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Conservation of wild animals by assisted reproduction and molecular marker technology

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Wild animals are an integral component of the ecosystem. Their decimation due to abrupt natural calamities or due to gradual human intervention would be disastrous to the ecosystem and would alter the balance in nature between various biotic components. Such an imbalance could have an adverse effect on the ecosystem. Therefore, there is an urgent need to put an end to the ever increasing list of endangered species by undertaking both *in situ* and *ex situ* conservation using tools of modern biology, to ascertain the degree of genetic variation and reproductive competence in these animals. This review highlights the development and use of molecular markers such as microsatellites, minisatellites, mitochondrial control region, cytochrome b and MHC loci to assess the genetic variation in various Indian wild animals such as the lion, tiger, leopard and deer. The review also presents data on the semen profile of the big cats of India. Reproductive technologies such as cryopreservation of semen and artificial insemination in big cats are also highlighted.

Keywords: Artificial insemination, Cryopreservation, MHC, mt DNA, Microsatellite, SNPs, Semen profile

"Like winds and sunsets, wild things were taken for granted until progress began to do away with them. Now we face the question whether a still higher 'standard of living' is worth its cost in things natural, wild and free. For us of the minority, the opportunity to see geese is more important than television, the chance to find a pasque-flower is a right as inalienable as free speech."
Aldo Leopold, excerpt from foreword of A Sand County Almanac, 1948.

Extinction of species is part of the natural process of evolution and is irreversible, however, it is now occurring at a much higher rate than speciation because of human activities such as habitat destruction and poaching. Such activities are known to fragment habitats and populations thus facilitating inbreeding of animals and genetic homogenization, which has negative effects such as poor reproductive performance, low fecundity, increased juvenile mortality and susceptibility to diseases. Thus, there is a need to arrest the accelerated depletion of the species by using multi-dimensional conservation efforts spanning the disciplines of politics, science, diplomacy and economics. In India, the situation is no way different. India is considered a hot spot of biodiversity country contributing about 7.6% of mammals, 12% of birds, 11% of fishes and 6% of flowering plants to the total world population. But, unfortunately, India also has 172 animal species considered globally threatened or 2.9% of the world's total number of threatened species[1]. Worldwide, extinction threatens 11% of birds, 25% of mammals and 34% of fish species[2,3]. Given the current trends and pace of extinction, there is an urgent need to conserve and propagate species both *in situ* (in nature) and *ex situ* (in intensively managed programmes in captivity, in zoos).

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 behavior 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 as assisted reproduction (AR). It involves application of techniques such as: semen collection, gamete and embryo cryopreservation, oestrus induction and artificial insemination (AI) and

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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 need 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 present review is an attempt to highlight research attempts made in India using Assisted Reproductive Technologies (ART) and molecular marker technology to achieve the long-term goal of conservation of wild animals.

ASSISTED REPRODUCTION IN CONSERVATION BIOLOGY

Evaluation of fertility status

Prior to undertaking any ART to conserve animals by way of improving their reproductive performance, it is absolutely essential that criteria be established for ascertaining the fertility status of animals. This is best done by evaluating the animals with respect to their semen profile, hormonal profile, reproductive cyclicity and general health parameters. Such evaluation in human beings, pets and domesticated animals does not pose any serious problem. In fact, over the years such evaluations have moved from the hands of the physician to laboratory trained personnel with automation making things even easier. However, though this knowledge base would be useful for evaluating the fertility status of wild animals, obtaining the data which would then form the basis for the analysis is not as easy, and in many cases it is difficult and sometimes even unachievable. For instance, collection of semen or blood which normally does not pose any problems in domestic animals, is unassumingly a difficult procedure involving anaesthesia and electroejaculation in wild animals. So also, steroid radioimmunoassay for studying the reproductive cyclicity is a big challenge in wild animals since it has to be done using scat samples and not serum, because to obtain the latter the animals need to be anaesthetized at regular intervals, which is not a recommended procedure. Another most challenging issue is the detection of oeatrus and ovulation in wild animals, which though can be done routinely in other animals, is not easily done in these animals. This part of the review would highlight the difficulties, the successes and failures related to AR in wild animals.

Semen collection

Electroejaculation (EE), first employed by Weisbroth and Young[4], has become the standard semen collection technique in wild species though it has been possible to collect semen from hand-reared cheetah[5], chimpanzees and Gorillas[6,7] without electroejaculation.

In EE, a rigid rectal probe containing silver, copper or stainless steel electrodes arranged in either circular or longitudinal configuration is used to pass either alternating- or direct-current from an electrostimulator (Fig. 1). A combination of ketamine hydrochloride (2.2 mg/kg body weight of animal) and xylazine hydrochloride (1.1 mg/kg body weight of animal) have been used as anaesthesia in mega cats (lion, tiger, leopard and jaguar) and at times to maintain a

