female
68	Long Billed Vulture	Not mentioned	Blood	Male
69	Bonnelli's Eagle	BW	Blood	Male
70	Bonnelli's Eagle	Not mentioned	Blood	Male
Nehru Zoological Park, Hyderabad				
71	Nicobar pigeon	1	Feathers	Male
72	Nicobar pigeon	2	Feathers	Male
73	Nicobar pigeon	3	Feathers	Male
74	Nicobar pigeon	4	Feathers	Female
75	Sarus crane	Not mentioned	Feathers	Male
1	African Grey Parrot	Feather Red	Feathers	♂ male
2	African Grey Parrot	Plain - Colorless	Feathers	♀ female
3	African Grey Parrot	Green	Feathers	Male
4	African Grey Parrot	Red	Feathers	Female

Sl. No.	Sample name	Markings	Source	Male / Female
5	Blue Fronted Amazon	Red	Feathers	Female
6	Blue Fronted Amazon	Green	Feathers	Female
7	Blue Fronted Amazon	Black	Feathers	Male
8	Timneh Parrot	Black	Feathers	Male
9	Timneh Parrot	Green	Feathers	Male
10	Petagonian Connure	Black	Feathers	Male
11	Petagonian Connure	Red	Feathers	Male
12	Blue Streaked Lory	--	Feathers	Male
13	Red Mulucan Lory	--	Feathers	Male
14	Chattering Lory	--	Feathers	Male
15	Blue and yellow Macaw	Ringed	Feather	Male
16	Blue and yellow Macaw	Not ringed	Feather	Female
78	Rosy pelican	Green color thread	Single Feather	Male
79	Rosy pelican	Brown color thread	Single Feather	Male
80	Rosy pelican	White color thread	Single Feather	Male
81	Rosy pelican	Without thread	Single Feather	Female
82	Eagle	Pink color thread	Single Feather	Male
83	Eagle	Violet color thread	Single Feather	Female
84	Eagle	Green color thread	Single Feather	Male
Mysore Zoo				
1	Flamingo	-	Feather	No DNA
2	Lesser Flamingo	-	Feather	Male
3	Lesser Adjutant Stork 1	-	Feather	Male
4	Lesser Adjutant Stork 2	-	Feather	Male
5	Sarus crane 1	-	Feather	Female
6	Sarus crane 2	-	Feather	Female
7	Sulphur-creasted cuckatoo	-	Feather	No DNA
8	Sulphur-creasted cuckatoo	-	Feather	Male
9	Moluccan cuckatoo	-	Feather	Male
10	Moluccan cuckatoo	-	Feather	Male
11	Green-winged macaw	-	Feather	Female
12	Green-winged macaw	-	Feather	Male
13	African grey parrot	-	Feather	Female
Thiruvananthapuram Zoo				
1	Cinereous vulture	-	Blood	Female
2	Lesser Adjutant Stork 1	-	Blood	Female
3	Lesser Adjutant Stork 2	-	Blood stain	Under process
MC Zoo, Punjab				
1	Sarus crane 1	-	Blood feather	Male
2	Sarus crane 2	-	Feather	Female
3	Sarus crane 3	-	Feather	Female
4	Sarus crane 4	-	Blood feather	Male
Sl. No.	**Sample name**	**Markings**	**Source**	**Male / Female**
5	Sarus crane 5	-	Feather	Female
6	Sarus crane 6	-	Blood feather	Male
7	Sarus crane 7	-	Blood feather	Male

13. In vitro maturation and fertilization in the Nilgai using oocytes and spermatozoa recovered post-mortem from animals that had died because of foot and mouth disease outbreak

The ability to rescue gametes from endangered or wildlife species and to subsequently produce viable embryos holds tremendous potential as a means to increase the population size of endangered or wildlife species. The objective of this study was to assess the developmental competence of gametes recovered from nilgai that had died because of foot and mouth disease outbreak. Oocytes collected from the ovaries of seven dead nilgais were allowed to mature in vitro and were tested for developmental potential by in vitro fertilization (IVF) with epididymal spermatozoa collected also post-mortem. The average number of oocytes (n = 517) recovered per ovary was 36.9, and the side (right or left), size and weight of the ovaries had no significant effect on the number and quality of oocytes recovered. In vitro maturation studies indicated that the proportion of matured oocytes (MII stage) at 18, 24 and 30 h was 55.6%, 63.4% and 63.6%, respectively. Furthermore, 43% of the matured oocytes cleaved following in vitro fertilization and 12% of the cleaved oocytes (6/49) developed to the 4–8 cell stage (Fig. 12). This study therefore demonstrated that gametes recovered from nilgais after sudden death possess competence to develop into embryos following IVM and IVF. These important observations have implications in conservation of endangered animals because the recovered gametes and the generated embryos from dead animals could be used for production of offspring of endangered animals. We sincerely wish that another outbreak of FMD does not occur. But if it does, we would try to obtain much more information on the reproductive status of the animal and improve the in vitro development competence of the oocytes such that it proceeds up to the blastocyst stage

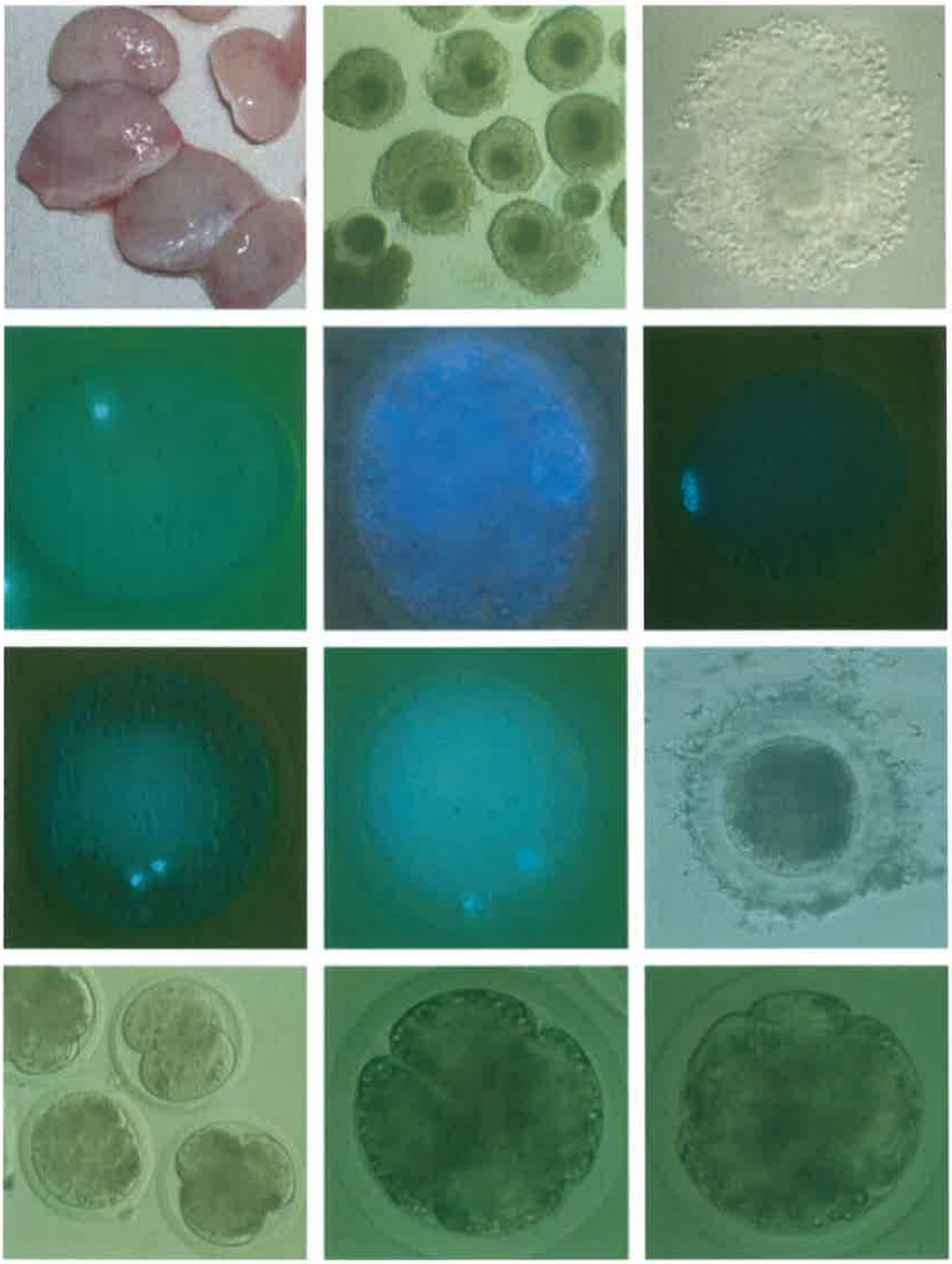

Fig. 12. In vitro maturation of oocytes recovered from the ovaries (a) of dead nilgai. Freshly collected culture grade oocytes (b) before IVM and various stages of oocyte maturation, such as the oocyte with cumulus cell expansion (c), the germinal vesicle stage (d), germinal vesicle breakdown (e), metaphase-I (f), telophase-I (g) and metaphase-II and the first polar body (h), are shown. Oocytes following IVM and IVF showing spermatozoa attached to the oocytes (i), 2-cell (j), 4-cell (k) and 8-cell (l) embryos. The oocytes were stained with Hoechst 33342.