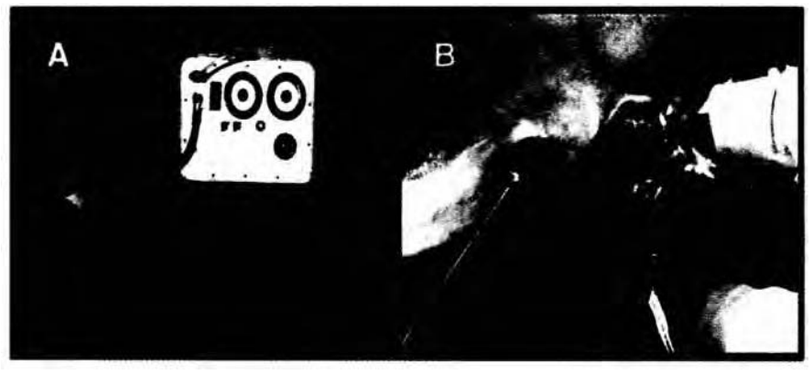

Fig. 1 — (A) Eelectrostimulator and rectal probes used for electroejaculation, B) Positioning of rectal probe and collection of semen from a lion.

surgical plane of anaesthesia, an additional dose of ketamine hydrochloride (50 to 100 mg) is given intravenously. During EE, the anaesthetized animal is placed in lateral recumbancy, faeces are removed manually from the rectum and the jelly-lubricated probe is inserted into the rectum of the animal such that the electrodes of the probe are oriented ventrally (against the male accessory sex organs). The stimulation regimen used for semen collection in wild cats[8] consists of a total of 80 electrical stimuli divided into three series consisting of 30 (10 stimulations at 2, 3 and 4 V: series 1), 30 (10 stimulations at 3, 4 and 5 V: series 2), and 20 (10 stimulations at 4 and 5 V: series 3) stimuli, respectively. Each stimulus is given in a 3-sec on and 3-sec off pattern with a rest of 3-5 min between each series of stimuli[8]. A moderately rigid extension of the hind limbs is indicative of an adequate electrical stimulus. Limb extension also can be used as an index of proper positioning of the probe. Using this procedure, it has been possible to induce electroejaculation in lions (100%), tigers (83%), leopards (85%) and spotted deer (100%).

In avian species, manual massage technique is commonly practiced for semen collection in domestic birds[9] and with slight modifications in uncooperative non-domestic birds like cranes[10], budgerigar[11] and pheasants[12]. We have been successful in collecting normal ejaculates consistently from the Blue rock pigeon (*Columba livia*) and the White backed vulture (*Gyps bengalensis*) by the manual massage technique[13].

Semen evaluation

The ultimate test of male fertility is conception. The fertilizing capacity of the spermatozoa can be evaluated by examining ova for fertilization following artificial insemination (AI). Unfortunately for the majority of wild animals the time of the ovulation is not known and AI techniques have not been perfected. Even getting the ova from wild endangered species for IVF studies is very difficult. These problems have enforced the use of laboratory tests for assessing the semen quality, which are then correlated to fertility. Traditional parameters of semen quality such as ejaculate volume, sperm motility, concentration and morphology have been used to assess the fertility status of mega cats of India and other wild animals. Table 1 shows the semen characteristics of lions, tigers[8] and leopards[14] of India. Briefly, in wild cats, ejaculate volume showed a wide range (0.5 to 9 ml), sperm concentration varied between 42 to 55 million

Table 1 — Semen characteristics and serum testosterone levels of captive tigers, lions and leopards from Nehru Zoological Park, Hyderabad, Sakkarbaug Zoo, Junagadh and Nandankanan Zoological Park, Bhubaneshwar in India

[Values are mean ± SD. Figures in parentheses indicate range of variation]

Parameter	Tigers (n = 16)	Lions (n =7)	Leopards (n=11)
Age (year)	8.8±2.7 (5.4-15.0)	9.3±4.2(5.1-15.8)	14.35 ± 3.93 (7.25-18.58)
Semen pH	7.66±0.09 (7.0-7.9)	7.85±0.67 (7.4-9.8)	7.39 ± 0.23 (7-7.7)
Ejaculate volume (ml)	1.41±.0.9 (0.3-3.7)	3.94±2.4 (1.25-9.0)	1.57 ± 1.26 (0.5-4)
Sperm concentration ($\times 10^6$/ml)	42.1±20.2 (12-84)	52.1±25.1 (20-95)	55.78 ± 38.67 (10-142)
Sperm motility (%)	46.9±14.9 (25-80)	63.1±18.0 (35-90)	57.05 ± 16.96 (20-90)
Normal spermatozoa (%)	74.82±11.7 (55-93)	77.09±10.4 (60-93)	71.92 ± 15.32 (38-90)
Abnormal spermatozoa (%)	25.18	22.91	—
1. Macrocephalic	0.67±0.47	0.73±0.71	0.94 ± 0.72 (0.1-2.7)
2. Microcephalic	0.70±0.49	0.39± 0.29	0.47 ± 0.38 (0.1-1.33)
3. Bicephalic	0.51±0.75	0.48±0.58	0.94 ± 1.10 (0-2.8)
4. Tricephalic	0.02±0.08	0.05±0.10	—
5. Detached head	0.95±0.08	0.87±0.98	—
6. Amorphous head	1.69±1.05	2.42±2.00	2.25 ± 1.81 (0.43-7.6)
7. Tightly coiled tail	15.29±10.14	11.47±4.80	13.59± 10.41 (1.4-39.28)
8. Bent neck	0.83±0.62	0.0	0.83 ± 0.98 (0.2-3.8)
9. Bent midpiece	0.42±0.16	0.57±0.80	—
10. Bent tail	2.80±1.64	4.73±4.49	3.01 ± 2.53 (0.2-9.5)
11. Biflagallate	0.09±0.22	0.16±0.15	—
12. Cytoplasmic droplet	0.91±0.66	0.70±0.66	0.95 ± 0.67 (0.17-2)
13. Combined defects**	0.30±0.08	0.34±0.49	2.66 ± 4.09 (017-1.14)
Serum testosterone (pg/ml)	1729±1134 (2250-4100)	1849±527(500-5000)	893.77 ± 317.75 (625-1562)

**Spermatozoa showing more than one defect.

per ml of ejaculate, percentage motility of spermatozoa ranged from 46 to 57% whereas the percentage of normal spermatozoa varied over a very narrow range (71 to 77%, Fig. 2A to C). The ejaculate volumes, percent motile spermatozoa and sperm concentration of tigers and leopards were less than that observed in lions.