14. Meiotic maturation of vitrified immature chousingha (*Tetracerus quadricornis*) oocytes recovered postmortem

Cryopreservation of gametes and embryos is becoming increasingly important in the conservation and management of wild/ endangered species and such gametes and embryos have been used successfully in artificial insemination and embryo transfer respectively. It is in this context that rescue of gametes from wild/endangered animals that have died unexpectedly is a worthwhile research tool for understanding the fundamental physiology of the species concerned and also for development of species-specific protocols for application of new emerging assisted reproductive technologies in endangered species. A few attempts have been made to explore the possibility of recovery, IVM and IVF of oocytes collected from the ovaries of antelopes In addition, cryopreservation of immature oocytes from wild/endangered animals which have died due to accidents or medical reasons would allow one to preserve oocytes until species specific protocols for assisted reproductive technologies become available for use. Studies have demonstrated that chilling and freezing of oocytes cause a number of cytological changes and thus affect fertilization rates. To date, oocyte cryopreservation has been performed in several mammalian species but the efficiency as judged by developmental potential of the oocytes is still very low . Two major methods have been used for embryo cryopreservation: conventional slow freezing and vitrification. Conventional slow freezing has been used successfully to preserve embryos of various laboratory and domestic species . But, cryopreservation by slow freezing was not successful when oocytes of pig, buffalo, horse and dog were used. Vitrification has thus become a viable and promising alternative to cryopreserve oocytes and embryos. However, the application of this technology to oocytes and embryos of wild/endangered species would be highly challenging since baseline data on the sensitivity of these to cryoprotectants is not known . It is in this context, the present study is an attempt to study the meiotic maturation of vitrified immature chousingha oocytes recovered at postmortem. This is the first report using chousingha oocytes.

The objective of this study was to assess the *in vitro* meiotic maturation of chousingha (four-horned antelope) oocytes following vitrification using open pulled straw (OPS) method. The average number of oocytes recovered per ovary was 65.6. The proportion of oocytes that matured was significantly lower in vitrified oocytes (29.4%) when compared with fresh oocytes (69.3%). The study provides evidence that it is possible to cryopreserve immature oocytes by vitrification collected from the ovaries of chousingha at postmortem and also demonstrates that these cryopreserved oocytes retain their potential to undergo *in vitro* meiotic maturation (Fig.13).

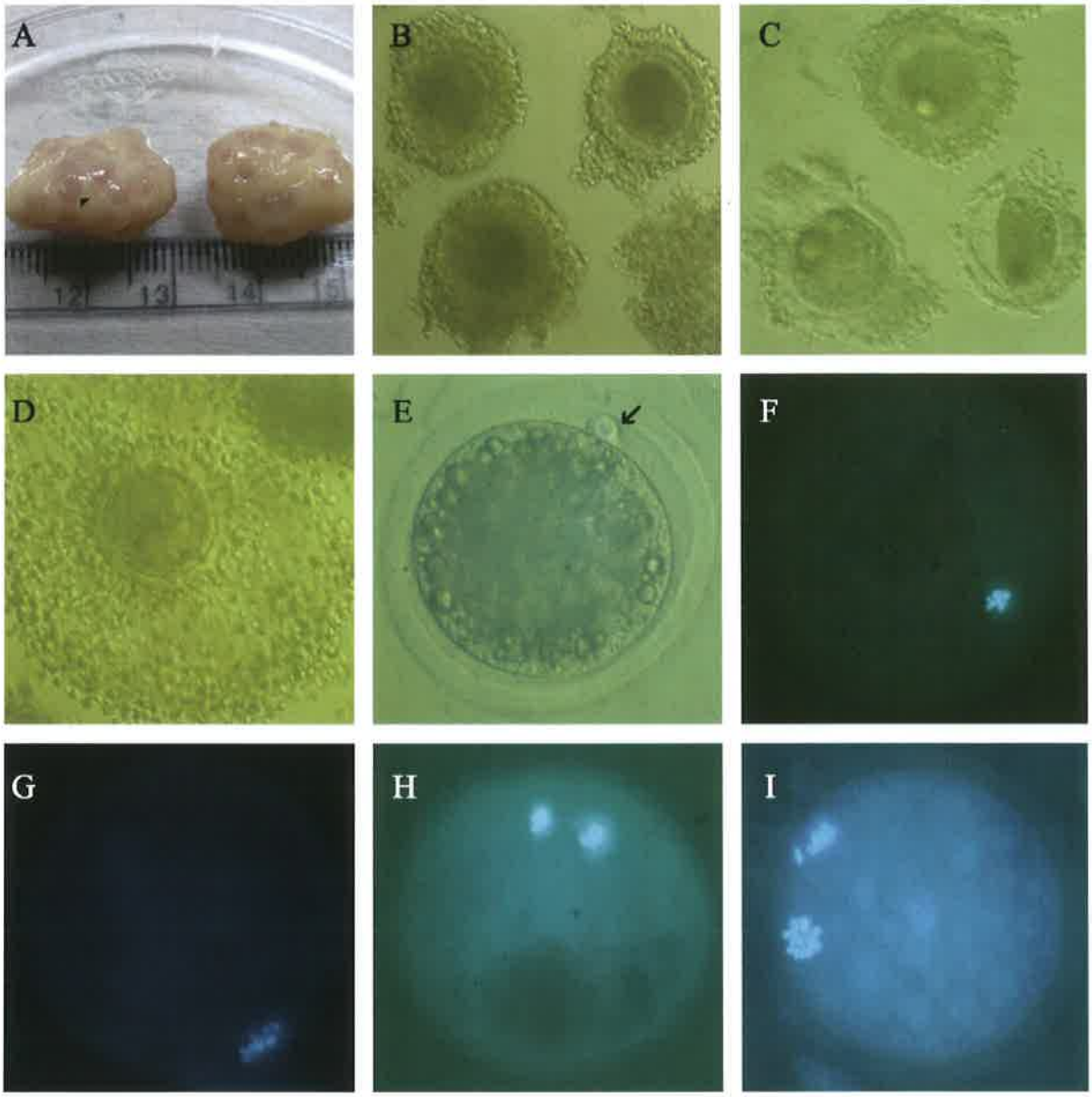

Fig. 13. Photomicrographs of the ovaries (A) recovered from a dead chousingha with follicles clearly visible on the surface (arrow head). Culture grade oocytes before vitrification (B) and after showing degenerative changes (C). Figures D–I show the following stages of in vitro maturation: oocytes with cumulus cell expansion (D), oocytes with first polar body (E), oocytes stained with Hoechst 33342 (F–I) depicting Germinal vesicle stage (F), metaphase I (G), telophase (H) and metaphase II with first polar body (I).

15. Developmental competence of oocytes recovered from post-mortem ovaries of the endangered Indian blackbuck (*Antilope cervicapra*)

The objective of this study was to assess the meiotic and developmental competence of oocytes recovered from post mortem ovaries of the Indian blackbuck. Oocytes collected from the ovaries of dead blackbucks, were allowed to mature *in vitro* and such oocytes were then tested for developmental potential by activation with ionomycin followed by 6-dimethylaminopurine treatment. The average number of oocytes recovered per ovary was 10.91 and the oocytes recovery did not depend on the presence or absence of corpus luteum, on the side, size and weight of the ovaries and on the type of oocytes recovered. The proportion of good quality oocytes showing cumulus expansion and extruded first polar body were 79.33 and 46.16 when cultured with gonadotropins. *In vitro* maturation studies indicated that the proportion of oocytes that reached MII stage was significantly higher when good quality oocytes (68%) were used compared to fair quality oocytes (48%) when cultured in presence of gonadotropins. Further, fifty seven percent of the *in vitro* matured oocytes cleaved and thirteen percent of the cleaved oocytes developed into blastocysts (Fig. 14). These findings suggest that the oocytes recovered from post mortem ovaries of blackbuck can be utilized for the production of embryos.