Our studies in spotted deer (*Cervus axis axis*) demonstrated that the ejaculate volume ranged from 0.2 to 7 ml, percentage motility was 35 to 80% with the sperm concentration showing a wide range (4 to 4000 million sperm per ml), whereas the normal sperm ranged from 50 to 80% (Fig. 2D).

Similar studies have been conducted in birds such as the Blue rock pigeon (*Columba livia*) and White-backed vulture (*Gyps bengalensis*) to study semen characteristics. The results showed that the Blue rock pigeon produces semen throughout the year except for a slight variation in semen characteristics during summer (Fig. 2E). In vultures, ejaculate volume ranged from 0.5 to 2 ml and sperm concentration varied from 5 to 200 million per ml (Fig. 2F).

Is sperm pleiomorphism an indication of inbreeding depression?

A close relationship seems to exist between genetic diversity and sperm pleiomorphism as reported in cheetah, where high proportion of pleiomorphic spermatozoa (>60%)[15,16] correlated with low levels of genetic polymorphism[15,17,18]. Earlier studies by Wildt *et al.*[15] showed that the Asiatic lions (Sakkarbaugh Zoo, Gujarat) are genetically monomorphic and there is a significant decrease in motile spermatozoa per ejaculate and an increase in pleiomorphic spermatozoa to 66%. Further, it was concluded that the Asiatic lion, which has experienced a severe population bottleneck and has been inbreeding ever since it was isolated as a small population, is a highly endangered species. However, our studies on Asiatic lions showed that the mean percentage of abnormal spermatozoa in lions was significantly lower (23%) than that reported by Wildt *et al*[15]. In our study spermatozoal pleiomorphism in tiger is about 25%[8] and is similar to the observations made by Wildt *et al.*[19] whereas in leopard, it was about 28%. Thus, the percentage of pleiomorphic spermatozoa in Indian lions, tigers and leopards is not alarming. A study carried out by our group on genetic variation showed that the Asiatic lions and Indian tigers showed 25.82% and 22.65% heterozygosity respectively[20]. These results were comparable to the genetic variability in 50 to 125-year-old skin samples from museum specimens of Indian tigers (21.01%). Thus, it can be concluded that the low genetic variability observed in the mega cats could be an inherent feature of these species and not the consequence of prolonged inbreeding[20].

Testosterone: An indicator of male fertility

Testosterone, the male sex hormone, is essential for normal spermatogenesis in mammals and reduced amounts would impair spermatogenesis. It has been observed that the serum testosterone level significantly correlated with semen characteristics in various species as in the African elephant[21], wildbeest and greater kudu[22], domestic cat [23] and Eld's deer[24]. Wildt *et al.*[15] reported that the inbred lions in Gir forest, which exhibited more than 60% abnormal spermatozoa had serum testosterone levels three-fold lower (<1 ng/ml) as compared to the outbred lions (~1.5 ng/ml) from the Serengeti population. However, in a recent study by us, it was observed that the mean serum testosterone level in the Asiatic lions was higher (1.85 ng/ml) compared to the report of Wildt *et al.*[15]. In fact, in most of the Asiatic lions, the levels of testosterone, the spermatozoal concentration,

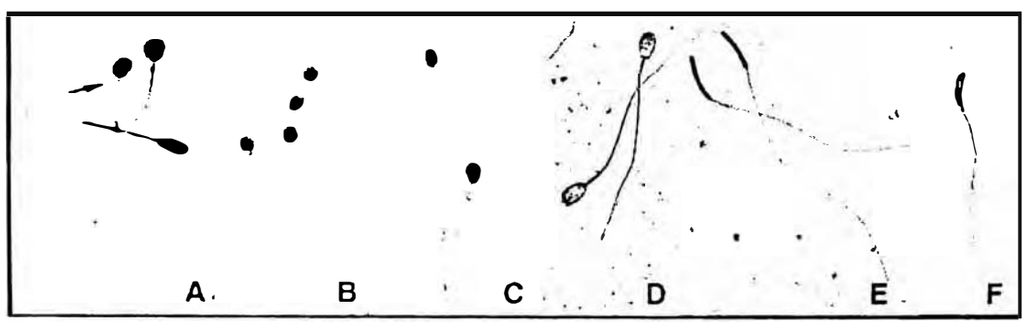

Fig. 2 — Bright field photomicrographs of spermatozoa of wild animals: (A) the tiger, (B) the lion, (C) the leopard, (D) the spotted deer, (E) the blue rock pigeon and (F) the white-backed vulture.

the percentage of motile spermatozoa were normal and the incidence of morphologically abnormal spermatozoa was low (<25%, Table 1). These results were comparable to the out-bred population of Serengeti, thus, implying that they are not inbred. In tigers, mean testosterone level was comparable with the lions (1.72 ng/ml)[8], but in leopards (from Indian zoos), it was lower than those in lions and tigers (0.89 ng/ml), but comparable to the North Chinese leopard[25].