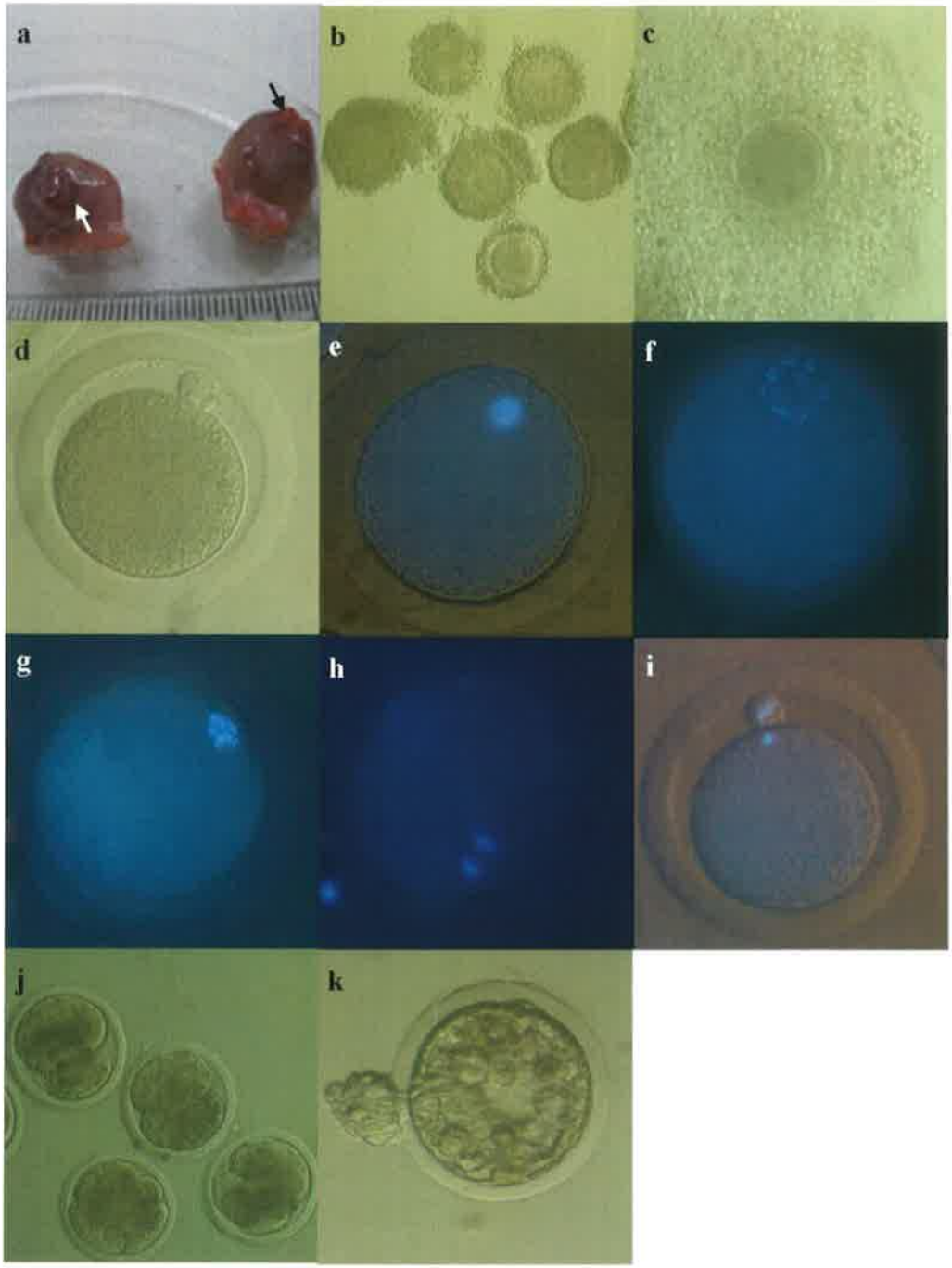

Fig. 14. *In vitro* maturation of blackbuck oocytes recovered from the ovaries (a) of dead Indian blackbucks. Corpora Lutea (black arrow) and visible follicles (white arrow) were visible in the ovaries. The freshly collected culture grade oocytes (b) before IVM and the various stages of maturation of the oocyte such as the oocyte with cumulus cell expansion (c), with first polar body (d), germinal vesicle stage (e), germinal vesicle breakdown (f), metaphase I (g), telophase I (h) and metaphase II and first polar body (i) are shown. *In vitro* development of 2-4 cell embryos (j) and Blastocyst (k) following parthenogenetic activation of IVM oocytes. The oocytes were stained with Hoechst 3342.

16. Chemical restraint and its antagonism in an endangered Blackbuck (*Antilope cervicapra*)

The blackbuck (*Antilope cervicapra*) is a small antelope listed in Schedule-I of the Indian Wildlife Protection Act, 1972. The studies were undertaken in blackbuck to develop assisted reproductive techniques as one of the conservation measures for a captive breeding program. However, because of its excitable nature, manipulation and handling of blackbuck is preceded by chemical immobilization. A suitable anaesthetic combination that provides adequate anaesthesia and analgesia is very important for the care and handling of an endangered species. Further, determining a suitable antagonist for immediate recovery from the anaesthetic effects is also important so as to avoid hazards of regurgitation, aspiratory pneumonia, distention of rumen in ungulates and to avoid predation in the wild. To our knowledge, chemical immobilization and its antagonism in the blackbuck is poorly documented.

A total of seventy-seven anaesthetic events were carried out on 22 captive adult blackbucks of either sex with a combination of 2 mg kg^{-1} ketamine hydrochloride and 0.25 mg kg^{-1} xylazine hydrochloride using a dart delivered from a blowpipe. Randomized anaesthetized animals received an intravenous injection of either yohimbine hydrochloride (0.125 mg kg^{-1} or 0.25 mg kg^{-1}) or tolazoline hydrochloride (1 mg kg^{-1} or 2 mg kg^{-1}) after 30 – 40 minutes of anaesthesia to antagonize the anaesthetic effects. Ketamine-xylazine induced smooth, rapid and reliable anaesthesia within 5 – 7 minutes of darting with no clinical adverse effects and causalities during or post-anaesthesia. Yohimbine failed to antagonize the anaesthetic effects of ketamine-xylazine in the blackbuck. On the other hand, tolazoline was found to be very effective in hastening recovery in dose-dependent manner within 1 – 2 minutes. This study documents the first report of ketamine-xylazine anaesthesia and its antagonism by tolazoline in captive Black buck (Sontakke *et al.* 2009a).

17. Optimization of anaesthesia and reversal for carrying out assisted reproductive techniques in spotted deer (*Axis axis*)

Chemical restraint is a valuable tool in wildlife research and management, as it is often needed for most of the manipulating procedures in wild animals. However, refinement of anaesthetic regimes is very essential in every species depending on the type and duration of handling procedure and also on the availability and cost-effectiveness of the anaesthetics. Further, the use of a suitable antagonist for reversing anaesthetic effects is very essential for smooth and quick recovery so as to avoid further physiological complications and also predation by wild animals in the wild.

Spotted deer (*Axis axis*) is an abundantly distributed cervid in India and hence it was chosen as a model system for other endangered species such as Manipuri-Brow antler deer, musk deer, Thiamin deer etc. for standardizing assisted reproductive techniques viz. semen collection by electroejaculation, ultrasonography and transcervical artificial insemination. However, being a highly temperamental animal, the spotted deer had to be anesthetized for the above procedures. In most of the wild ungulates, Hellabrunn's anaesthetic mixture (ketamine: xylazine in a ratio of 1: 1.25) is routinely being used to immobilize for short-term procedures such as blood collection, radio collaring, minor surgical manipulations and also for translocation of animals. However, our preliminary trials on semen collection by electroejaculation technique using this combination failed to induce electroejaculation and semen collection in this species. Hence anaesthesia was standardized in both the species.

A study was conducted on 10 males and 25 females to determine the optimal dose of anesthetics and cardiopulmonary effects of xylazine alone and in combination with ketamine and to evaluate the efficacy of different doses (5, 8 and 10 mg) of yohimbine as an antagonist. Deer were immobilized with either 3.5 mg/kg xylazine, or three different combinations of ketamine and xylazine. Anesthesia was reversed with 5 to 10 mg of yohimbine hydrochloride. A combination of 2.5 mg/kg ketamine and 0.5 mg/kg xylazine proved to be the best anesthetic combination, both for rapid induction of anesthesia and electroejaculation in male deer, whereas in female, 1.5 mg/kg ketamine and 1 mg/kg xylazine combination was the best for induction of anesthesia without any adverse effects. All yohimbine doses reversed anesthesia within 1 minute and the duration of immobilization had no effect on the recovery time. (Sontakke et al. 2007).

18. Optimization of anaesthetic reversal in captive Indian felids (lions, tigers and leopards)

Determining a suitable antagonist for reversing anaesthetic effects in endangered wild felids is very essential since uncontrollable recoveries due to prolonged sedations are known to cause profound respiratory depression and sometimes may result in death and moreover, the sedated free-ranging animals could become easily predated upon in the wild. Yohimbine has been used effectively to reverse the anesthetic effects in wild ungulates. However, to our knowledge, scanty information is available about reversal in wild felids.

The present study was undertaken in 52 captive lions (*Panthera leo persica*), 55 leopards (*Panthera pardus*) and 16 tigers (*Panthera tigris*) from various zoos of India to evaluate the effectiveness of yohimbine hydrochloride as an antagonist of ketamine-xylazine anesthesia. Anesthesia was reversed at various time periods with an intravenous administration of two different dosages of yohimbine hydrochloride. Yohimbine effectively reversed the anesthetic effects in all animals within 10 – 15 minutes of injection without any excitatory behavior compared to control animals. Further, it was observed that the duration of anesthesia had no significant effect on the recovery in any of the species. (Sontakke et al. 2007).

II. DNA FINGERPRINTING AS A TOOL TO MONITOR GENETIC VARIATION IN ENDANGERED ANIMALS

1. Development of markers to study genetic variation in Leopards (*Panthera pardus*)

Indian big cats, namely lions, tigers and leopards (n=93) were characterized using 31 microsatellite loci identified from a partial genomic library of the Asiatic lion (*Penthera leo persica*). These markers revealed a considerable extent of genetic variation in Asiatic lions, which was undetectable by other molecular markers. When out of 31, ten most polymorphic loci from Asiatic lions were compared among the three big cats, leopards and tigers showed low allelic diversity as compared to the Asiatic lions. The low allelic diversity in leopards and tigers may be attributed to an ascertainment bias that may have resulted from the use of heterologous PCR primers. The microsatellite markers used in this study were developed for the Asiatic lions, and they produce a significant number of alleles in this species. But, they produce consistently fewer alleles in leopards and tigers. This reduction in diversity and the ascertainment bias is expected when using heterologous primers.

To check this theory of ascertainment bias, a partial genomic library of leopard was developed and screened. Thirteen (13) new microsatellite markers were developed,

characterized and validated. Genetic diversity was quantified as the total number of alleles at each locus and over all loci as observed heterozygosity (H_o), and expected heterozygosity (H_E) at each locus. Four of the 13 loci showed significant deviation from Hardy Weinberg equilibrium ($P<0.001$) and this may have been a consequence of the small number of samples genotyped. Percent heterozygosity (0.32) observed in the Asiatic lions, using leopard markers, was found to be significantly low as compared to percent heterozygosity (0.68) observed using markers developed from Asiatic lion. The average number of alleles per locus was also observed to be less when markers from leopard library were used for assessment of Asiatic lion population. However, the level of heterozygosity in leopards was high (0.54), using markers from leopard as compared to a heterozygosity of 0.34 using markers from Asiatic lion. The markers developed from leopard genomic library were less polymorphic and less informative in lions but more polymorphic and more informative in leopards, in comparison to the microsatellite markers developed from Asiatic lion. This confirms the need for the development of species-specific genetic markers. The mutation rates of various genetic loci coding for microsatellites vary considerably within and amongst the species and sequences that are hypervariable, are different in different species.

In addition to the microsatellite loci characterized form leopard genomic library, a CA-rich locus LD 62 (~600bp) was also identified, that shows PCR-amplification in big cats belonging to *Panthera* genus only, *i.*e. lion, tiger, leopard, and jaguar. The sequence variations were clearly distinct and would discriminate between different big cats. In tigers, a deletion of 20bp was observed (Figure 15). This locus can efficiently be used for forensic identification of Indian big cats and their body parts.

Panthera sp.	NUCLEOTIDES POSITION
	111111111122222222223333333333477777777788888888899912345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012
Leopard	CTCACACACACACACACACACACACACACACACACACACTCGTGTTCACATATATACACATGCACG
Tiger	..................----------.....----------..
African lion	..................----------.....----------..
Asiatic lion	..................----------.....----------..
Jaguar	..................----------.....----------..

Fig. 15. Alignment of LD 62 sequences in different members of Genus *Panthera*. Only variable positions are shown. Note the 20-bp deletion in tiger and variable length of the CA-repeat in all the members. ' - ' indicates deletion.

2. Conservation genetics of Indian wild buffalo

The existing populations of wild buffalo in India are highly endangered because of habitat loss and degradation due to anthropogenic interference. Furthermore, the loss of genetic purity because of cross breeding with the domestic and feral animals is the other major threat. The wild buffalo can occupy and disperse through a wide range of habitat types and is physically capable of moving over large distances. Wild buffalo males are reported to kill domestic buffalo males to mate with domestic females. There are reports from Assam region that a majority of domestic females had calves sired by wild males. Hybrid males that remain in the wild are likely to subordinate to wild males, and hence may not breed, but hybrid females would be expected to breed with wild males. To the extent that hybrid progeny of wild males actually returned to the villages, the gene pool of the wild populations will have suffered little introgression from domestic buffalo. Such events indicate the genetic proximity of wild and domesticated buffalo and subsequently, mixing of their germplasm. The overall concern of this study is to study and find measures to counter this threat. Since it is difficult to distinguish between wild buffalo, feral, domesticated and their hybrids, the actual conservation status is uncertain and the possibility of extinction of wild populations is quite real. Therefore, there is an urgent need to review the genetic status of this endangered species for ascertaining its genetic purity and to assess the level of genetic diversity in the existing populations, using tools of molecular genetics.

We have successfully generated the partial sequences of cytochrome b and hypervariable region II of d-loop for more than 75 % of the faecal samples. Hypervariable region I of d-loop and 16S have also been PCR amplified and sequencing has carried out. Simultaneously, work is also progressing on the microsatellite analysis. Four out of seven microsatellite loci identified for genotyping have been standardised and are being used to screen all the samples received. Most of the domestic samples from Kaziranga showed distinct variations as compared to their wild counterparts. Still some of the domestic samples were found to be sharing haplotypes with wild samples. However, as far as the samples from Udanti wildlife sanctuary are concerned, there was no clear cut distinction between wild and domestic samples. The samples from Udanti wildlife sanctuary were found to be more close to the wild samples from Kaziranga. But these samples showed overall distinct variations from Kaziranga samples, may be because of the geographical distance. The preliminary observation indicates gene flow among domestic and wild animals in recent past.

3. Genetic diversity in a captive population of Indian grey jungle fowl

Indian subcontinent is rich in avian diversity with its wide geographical and altitudinal variations extending from sea level to the summits of Himalayas, which provides different ecosystems for reproduction and hence species diversity. Galliformes classification and distribution has been well documented and widely studied in global arena. The jungle fowl is classified into four species: red jungle fowl (*G. gallus*), grey jungle fowl (*G. sonnerati*), green jungle fowl (*G. varius*) and ceylon jungle fowl (*G. lafayettei*). The grey jungle fowl, also known as Sonnerat's jungle fowl, is a resident species of Indian peninsula. Grey jungle fowls are threatened by hunting for food and habitat loss. Another factor leading to loss in genetic diversity is uncontrolled breeding of poultry to meet the global demand. Phylogenetic positioning of grey jungle fowl has also been affected due to lack of breeding management among the different species of the fowls.

In the present study, a precise investigation on genetic diversity was performed in a captive grey jungle fowl population maintained at Sri Venkateswara Zoological Park, Tirupati, Andhra Pradesh, using nuclear and mitochondrial DNA markers. We used five microsatellite markers to investigate the genetic variations within the population, out of which four microsatellite loci viz. MCW 5, MCW 4, GUJ0063, GUJ0084 were found to be highly polymorphic in all the samples of grey jungle fowl with maximum number of alleles observed in the locus MCW 5. A partial sequence of 520 bp of the control region of mtDNA was also obtained from 33 individuals of a captive population of grey jungle fowl. Both nuclear and mtDNA analyses showed high level of genetic diversity among the captive population of *G. sonnerati* which can be used for controlled, conservation breeding in future. The outcome of this study would help in identifying genetically healthy founder population for the purpose of conservation breeding of this species.

4. Evolutionary and molecular systematic studies in the Indian deer

Of the 40 existing deer species in the world, nine occur in India. Most of these species are listed in IUCN and/or CITES as vulnerable or threatened and also in schedule I-III under Wildlife Protection Act of India (1972). The phylogenetic status of the endemic Indian deers is not fully resolved. To date phylogenies have been proposed on the basis of comparative morphology, chromosomal and biochemical data, behaviour and fossil record. However, modern genetics provides many tools for deducing genetic relationships and inferring phylogeny based on molecular genetic markers. The present work has been initiated to study evolution and molecular systematics in the Indian deer species.

The Indian mouse deer (*Tragulus memmina*, Family: *Tragulidae)* is known to exist 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 the phylogenetics and evolution are still lacking.

Sequences for mitochondrial genes for cytochrome b, 12S rRNA and 16S rRNA, were generated. These sequences were further analysed to make Neighbour-joining trees using various parameters to show the phylogenetic positioning and relationships with respect to other deer species. The topology of three neighbour joining phylogenetic trees was similar as far as the positioning of Indian mouse deer (*Tragulus memmina*) is concerned (Fig. 16). All the trees showed clear distinction between *Tragulina* and *Pecora* supporting the widely accepted classification done by Flower in 1883. It also shows that tragulidae diverged first with respect to all other ruminant families. The family Tragulidae was clearly distinct from the other deer families namely *Cervidae, Antilopinae, Moschidae and Muntiacae.* All the members of *Tragulidae* form a separate clade. This supports the earlier morpho-paleontological studies conducted in the order Ruminantia.

There were remarkable differences between Indian mouse deer (*Tragulus memmina*) and other members of *Tragulidae* namely *T. napu* and *T. javanicus* indicating that it is a genetically distinct species. However, this was grouped with the other two *Tragulus* species namely *T. javanicus* and *T. napu* showing the monophyletic nature of the family. However, there is still a greater need for extensive research to study the molecular evolution of the Indian mouse deer.

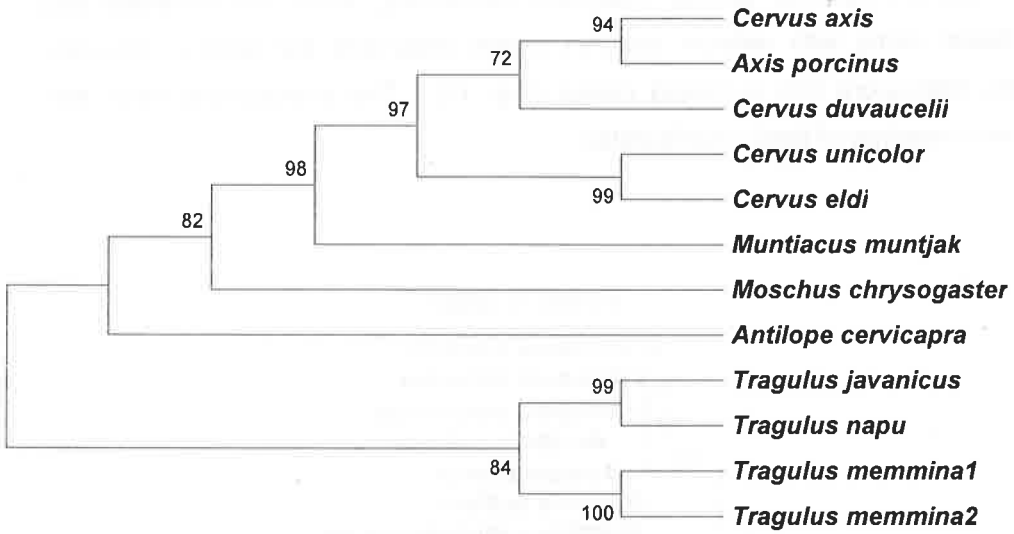

Fig. 16. An unrooted neighbour-joining tree of cytochrome b sequences of different deer species constructed using Kimura- 2 parameter and bootstrap values (>50%).

5. Phylogenetic studies on Indian Scletranian corals

The most well-known members of Phylum Cnidaria are the stony coral that contribute largely in building and formation of some of the richest, the most beautiful, colourful and complex ecosystems on the planet, the coral reefs which also serves as breeding areas for a magnitude number of fish, some of which are commercially important. These true stony corals belong to order Scleractenia. The diversity and taxonomy of genus Acropora has been investigated extensively using morphological characters and fossil records. However this approach has proved insufficient, because of several complex ecological and life-history traits of this genus. Keeping this in mind an attempt was made to study the phylogenetic relationships of Indian Scleratenian using mt DNA sequences.

Phylogenetic relationships within and among three genera of family Acroporidae, namely *Acropora, Montipora and Asteopora* were examined with special emphasis on Indian populations. Analyses using mitochondrial DNA (mtDNA) sequences highlight the usefulness of a molecular approach for examining the phylogenetics of corals. Sequences of mtDNA cytochrome b gene (690 bp) were analyzed. The phylogenetic analysis based on various algorithms, including neighbour joining, maximum parsimony, maximum likelihood and minimum evolution along with network analysis clearly separated the genera *Acropora, Montipora*, and *Astreopora* into 3 distinct clades (Fig. 17). The phylogenetic trees also established the monophyly of family Acroporidae.