Computerized evaluation of semen

Computer assisted semen analysis (CASA) is routinely used to eliminate subjective nature of routine semen evaluation and to facilitate rapid analysis of various motility parameters of spermatozoa which are normally very difficult to quantify by visual examination. Apart from determining the sperm count and the number of motile spermatozoa, CASA provides data for various motility characteristics of spermatozoa.

CASA (HTM-IVOS, Version 10, Hamilton Thorne Research Inc. Danvers, MA, USA) has been standardized to evaluate the spermatozoal motility parameters of wild cats (tiger, lion and leopard)[14,26] and such studies formed the basis for evaluating male fertility in wild animals. CASA analysis showed that the trajectories of spermatozoa from neat semen were linear and travelled more distance than the spermatozoa of cryopreserved semen (Fig. 3). However, such changes did not affect the fertilizing ability of cryopreserved spermatozoa of tiger, lion and leopard as evidenced by zona-free hamster oocyte penetration assay[14,26].

Similarly, CASA was also used for semen analysis of the Blue rock pigeon as a model for avian species and the motility parameters and motility pattern of fresh- and cryopreserved-spermatozoa of pigeon were studied (communicated).

Hamster zona-free oocyte penetration test

The hamster zona-free oocyte penetration test is extensively used to explore the fertilizing capacity of spermatozoa. This heterologous sperm penetration assay has been developed for widespread use in the prediction of IVF success in humans[27] and also in numerous wild species including dolphin[28], marmoset[29], rhesus macaque[30], budgerigar[11], lion-tailed macaque[31], tiger[32], and cheetah[5]. This test may find its greatest usefulness in the evaluation of various cryopreservation protocols.

Our studies revealed that the spermatozoa of lion, tiger and leopard were capable of binding and penetrating zona-free hamster oocytes (Table 2). The higher percentage of penetration of tiger spermatozoa could be due to less percentage of pleiomorphic spermatozoa (10%), as against 40% in lions[8]. Earlier studies had indicated that in domestic cats and leopards with more than 60% normal spermatozoa, a greater number of zona-free hamster oocytes or zona-intact cat oocytes were penetrated compared to ejaculates of teratospermic cats which had more than 60% pleiomorphic spermatozoa[23,33,34], thus indicating that teratospermia affects gamete interaction. IVF studies in puma and tiger also showed that teratospermia may be responsible for poor fertilizing ability of spermatozoa[35,36]. In our experiments, it was observed that both the neat and cryopreserved spermatozoa of lions, tigers and leopards successfully penetrated the zona-free hamster oocytes (Table 2).

Cryopreservation of semen

Semen cryobanking is a great boon to the conservation and management of wildlife, especially endangered species. Preserved semen could be used as and when needed and more importantly to preserve genetic heterozygosity of a rare population held in captivity. Frozen repositories help ensure wild populations against natural-and human-induced

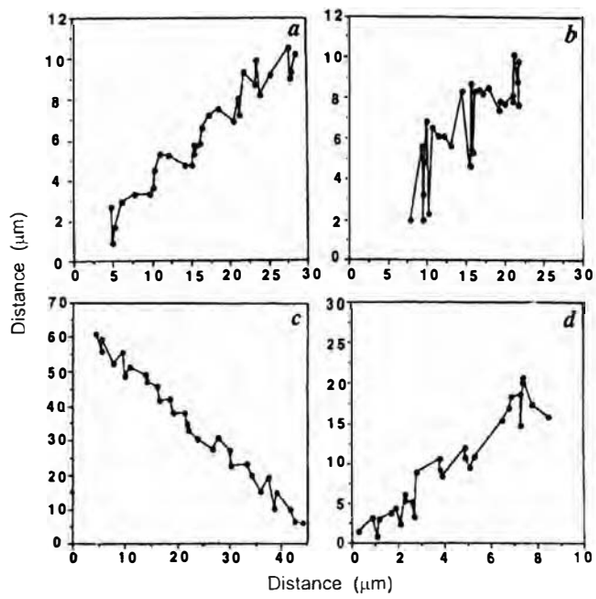

Fig. 3—Motility track of a tiger (a and b) and a lion (c and d) spermatozoon prior to (a and c) and after cryopreservation (b and d) as obtained directly on HTM-IVOS motility analyzer.

Table 2 — *In vitro* penetration of zona-free hamster oocytes by tiger, lion and leopard spermatozoa

[Values are mean ± sd. Figures in parentheses indicate variation in the number of sperm that penetrated the oocytes]

Animal	Nature of semen sample	No. of oocytes used	No. of oocytes penetrated (%)	Av. number of sperm/oocyte
Lion	Neat	96	73.75 ± 2.5	23.82 ± 1.01 (7-53)*
Tiger	Cryopreserved	33	87.25 ± 2.98	9.35 ± 0.83 (2-20)
Leopard	Neat	42	33	24.29 ± 0.9
	Cryopreserved	36	29	13.22 ± 4.02

catastrophes and also help to introduce disease-resistant genes from the wild population. Although cryopreservation protocols of cattle and human spermatozoa are very well established, protocol developed for one species is not universally applicable to all animals and needs to be modified and standardized for each species[37,38]. These protocols depend on various factors such as semen diluent, cryoprotectant, the freezing regime and the storage (straws, ampules or pellets)[37,39].

Studies have been carried out to standardize protocols for cryopreserving the semen of lions, tigers, and leopards. The method followed is essentially the same as that used for human semen with minor modifications. The extender used was Tris-Egg Yolk buffer (TYB) and the cryoprotectant was glycerol.

Table 3 summarizes the results of semen cryopreservation for tigers, lions and leopards. The data indicated a decrease of 15-50% in percentage motility of tiger, lion and leopard spermatozoa following freeze-thawing, which is in agreement with the reports of Donoghue *et al.*[40] and Byers *et al.*[41] in the tiger. Moreover, cryopreservation of spermatozoa also results in damage to the acrosome of spermatozoa, thus affecting its fertilizing ability[8,14].

Successful attempts have also been made to cryopreserve the semen of spotted deer, Blue rock pigeon and White-backed vulture.

Table 3 — Quantitative motility (%) of the spermatozoa of tigers, lions and leopards, prior to and after cryopreservation

[Values are mean ± SD]

Species (number)	Motility before cryopreservation	Motility after cryopreservation
Tiger (11)	53.75 ± 15.75	24.12 ± 8.39
Lion (5)	61.42 ± 22.49	30.4 ± 17.98
Leopard (6)	59.37 ± 8.63	32.14 ± 9.14

Non-invasive artificial insemination

Induction of oestrus and ovulation in Mega cats

The use of exogenous gonadotropins to mimic the natural female cycle and induce ovulation in cats was carried out as early as 1930s[42,43]. Our standardized protocol for induction of oestrus and ovulation in big cats involves two doses of eCG 24 hr apart followed by hCG 80 hr later. Approximately 40 hr to 45 hr post hCG, the females were inseminated transcervically. This protocol induced behavioral oestrus in 80% of females and faecal progesterone data suggested that 75% of animals successfully ovulated following gonadotropic treatment (unpublished data).

Induction of oestrus in spotted deer

Oestrus synchronization has been widely used in ungulates with variable success[24,44]. However, the exact time of ovulation has been very difficult to assess, thus the routine use of AI and ET in these animals is quite limited[45,46]. Moreover, behavioural cues are not reliable indicators of oestrus detection in these animals. Commonly acceptable procedure for oestrus synchronization in ungulates involves the use of CIDR intravaginal devices, MAP-pessaries or $PGF_{2\alpha}$.

Attempts are in progress to synchronize oestrus in spotted deer using Crestar ear implant (Intervet), a progestin slow-releasing device followed by administration of eCG at the time of implant removal.

Transcervical artificial insemination in mega cats

Incompatibility between male and female preventing the natural mating and aggression towards female are common behavioural problems in mega cats and in such cases AI seems promising. Recent developments in assisted reproductive techniques such as induction of ovulation, artificial insemination, IVF and embryo transfer have helped in effective conservation of many critically endangered animals[24,47-49]. The technique of AI, although routinely practiced in domestic animals, has not been commonly applied to wild animals. Success has been reported using laparoscopic intrauterine (surgical)

insemination in some of the wild animals such as tiger[50], cheetah[51], puma[52], snow leopard[53] and Eld's deer[24]. In our laboratory, attempts are being made to standardize non-surgical (non-invasive) AI following exogenous gonadotropins in lion and leopard. Female cats following hormonal induction of ovulation were sedated, placed in lateral recumbancy and the cervix was dilated with a speculum. Freshly collected neat semen (sperm count 30-60 million/ml, 60-70% sperm motility and <15% sperm abnormalities) was deposited near the cervical-os using cattle AI-sheath attached to a 20 ml disposable syringe. However, no pregnancy has been achieved so far. Our studies suggest that the failure of AI is probably not due to lack of ovulation but due to either the unavailability of spermatozoa at the site of fertilization or a lack of proper management after insemination.

Detection of ovulation by estimation of progesterone in faeces

Monitoring ovarian cyclicity is a prerequisite for Species Survival Plan (SSP) and for success of captive breeding programmes and this is normally monitored by estimating steroid hormones. However, regular blood sampling is impractical, since, most of the wild animals need to be restrained chemically. The only alternative is the detection of hormonal metabolites in faeces and has been established as an aid for captive breeding programs in various wild animals such as leopard[54], cheetah[55], ocelot[56] and red wolf[57].

Radioimmuno assay (RIA) for progesterone (Coat-A-Count), which is a solid phase assay for direct measurement of progesterone of human serum/plasma was tried on faecal progesterone metabolites in lions (n=13) and leopards (n=6) following exogenous gonadotropin treatment. 70% of lions showed distinct rise in faecal progesterone levels following hCG administration (6 to 12 μg/g) compared to basal levels (<0.5 μg/g) before eCG injection. In leopards, 66% of animals showed similar rise in progesterone levels. Thus, ovulation occurred in majority of the treated animals. These findings suggested that the failure of AI was surely not due to lack of ovulation, but may be attributed to some other factors, still unidentified.