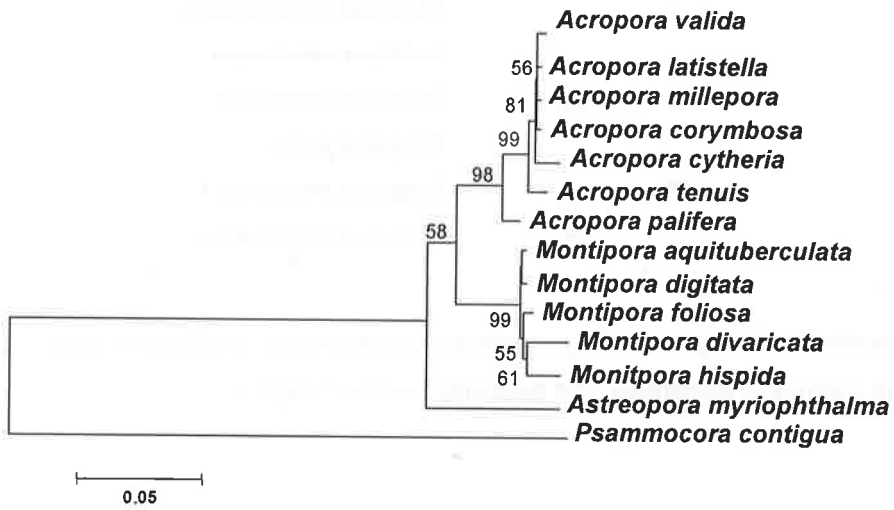

Fig. 17. A neighbour-joining tree of cytochrome b sequences of different scletranian coral species of family *Acroporidae* constructed using Kimura- 2 parameter and bootstrap values (>50%). *Psammocora contigua* was used as an out-group.

6. Conservation breeding programme

CZA has initiated a conservation breeding programme, under which 64 endangered species have been identified and an effort has been initiated to identify and maintain a founder population for each of these species in captivity. LaCONES is an important partner in this initiative and has been conducting genetic analysis for the origin, purity and heterozygosity status. This work involves identification or development of nuclear microsatellite and mitochondrial markers. A large number of blood, tissue and feather samples of different species have been received for genetic health checkup, for eg. Lion Tailed Macaque *(Macaca silenus*), Asiatic Lion (*Panthera leo persica),* Wild Dog (*Canis spp.*), Nilgiri Langur (*Trachypithecus johnii),* Tiger (*Panthera tigris tigris*), Clouded Leopard (*Neofelis nebulosa*), Spectacled Langur (*Presbytis obscura*), Pig Tailed Macaque: *Macaca nemestrina* (pigtail macaque), Tibetan Wolf (*Canis lupus*), Mouse deer (*Tragulus memmina*), Stump Tailed Macaque (*Macaca arctoides*), Hoolock Gibbon (*Hoolock sps*), Binturong (*Arctictic binturong*), Manipuri brow-antlered deer (*Cervus eldi eldi*), Red Jungle Fowl (*Gallus gallus gallus*), Indian Pangolin (*Manis crassicaudata*), Serow (*Capricornis thar*),Star tortoise (*Geochelone elegans*), Snow leopard (*Uncia uncia*) and Elephant (*Elaphus maximus*). A few of the studies completed are reported below :

i. Genetic management of captive tigers (*Panthera tigris tigris*)

As part of the *ex-situ* conservation program taken up by the Central Zoo Authority in several endangered species, we at LaCONES are involved in the genetic aspects of creating and maintaining founder populations in several zoos. We received blood or scat samples of captive tigers from Pune, Assam and Sundarbans for their genetic profiles and to know the levels of inbreeding in these animals, details of which are given below in Table 5.

ii. Genetic testing for the pure/hybrid status of lions

We have received eleven lion blood samples (Table 6), for testing their pure Asiatic origin and heterozygosity status, using DNA markers. The DNA was isolated from these blood samples. The DNA obtained was subjected to PCR amplification and sequencing using mitochondrial DNA cytochrome b gene specific primers (Gaur et al. Unpublished) to establish the pure Asiatic or hybrid origin of these samples. Only the two samples received from National Zoological Park, New Delhi, were found to be of pure Asiatic origin, the remaining were of hybrid origin (Table 6).

Table 5. Genetic profile of captive tigers from Pune, Assam and Sundarbans

Loci	Ragini (Assa	Sultan (Pune)	Rajiya (Pune)	Nandini (Pune)	Tanaji (Pune)	Damini (Pune)	Gypsy (Pune)	Mukta (Pune)	Mastani (Pune)	Jagir (Pune)	Priya (Pune)	SWB1	SWB2	SWB3	SWB4
1	225	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	225	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	206	205	205	223	205	223	224	216	227	223	223	224	-	-	-
	242	223	227	227	227	227	228	223	234	227	223	236	-	-	-
2	160	168	175	188	168	176	172	167	176	175	172	176	-	-	-
	160	188	186	188	176	187	176	186	176	179	176	180	-	-	-
4	158	153	153	153	156	154	157	154	157	156	156	150	-	-	-
	158	156	157	156	156	157	157	157	157	156	156	158	-	-	-
5	209	204	204	204	204	204	204	204	207	207	207	200	-	-	-
	224	207	207	207	207	204	207	207	218	207	207	204	-	-	-
6	262	261	261	261	261	261	261	261	261	261	261	-	252	252	258
	277	269	261	269	261	269	269	269	269	265	265	-	270	252	258
7	154	158	158	158	158	158	158	158	158	162	158	150	173	164	170
	167	158	162	158	162	162	162	158	162	170	166	158	184	170	170
8	179	178	178	178	178	178	178	178	178	178	178	193	-	185	-
	184	178	186	178	186	178	186	186	192	184	178	193	-	185	-
9	202	197	196	196	196	212	197	196	196	212	212	-	-	198	198
	202	212	212	212	212	212	197	212	196	212	212	-	-	198	198
10	152	152	152	152	156	152	152	152	147	147	152	147	156	147	155
	156	156	160	156	160	160	160	152	160	152	152	147	156	165	155
11												-	201	179	170
												-	201	206	177

Table 6. Hybrid status of lions from Assam State Zoo, Nandankanan Zoological Park, Bhubaneswar, Van Vihar National Park, Bhopal and National Zoological Park, New Delhi

S.No.	Samples	Sex	Zoo	Result
1.	Blood (Montu)	Male	Assam State Zoo	Hybrid
2	Blood/0006A2B4E8	Male	Nandankanan Zoological Park, Bhubaneswar	Hybrid
3	Blood / 0006A2AOCF	Female	Nandankanan Zoological Park, Bhubaneswar	Hybrid
4	Blood (Shiva)	Male	Van Vihar National Park, Bhopal	Hybrid
5	Blood (Shivani)	Female	Van Vihar National Park, Bhopal	Hybrid
6	Blood (Bhawani)	Female	Van Vihar National Park, Bhopal	Hybrid
7	Blood (Jamuna)	Female	Van Vihar National Park, Bhopal	Hybrid
8	Blood (Moly)	Female	Van Vihar National Park, Bhopal	Hybrid
9	Blood (Lily)	Female	Van Vihar National Park, Bhopal	Hybrid
10	Blood (Meera)	Female	National Zoological Park, New Delhi	Pure Asiatic
11	Blood (Gaghas)	Male	National Zoological Park, New Delhi	Pure Asiatic

iii. Genetic testing of Eld's deer samples from Nehru Zoological park, Hyderabad, Assam State Zoo, Guwahati, Zoological Garden, Alipore, Kolkata and from National Zoological Park, New Delhi

Brow-antlered deer fecal samples were received from the above zoos to assess heterozygosity, kinship, etc. Genomic DNA was extracted from fecal samples using phenol-chloroform extraction procedure. These DNA samples were subjected to PCR amplification using 7 deer-specific microsatellite primers and genotyping was done as described in Gaur *et al.* (Molecular Ecology Notes, 2003). All the samples amplified with markers 1 to 5 and majority with all the 7 markers (Tables 7-10)

It is concluded that deer NZPF4, NZPF5, NZPM3, NZPM5, ASZF4, ASZF5, ASZM4, ASZM5, ZGAF3, MZGM3, MZGF1 and MZGF3 are heterozygous at more number of loci, as compared to the other deer samples and thus are genetically more vibrant when compared to other deer samples (Tables 7-10).