Molecular sexing in monomorphic bird species

Sexing in birds is often a difficult task due to the fact that more than half of the existing bird species are monomorphic, i.e. males and females are phenotypically identical. This problem can hinder assisted breeding of wild bird species. DNA-based sex identification provides a solution[58]. In birds, the heterogametic sex is the female (WZ), while males are homogametic (ZZ). The test is based on two-conserved CHD (chromo-helicase-DNA-binding) genes located on the avian sex chromosomes of most of the bird species[59-61]. This Polymerase Chain Reaction (PCR) test employs two primers, which anneal to conserve the exonic regions and amplify across an intron, which usually differs in length between the CHD-W and CHD-Z genes[58]. Thus yielding one amplified DNA fragment in male and two in female as seen by gel electrophoresis. As a part of our ongoing research in assisted reproductive techniques in birds this test was successfully used for the sexing of the Blue rock pigeon (*Columba livia*) and the White-backed vultures (*Gyps bengalensis*).

MOLECULAR GENETIC MARKERS IN CONSERVATION BIOLOGY

Molecular genetic markers play a major role in evolutionary biology and have increasingly become popular in conservation[62] because of their rapid and accurate decision-making regarding the assignments of conservation priorities. The study of genetic markers may provide useful information at different levels: detecting genetic variations, population structure, levels of gene flow, phylogenetic relationships, patterns of historical biogeography, analysis of parentage and relatedness, recognition of evolutionary and taxonomic status of a species and finally the assignments of conservation priorities in terms of Evolutionarily Significant Units (ESUs) representing independently evolving clades[62-66]. In this part of the review, some recent data on the use of molecular markers in conservation biology is highlighted.

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

Microsatellites are short tandem DNA repeat sequences, found scattered throughout the eukaryotic genome, and exhibit unusual degree of polymorphism[67,68]. Their abundance, polymorphic nature and amenability to amplification by PCR make microsatellites ideal markers for studies on linkage mapping, forensics, population genetics and mating systems in the natural populations. Unlike the other genetic markers such as restriction fragment length polymorphism (RFLP), DNA fingerprints and protein polymorphism, it is possible to screen a large number of samples and several microsatellite loci, quickly.

The Asiatic lion (*Panthera leo persica*) once widespread throughout Southwest Asia is today restricted to a single location in the wild, the Gir forest in Gujarat, and is critically endangered. With the entire wild population of Asiatic lions being confined only to one area, it is highly vulnerable to biological, climatic or man-made catastrophe. Critical genetic analysis of this species is, therefore, warranted in order to ascertain the degree of genetic polymorphism and to recommend steps for conservation of the population.

Shankarnarayanan *et al.*[20] used microsatellite loci originally developed in domestic cat[69], to study the genetic variation in the Asiatic lions. However, none of the microsatellites showed variation as all the Asiatic lions were found to be monomorphic and homozygous at the five loci analysed. Although a large number of cat microsatellite markers are already available[70], identification of polymorphic microsatellite markers of Asiatic lion would provide a more thorough way of gauging the intra- and inter-generic variation amongst big cat populations. The microsatellite markers, developed so far, from the genomic library of the pure Asiatic lion, were highly polymorphic (Fig. 4) and efficient enough to analyse the genetic variability in the present lion population[71]. The results also suggest that the level of heterozygosity between pure Asiatic and hybrid lions was comparable (Table 4). These markers have also shown a high degree of polymorphism in leopard and tiger populations (unpublished data). Further efforts are underway to generate more number of such polymorphic markers so as to accurately evaluate the extent of relatedness and level of inbreeding in big cats.

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

The Eld's deer, or brow-antlered deer (*Cervus eldi*) is a highly endangered Southeast Asian cervid. Eld's deers were once distributed throughout Asia, their range extending from Manipur in eastern India to Indo-china and southern China. Due largely to

Fig. 4 — Allelic variation in the Asiatic lion at locus Ple 55 as seen by agarose gel electrophoresis

Table 4 — Seven polymorphic microsatellite loci isolated and characterised from *Panthera leo persica*[71]

Locus	Repeat motif	Primer Sequence (5′ – 3′) *	Ta (°C)	Size range (bp)	N	Asiatic lions (n = 15)		Hybrid lions (n = 13)		Accession numbers
						H_o	H_e	H_o	H_e	
Ple23	$(CA)_{20}$	F:GCTGCTCAAACAGGCTTCAC R:CGCACACATCCGCTTCTACT	60	176-186	8	1.00	0.58	1.00	0.86	AY095486
Ple24	$(GA)_{12}$	F:GCTTCATGACTGAGCGTGAG R:AACCACAGGCACTTCCTGAC	56	190-232	6	0.06	0.41	0.23	0.50	AY095487
Ple30	$(CA)_{13}$	F:GTGTTACGGTGCGTTTTGTG R:TGGCAACTCAGTCCACGTAA	57	224-239	8	0.86	0.69	0.53	0.84	AY095488
Ple46	$(CA)_{22}$	F:GAGGACGGTCTGGTGGAGT R:AACTTTAACCCACTGCTGCC	57	111-119	6	0.93	0.79	1.00	0.58	AY095489
Ple51	$(GA)_{28}$	F:TCTCTCTCTGCTCCTCCCAG R:CCCTAGCATCCTGCTCAGTC	56	174-187	8	0.86	0.85	1.00	0.79	AY095490
Ple55	$(CA)_{16}$	F:AGAGAGGGAACAGAGAGTG R:CAGGTGTGGCTCCTTAAAC	59	148-163	11	0.86	0.71	0.53	0.70	AY095491
Ple57	$(CA)_{20}$	F:CAGAGTGCGAGTGTGGACAT R:CATGGAAATGACTTGGGGAC	60	128-156	7	1.00	0.58	1.00	0.82	AY095492

*All forward primers were fluorescence labeled. T_a is the annealing temperature, N is the number of alleles per locus, H_O and H_E are observed and expected heterozygosity, respectively.

hunting
and habitat destruction, these deer now persist only in small, fragmented populations[72,73]. The Indian subspecies (*C. e. eldi*) was considered extinct until a small population was rediscovered in the early 1950s[74]. The population of *C. e. eldi* or the Sangai deer has since increased to approximately 150 individuals all restricted to Indian zoos and Keibul Lamjao National Park in Manipur. *C. e. eldi* inhabit low-lying swamps[75] and live on floating mats of dense vegetation, known as "phum" or "phumdi". The species is currently listed in Appendix I of the Convention on International Trade in Endangered Species (CITES) and is considered endangered by the World Conservation Union (IUCN).