Table 7. Microsatellite-based genotypes of ten Brow-antlered deer from National Zoological Park, New Delhi

LOCI	ANIMALS IDs									
	NZP F1	NZP F2	NZP F3	NZP F4	NZP F5	NZP M1	NZP M2	NZP M3	NZP M4	NZP M5
1	207	207	207	207	207	207	207	207	207	207
	209	209	209	209	209	209	209	209	209	209
2	136	138	128	124	128	138	128	136	136	136
	136	138	136	136	136	138	138	138	136	138
3	307	307	307	307	307	307	307	307	307	307
	307	307	307	307	307	307	307	307	307	307
4	239	239	239	239	239	239	239	239	257	239
	257	257	241	257	241	241	239	257	257	257
5	251	251	251	251	251	251	251	251	257	251
	251	251	251	251	257	257	251	257	257	257
6	183	183	185	183	183	183	185	183	183	183
	183	183	185	185	183	185	185	185	185	185
7	308	314	308	308	308	314	314	308	308	308
	308	314	314	314	314	314	314	314	308	308

Table 8. Microsatellite-based genotypes of ten Brow-antlered deer from Assam State Zoo, Guwahati

LOCI	ANIMALS									
	ASZ F1	ASZ F2	ASZ F3	ASZ F4	ASZ F5	ASZ M1	ASZ M2	ASZ M3	ASZ M4	ASZ M5
1	209	209	209	209	211	203	203	209	209	209
1	209	209	209	211	211	203	203	209	209	211
2	137	135	135	135	135	149	135	137	135	137
2	137	135	135	137	135	149	135	137	137	137
3	112.	116	112	116	114	116	114	114	114	116
3	112	116	112	116	116	116	114	114	116	118
4	239	239	239	239	257	207	239	233	239	239
4	239	239	239	257	271	239	239	233	257	257
5	172	172	172	138	170	172	136	120	120	120
5	172	172	172	138	172	172	136	120	136	120

Table 9. Microsatellite-based genotypes of four Brow-antlered deer from Zoological garden, Alipore, Kolkata

LOCI	ANIMALS			
	ZGA F1	ZGA F2	ZGA F3	ZGA F4
1	207	207	211	215
1	207	207	215	215
2	137	137	135	134
2	137	137	137	135
3	152	152	152	152
3	154	154	154	154
4	239	251	253	233
4	239	251	253	233
5	160	120	120	120
5	160	120	120	120

Table 10. Microsatellite-based genotypes of seven Brow-antlered deers from Alipore Zoological garden, Kolkata

Loci	Animals						
	MZGF1	MZGF2	MZGF3	MZGM1	MZGM2	MZGM3	MZGM4
1	209	209	209	211	209	209	209
	211	209	211	211	209	211	209
2	124	124	124	124	124	124	124
	130	130	128	128	128	130	124
3	152	152	158	158	152	152	152
	154	152	160	160	152	154	154
4	116	116	116	120	126	116	116
	116	120	126	126	126	120	116
5	241	241	241	257	257	241	241
	257	257	263	261	257	257	257
6	183	183	183	183	185	183	183
	185	185	185	183	185	185	185
7	308	308	308	308	308	308	308
	314	308	314	314	308	314	308

iv. Genetic testing for the species status in of wolves from Pune Zoo

Three blood samples of wolves were sent to know their exact species (Table 11). The DNA isolated from the three samples was subjected to PCR amplification and sequencing using the universal primers for 16S rRNA gene of mitochondrial DNA (Palumbi *et al.* 1991) to generate the species-specific molecular signature. On comparison of the partial 16S rRNA gene of mitochondrial DNA generated from the source of exhibits A, B and C with the molecular signatures of the known wolf species (available in database of molecular signatures generated and maintained by CCMB), it is concluded that the molecular signatures obtained from the exhibits A, B and C showed 100% sequence similarity with the sequence of Indian Grey wolf i.e. *Canis indica*. The above test concludes that all the 3 blood samples/exhibits A, B and C belongs to same species that is the Indian Grey Wolf.

Table 11. Details of wolves sample received for testing species status

Sample markings	Sex / Sample type	Exhibit	CCMB Code
M	Male/Blood	A	PZ-1
F1	Female/Blood	B	PZ-2
F2	Female/Blood (ARC)	C	PZ-3

v. Genetic testing of Clouded leopards

Fifteen Clouded leopard blood samples were received from Sepahijala Zoological Park, Tripura, to assess the genetic health of these animals for future involvement in the Conservation Breeding program of CZA. Genomic DNA was extracted from blood samples of fifteen Clouded leopards using phenol-chloroform extraction procedure. DNA samples were subjected to PCR amplification using microsatellite primers and genotyping was done as described in Gaur *et al.* (Molecular Ecology Notes, 2003 & 2006).

Genotypes of fifteen Clouded leopards for five polymorphic microsatellite loci are given in Table 12. Clouded leopards with Serial nos. 1, 5, 6, 12 and 13 are heterozygous at more number of loci (3 - 4 loci out of 5, > 50 %), as compared to the other samples. On the comparison of the genotypes of fifteen Clouded leopard samples, it is concluded that the Clouded leopards with Serial nos. 1, 5, 6, 12 and 13 are genetically more vibrant when compared to other samples and may be used selectively for conservation breeding.

Table 12. Microsatellite-based genotypes of fifteen Clouded leopards from Sepahijala Zoological Park, Tripura.

ANIMAL IDENTITY		LOCUS									
		L1	L1	L2	L2	L3	L3	L4	L4	L5	L5
1.	Jyamona (F), CL-11 ID No-006B7360D	222	232	151	151	136	140	115	119	168	168
2.	Rani, (F) CL-12 ID No-0006B8AC34	222	232	151	151	136	136	117	117	168	168
3.	Taju (M), CL-13, ID No-0006B7E31B	232	232	151	151	136	136	115	117	168	168
4.	Ghoura, CL-3 ID No-0006B7F07E	222	232	151	151	136	136	119	119	168	168
5.	Rashime, CL-2 ID No-0006B887B1	222	232	151	153	136	136	115	119	166	168
6.	Saakik, CL-1 ID No-0006B8AEFC	226	234	151	151	136	140	117	119	168	168
7.	Nandan (M), CL-10 ID No-0006B73987	232	232	151	151	136	136	117	119	168	168
8.	Parul (F), CL-9 0006B88836	230	230	151	151	136	136	117	119	168	168
9.	Sanjet (M), CL-5 0006B7E0CA	222	232	151	151	136	136	119	119	168	168
10.	Pallbi (F), CL-4 0006B8952A	232	232	151	151	136	136	115	119	168	168
11.	Rahul, CL-14 0006B899A8	232	232	151	151	136	136	115	115	168	168
12.	Nibash (M), CL-8 0006B88A82	230	232	151	151	136	140	117	119	166	168
13.	Ashok (M), CL-7 0006B883AC	232	232	151	153	136	136	115	119	166	168
14.	Rahana (F), SL No-15	230	230	151	151	136	140	119	119	168	168
15.	Priti CL-6 ID No. 0006B886C0	222	232	151	151	136	136	115	115	168	168

vi. Molecular Sexing of Vultures and Eagles

Blood and feather samples were received from Pune Zoo, Pune and Sepahijala Zoo, Tripura (Table 13). The DNA was isolated from these samples and was subjected to PCR amplification using procedure of Griffiths *et al.* (1998) to determine the sexes of the above-mentioned samples. The DNA analysis showed that the blood samples from Pune Zoo were of the female birds, while the feathers received from Sepahijala Zoo were from the male birds.

Table 13. Sexing of Vultures and Eagles received from Pune Zoo, Pune and Sepahijala Zoo, Tripura

S. No.	Sample	Zoo	Zoo Identification	Genetic Identity
1.	Blood (Vial 1)	Pune Zoo, Pune	Long Billed Vulture	Female
2.	Blood (Vial 2)	Pune Zoo, Pune	Bonnelli's Eagle (BW)	Female
3.	Blood (Vial 3)	Pune Zoo, Pune	Bonnelli's Eagle	Female
4.	Feather	Sepahijala Zoo, Tripura	Red spot over right leg	Male
5.	Feather	Sepahijala Zoo, Tripura	Red spot over left leg	Male
6.	Feather	Sepahijala Zoo, Tripura	White spot over both legs	Male

III. CRYOBANKING OF CELLS, TISSUES AND GENES OF ENDANGERED ANIMALS.

1. Genome Resource bank (GRB)

A Genome Resource bank is a systematic collection, cryopreservation and use of biological material like DNA, tissues, blood products, gametes, and embryos from a given species. It is a frozen repository that provides insurance against loss of genetic diversity by retaining viable germplasm from founder animals for future generations. A GRB can be used as a strategic method for managing exchange of genetic diversity among wild populations. At the GRB, LaCONES, we have a collection of 288 properly quantified and catalogued DNA samples of Mammals (40), Birds (215), Reptiles (08) and Corals (25).

2. Establishment of fibroblast cultures of endangered animals

Establishment of cell cultures of endangered animals could serve as a bioresource for resurrection of wild animals if the need arises. Such cell cultures once developed could be stored in liquid nitrogen for posterity. The modern technique of somatic cell nuclear transfer would be the frontline technique to utilize these cell lines to clone or resurrect a species. With this in mind, attempts have been made to establish fibroblast cell cultures from various wild animals. Samples were obtained by skin biopsy either from live or dead wild animals, and fibroblast cell cultures were established. At present fibroblast cell lines of 17 wild animals have been developed and cryopreserved at LaCONES (Table 14 and Fig. 18).

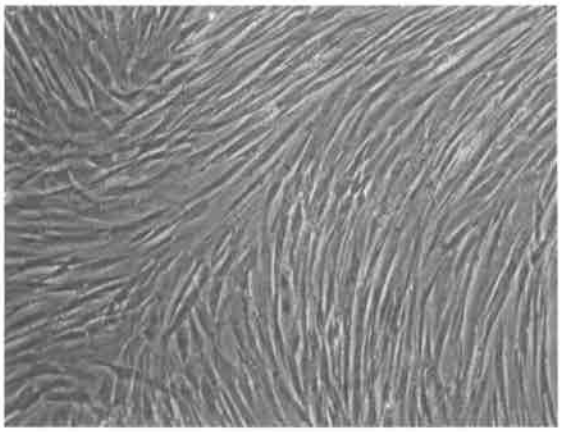

Fig. 18. A confluent monlayer of fibroblast cells developed using an ear skin explant from a wild ass at postmortem

Table 14. Fibroblast cultures from endangered wild animals

Serial number	Species	Number of animals
1.	Barasingha	5
2.	Chousingha	6
3.	Jungle cat	4
4.	Lion	5
5.	Malabar Squirrel	1
6.	Nilgai	16
7.	Porcupine	1
8.	Spotted deer	4
9.	Thamin deer	2
10.	Wild ass	1
11.	Wild dog	2
12.	Star tortoise	1
13.	Leopard	5
14.	Barking deer	1
15.	Mouse deer	2
16.	Blackbuck	9
17.	Bison	2
2.	Tiger	2
10.	Lion tailed macaque	1
12.	Hyena	1
14.	Palm civet	2
15.	Pig tailed macaque	1

3. Cell Cycle synchronization

Attempts were made to optimize conditions for cell cycle synchronization of bison ear fibroblasts at G0 G1 using different approaches such as using cells in confluency, after contact inhibition, serum starvation or treatment with dimethyl sulfoxide (DMSO) (0.5%, 1.0% and 2.0%), sodium butyrate (NaBu) (0.5, 1.0 and 2.0 mM), cytochalasin-B (CB) (7.5 µg/ml), cycloheximide (CHX) (7.5 µg/ml) and 6-dimethyl aminopurine (6-DMAP) (2.0 mM). A small piece of an ear of an adult female bison collected post-mortem 10 h after death was used for the preparation of fibroblast cells (Fig. 19). Higher proportion of G0 G1 phase was obtained when cells were subjected to serum starvation for 48 h (85.4%). In conclusion, this study shows that cells subjected to serum starvation for 24–48 h or confluent monolayer, or cycling cells treated with 1.0% DMSO or 2.0 mM NaBu for 24 h showed best synchronization in G0 G1 phase of cell cycle. Such synchronised cells could be used as a donor source for somatic cell nuclear transfer.

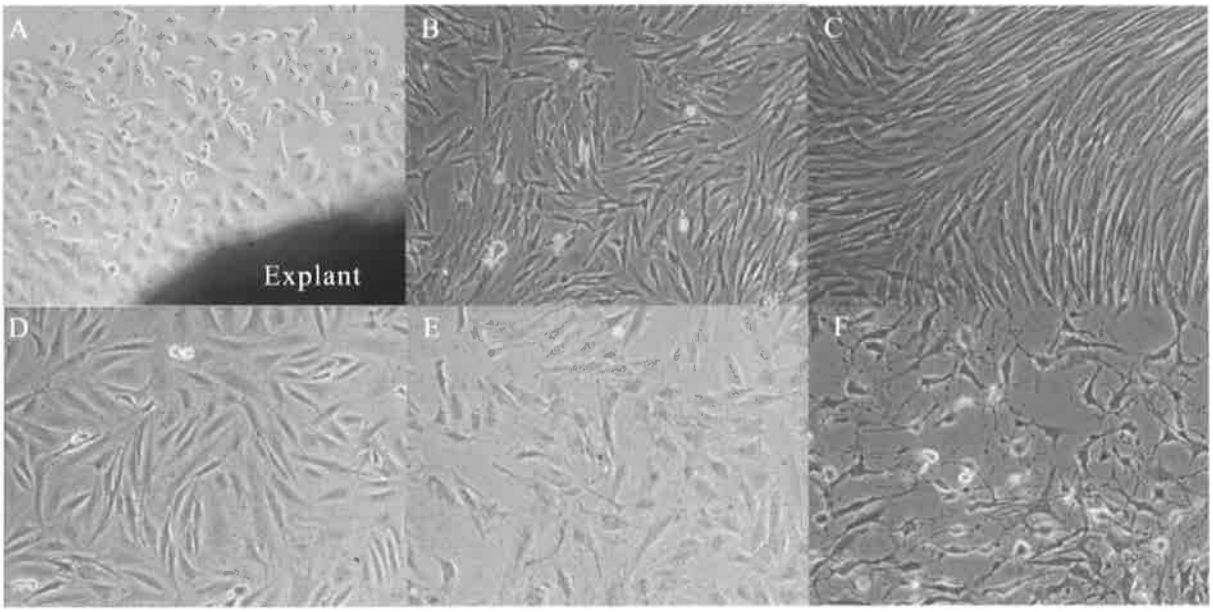

Fig. 19. Culture of bison fibroblast cells (100X). (a) Primary cells from an explant of bison ear; (b) fibroblast cells at 70–80% confluency; (c) fibroblast cells at 90–100% confluency; (d–f) representative images of cycling stage bison fibroblast cells treated with 1% DMSO for 24 h (d) showing normal morphology; cells treated with 7.5 µg ml cycloheximide for 24 h (e) and 2.0 mM 6-dimethylaminopurine (f) for 24 h showing alterations in cell morphology.

IV. WILDLIFE DISEASES

1. Avian pox infection in different wild birds in India

Avian pox infection is a re-emerging disease causing infection in fowl, turkey, pigeon, duck and quail and it has been reported to cause high morbidity and mortality in these birds. In the present study, avian pox viruses recovered from different infected wild birds during 2008-2009 were analysed using two different genes namely *fpv*167 and *fpv*140 to determine if one or more avian poxviruses were spread between different species of wild birds, to ascertain whether avian poxvirus isolated from wild birds of India were identical to those reported in Indian domestic birds, and also to establish the phylogenetic relationships between the avian poxviruses from wild birds from India with all other avian poxviruses previously reported from different parts of the world.

Amplicons of *fpv*167 and *fpv*140, of 578 bp and 1800 bp respectively, characteristic of the avipox viral genes, were amplified by PCR using DNA from viruses isolated from eight Indian wild birds. BLAST and phylogenetic analysis of the sequences of the *fpv*167 and *fpv*140 amplicons indicated that *Fowlpox virus* (FWPV) was the nearest phylogenetic neighbour to the viral isolates, from two Indian peacocks (*Pavo cristatus*), two golden pheasants (*Chrysolophus pictus*), one silver pheasant (*Lopahura nycthemera*), one sparrow (*Passer domesticus*). However, the two isolates from the Indian little brown dove (*Stigmatopelia senegalensis*) and the common wood pigeon (*Columba palumbus*) formed a separate cluster with turkeypox (TKPV) and pigeonpox virus (PGPV) isolates when the phylogenetic tree was constructed using the sequence of *fpv*167 (Fig. 20). When the phylogenetic analysis was done using the *fpv*140 gene sequence both isolates formed a cluster with isolates of PGPV (Fig. 21). Thus the results support that *fpv*140 gene along with the *fpv*167 gene should be used for phylogenetic analyses of avipoxviruses for better discrimination of the viruses. Additionally, avian poxvirus isolated from wild birds of India were identical to those reported in Indian domestic birds, and phylogenetically related to avian poxviruses reported from different parts of the world. To our knowledge, this is the first molecular characterization of avian poxviruses infecting different wild birds in India. The study shows that FWPV and PGPV cause infection in wild birds irrespective of the species of birds indicating that these viruses are not species specific. Thus these viruses, which are not host specific have the ability to cause infection in game birds, endangered birds and domestic birds and therefore could spread easily. To our knowledge, this is the first molecular characterization of avian poxviruses infecting different wild birds in India.

2. Molecular prevalence and characterization of *Hepatozoon ursi* sp. infection in Indian sloth bears (*Melursus ursinus*)

Hepatozoonosis is a parasitic disease caused by protozoans affiliated to the genus *Hepatozoon* (phylum Apicomplexa, suborder Adeleorina, family Hepatozoidae), which infect a wide variety of mammals, birds, reptiles, and amphibian hosts. The sloth bear (Melursus ursinus) inhabits India, Sri Lanka, Nepal, Bhutan and Bangladesh. In India, it ranges from the foothills of the Himalayas to the southern tip of the Western Ghats. The International Union for Conservation of Nature (IUCN) 2008 Red List of Threatened Species (IUCN 2008) lists Indian sloth bear as vulnerable. However, in India, the Indian sloth bear has been listed as a Schedule I (meaning it is endangered) animal of the Indian Wildlife Protection Act, 1972, because of a serious threat to its survival in the wild due to poaching, trade and loss of habitat. The objective of this study was to investigate whether the Indian sloth bear are infected with a *Hepatozoon* species and know the prevalence of infection and then to identify the species based on phylogenetic analysis of the partial 18S rRNA gene sequence.