The analysis of mitochondrial DNA (mt DNA) has revolutionised the evolutionary, conservation and population studies of a large number of species. The maternal inheritance, the high copy number per cell, and the faster rate of sequence evolution, have rendered mt DNA a special value as compared to the nuclear or chromosomal DNA. Mutations accumulate several times faster in the mt DNA than in the chromosomal DNA. In our recent studies, we used mt DNA control region sequences to examine the genetic structure of Eld's deer populations and to test whether current intra-specific taxonomy is congruent with evolutionary history[76]. Population genetic parameters, including nucleotide diversity and haplotype diversity[77,78] were calculated using Arlequin Ver. 2.0[79]. Arlequin was also used to calculate the number of transitions and transversions in the data set. Taken together, these data may assist conservationists and animal managers in developing genetic management strategies that help to ensure the long-term survival of Eld's deer.

All the subspecies of Eld's deer clustered together, when a neighbor-joining tree of different species was made (Fig. 5). However, the Sangai deer was found to be clearly distinct from the other two subspecies of Eld's deer. The results showed that the genetic data for *C. e. eldi* are consistent with a substantial loss of genetic diversity. Low nucleotide diversity suggests that this subspecies probably had small effective population size in its recent history (Table 5). While the sample size in this study is small and nonrandom, the lack of variation among these individuals may be a cause for concern for the subspecies as a whole. *C. e. eldi* presents the most difficult, and perhaps most controversial, conservation management challenge. This subspecies appears to have a markedly different ecology and may also be genetically distinct from the other two subspecies. In our opinion, *C. e. eldi* deer are highly inbred and would benefit from the incorporation of new genetic material. Because of the critically endangered status of *C. e. eldi*, there is an urgent need to develop non-invasive methods for their DNA based genetic characterization.

Major Histocompatibility Complex (MHC) variation in Asiatic lions

MHC is a large multigene family involved in the humoral and T-cell mediated immune responses of vertebrates. These molecules play an important role in immune recognition and defense and are extremely polymorphic in most species. Detailed sequence analysis of the gene encoding these molecules shows the majority of the polymorphism is exhibited in the antigen-binding domain. Therefore, we undertook the study of genetic variation in MHC loci of Asiatic lions, which may ultimately pave the way for identifying the reasons for susceptibility/resistance of

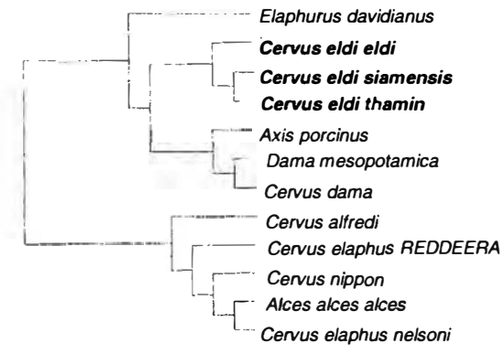

Fig. 5 — Neighbour joining tree showing relationship of *Cervus eldi eldi* with other deer species. All subspecies of Eld's deer cluster together.

Table 5 — Control region characteristics for the three subspecies of Eld's deer (*Cervus eldi*), including the number of polymorphic sites, haplotype diversity, and nucleotide diversity (Balakrishnan *et al.*[76])

Population	Samples	Haplotypes	Polymorphic sites (s)	Haplotype diversity	Nucleotide diversity
C. e. thamin	35	10	29	0.81	0.014
C. e. siamensis	8	4	21	0.82	0.024
C. e. eldi	5	1	0	0	0

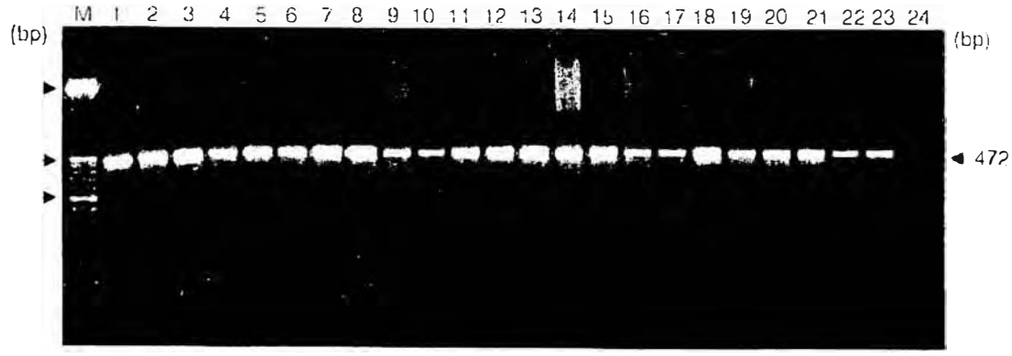

Fig. 6 — Agarose gel showing high performance and universal nature of our primers among a vast range of animal species. Description of the lanes 1to 23 are given in Table 6, Lane 24 is the negative control for PCR (no template DNA) and Lane M is the molecular weight marker.

these wild cats to diseases.