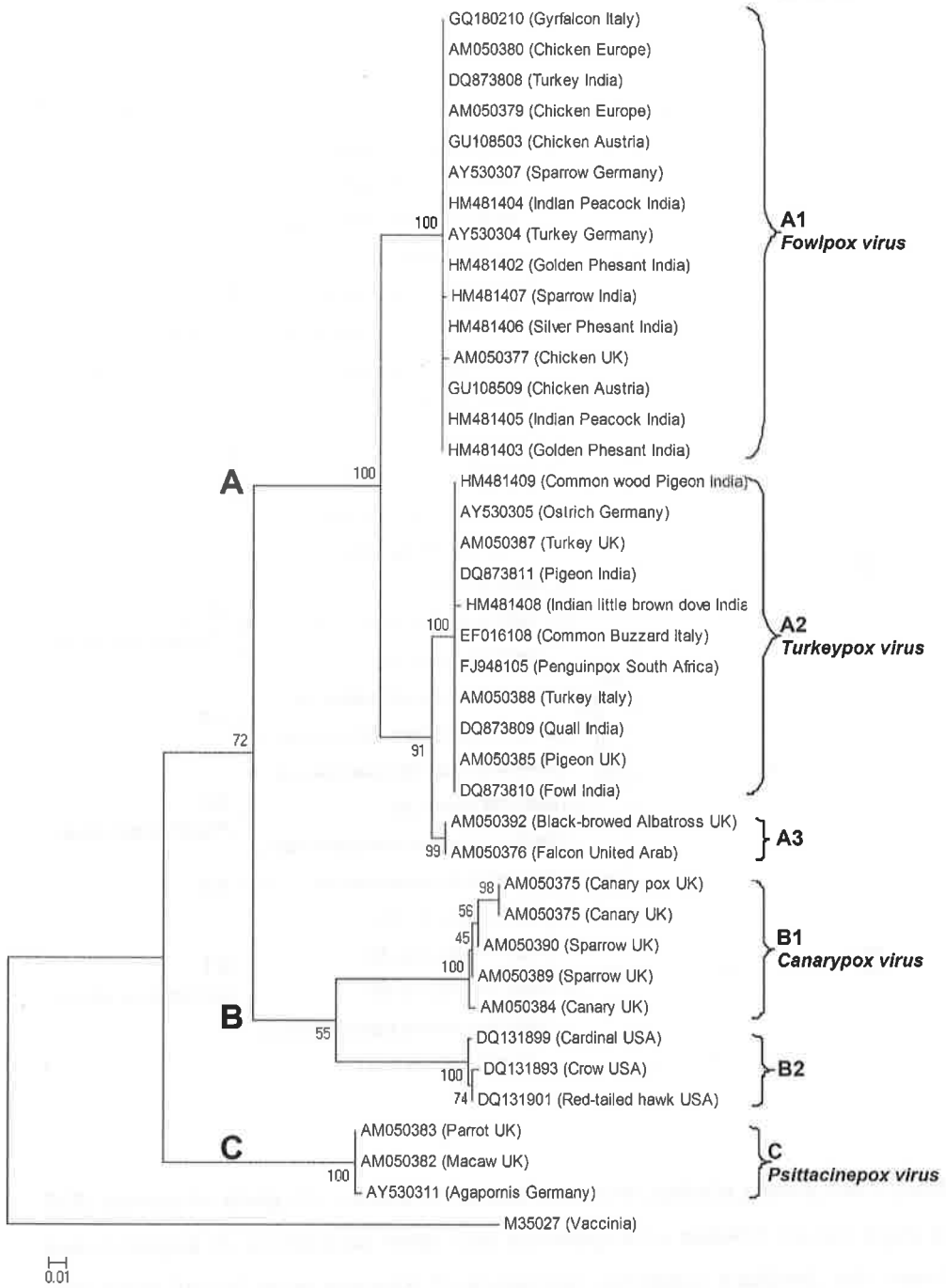

Fig. 20. Neighbour-joining phylogenetic tree based on the *fpv*167 gene sequence (483 bp) of the eight Indian isolates of avipoxvirus and other sequences of avipoxviruses retrieved from the GenBank database. Sequence of Vaccinia virus *fpv*167 gene was used as an out-group. Aviapoxvirus clades A1, A2, A3, B1, B2 and C are labelled. Booststrap values for 1000 replicates are shown at the respective nodes. Bar indicates 1 substitution per 100 nucleotides

Fig. 21. Neighbour-joining phylogenetic tree based on the *fpv*140 gene sequence (802 bp) of the eight Indian isolates of avipoxvirus and other sequences of avipoxviruses retrieved from the GenBank database. Sequence of Vaccinia virus *fpv*140 gene was used as an out-group. Aviapoxvirus clades A1, A2, A3, A4, A5 and B are labelled. Booststrap values for 1000 replicates are shown at the respective nodes. Bar indicates 1 substitution per 100 nucleotides

We used partial 18S rRNA gene sequence to detect the occurrence of *Hepatozoon ursi* sp. in Indian sloth bears and to characterize the parasite based on phylogenetic analysis (Fig. 22). Out of fifty four blood samples of Indian sloth bears (captive and wild), *Hepatozoon* infection could be detected in 38 (70%) using *Hepatozoon* specific PCR suggestive of high prevalence of in Indian sloth bears. Sequencing of partial 18S rRNA gene of the positive samples and BLAST analysis indicated that the nearest phylogenetic neighbour was *Hepatozoon ursi* sp. with which they exhibited 99-100% similarity (Fig. 22). Additionally, *Hepatozoon* sp. isolated from wild sloth bears of India were identical to those in captive sloth bears and

phylogenetically related to *Hepatozoon ursi* sp. reported from Japanese black bears from Japan. Considering the high prevalence (70%) of *H. ursi* infection in rescued Indian sloth bears maintained at three different geographical locations in India and in free ranging wild sloth bears it would appear that *H. ursi* may be one of the common parasites in Indian sloth bears.To our knowledge, this is the first report on the molecular characterization of *Hepatozoon ursi* sp. infection in Indian sloth bears. *Hepatozoon* sp. may be a potential pathogen and an opportunistic parasite in immuno-compromised animals and could thus represent a threat to endangered Indian wild felids and canids.

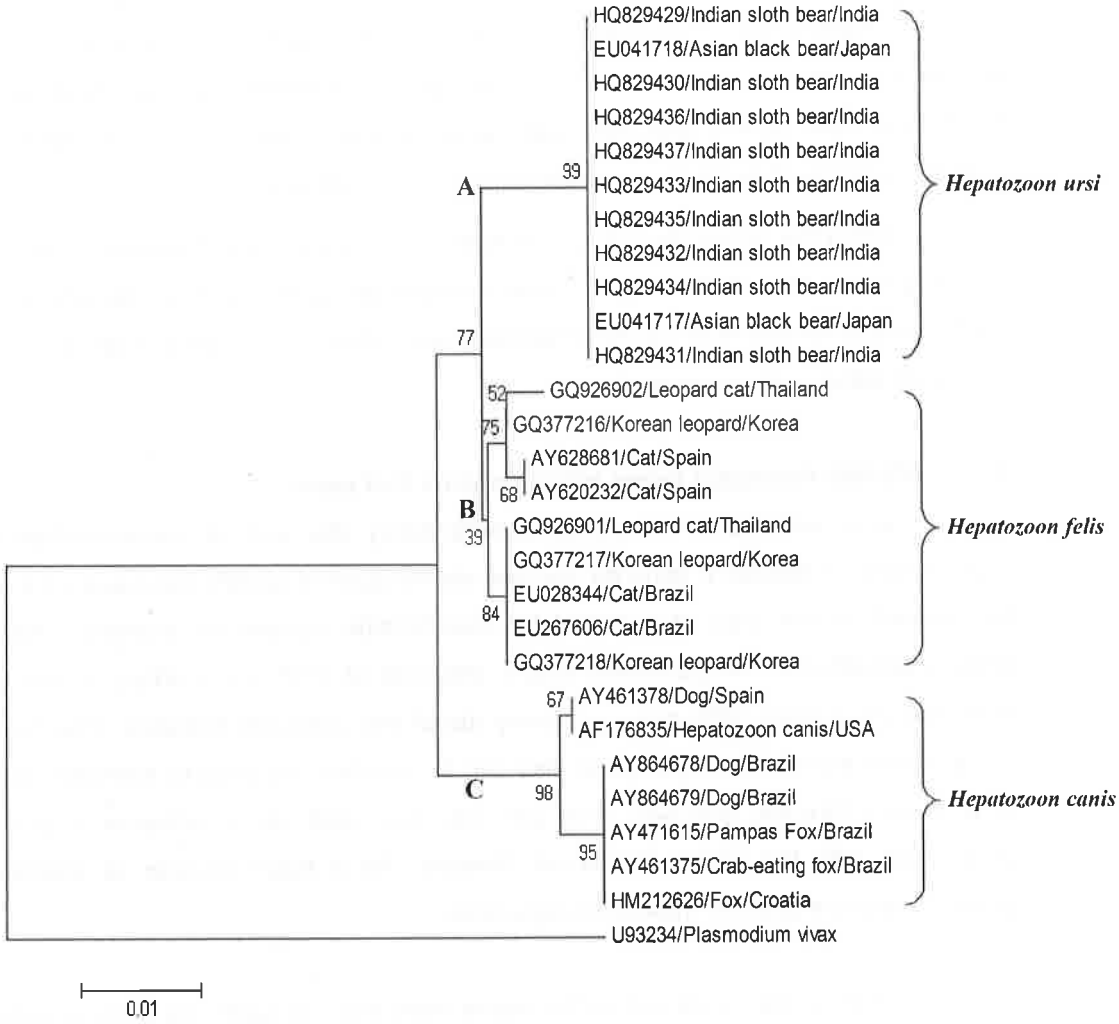

Fig. 22. Phylogenetic analysis of the *Hepatozoon* sp. partial 18S rRNA gene sequence amplified from the blood of Indian captive and free-ranging sloth bears using primers BTH-F1 and BTH-R1. The phylogenetic tree was constructed using partial 18S rRNA gene sequence corresponding to a stretch of 625 nucleotides common to all the sequences obtained in the study and retrieved from the database. 18S rRNA gene sequence of Plasmodium vivax was used as the out-group. Bootstrap values are shown at the nodes. Jukes-cantor algorithms were utilized, and phylogenetic trees were constructed by Neighbour-joining method using MEGA 4.1 program.

V. WILDLIFE FORENSICS

1. Wildlife forensics using universal primers

LaCONES provides wildlife forensic services to the nation for the purpose of investigating wildlife related crimes. A pair of 'Universal Primers" developed at CCMB (US/PCT Patent No. 7141364) is being routinely used for PCR-based species identification from a large varieties of biological specimens such as meat, cooked meat, skin, horn, bones, tusk, hair, nails, snake poison, scales, faeces etc. seized and forwarded by forest and police departments and the judiciary.

During the period April 2007 to March 2012, a total of 413 wildlife crime cases involving more than 813 samples were received and DNA analysis reports for 376 cases have been submitted to the forwarding authorities and revenue of about Rs. 20.25 lakhs was generated.

2. Wildlife forensics using mitochondrial COI gene

Efforts were also aimed at standardizing the use of mitochondrial COI (Cytochrome c Oxidase I) gene for species identification in wildlife forensics. COI gene has served as the core of a global bio-identification system for animals. The COI primers amplified a comparatively longer segment of DNA (i.e. 660bp), which gave authentic information with more accuracy about the unknown samples. The present study clearly showed that COI gene also can be successfully used to establish identity of unknown forensic samples and can also be used as a universal marker in conjunction with the Cyt b 'Universal Primers', for a large number of species to generate species-specific molecular signatures.

Further, a data bank for all the cases dealt with, till date has been developed and multiple information related to the cases, report status (ready/pending for payment etc.), correspondence, reminders, payment status (money due/paid), sequences in fasta/electopherogram format, blast results & sequence alignments can be retrieved with several filter options at a click of mouse.

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