To study these loci, primers were designed from the published sequences[80] of exons of α_1 and α_2 domains of MHC-I loci of the domestic cat. These exons were selected since they code for the extracellular component of the MHC receptor involved in antigen and T-cell recognition and their flanking regions are conserved across species including humans.

PCR amplification and sequencing of the cloned amplicons has provided many single nucleotide polymorphisms (SNPs) and revealed abundant polymorphism at these loci not only between different individuals but also amongst the clones from the same individual in spite of the population bottlenecks these wild cats were being subjected to. A BLAST search for the translated products also showed 10-30% variation with domestic cat sequence. These sequences show homology with different classes of HLA also. Therefore, as not much literature is available in animals, these results can give us tools to correlate it with the available human literature.

Earlier RFLP studies[81] using MHC class-I probe failed to demonstrate variation in the immune loci of Asiatic lion. However, this seems unlikely since the Asiatic lions still represent a healthy wild population with wide genetic variability and still possess good polymorphism at MHC loci. Our results, however, testify the presence of high degree of genetic variation at MHC I loci which is very much comparable with the HLA polymorphisms (0.6-18%), including all different classes. These variations may thus be directly related to the immune potential of the organism. Now, we are planning to apply similar approach to other wild animals also.

Mitochondrial DNA and wildlife identification: A forensic perspective

Biodiversity protection and wildlife forensic identification are both linked to the stability of natural ecosystem by means of establishing identity of confiscated animal remains for wildlife law enforcement. Various approaches, which are either, based on morphological markers[82] or biochemical traits, such as, the bile characteristics[83], blood haem analysis[84,85] etc. have also been employed for establishment of identity of forensic samples. These approaches have various limitations for use in wildlife forensics since these markers are limited in number and cannot be practically applicable to mutilated remains with decomposed morphology and biochemical markers. Further, the molecular approaches[86-88] requires prior information of the species to establish the identity. Since, prior information about the origin of confiscated animal parts and product is never available in forensics, these methods are also not useful and practical in wildlife identification. We have developed a novel approach utilizing the immense potential of mitochondrial cytochrome b gene to reveal the identity of an unknown sample to the level of family, genus and species using a pair of novel universal primer mcb398 and mcb869[89] to amplified and sequence the PCR amplicons.

The universal nature and high performance of our primers amongst a vast range of animal genera has been validated by examining their potential to establish the correct identity of specimens from known animal sources representing various mammalian genera (with distribution among major mammalian groups), reptiles and birds (Table 6). The band of expected size was obtained from all the animals tested (Fig. 6). The molecular signatures revealed from these animals established the accurate identity of the animals. Table 6 summarizes the results of PCR success and BLAST scores of the

Table 6 — Various animal species included in the study to validate the universal nature of the primers, used in the ongoing study

Animal Species tested	PCR success[§]	*NCBI accession number	Highest BLAST scores			†NCBI accession number	Specimen identity revealed as
			Bits score	E-value	Nucleotide similarity (%)		
Mammal							
Rodent							
Mus musculus	Lane 14	AF540912	835	0.0	100%	BC006023	*M. musculus*
Mesocricetus auratus	Lane 8	AF540913	835	0.0	100%	AFI19265	*M. auratus*
Carnivore							
Canis familiaris	Lane 5	AF540914	835	0.0	100%	CFU96639	*Canis sp.*
Cetacean							
Platanista gangetica (3) #	Lane 9-11	AF540915-17	827	0.0	99%	AF304070	*Platanista sp.*
Ruminant							
Bubalus bubalis (2) #	Lane 22 & 23	AF540918-19	835	0.0	100%	AY079132	*B. bubalis*
Ovis aries	Lane 3	AF540920	827	0.0	99%	AF010406	*Ovis sp.*
Antelope cervicapra (2) #	Lane 1, 2	AF540921-22	819	0.0	99%	AF036283	*Antelope sp.*
Suina							
Sus scrofa	Lane 4	AF540923	835	0.0	100%	AF304200	*S. scrofa*
Proboscidean							
‡*Elephas maximus*	Lane 16	AF540924	511	e-142	88%	D50846	*Elephas maximus*
Sirenian							
Dugong dugong	Lane 18	AF540925	811	0.0	99%	AY075116	*Dugong sp.*
Primate							
Pan troglodytes	Lane 6	AF540926	795	0.0	99%	X93335	*Pan sp.*
Homo sapiens	Lane 7	AF540927	827	0.0	99%	AF382011	*Homo sp.*
Avian							
‡*Ploceus benghalensis (3)* #	Lane 19-21	AF540928-30	565	e-158	92%	AF255709	Ploceinae
‡*Gallus gallus*	Lane 17	AF540931	793	0.0	98%	AY029583	*Gallus sp.*
Reptile							
‡*Naja naja*	Lane 15	AF540932	492	e-136	89%	AF217835	*Naja sp.*
‡*Lepidochelys sp.* (2) #	Lane 12 & 13	AF540933-34	486	e-134	89%	CMU81352	Cheloniidae

[§]Refer to Fig. 6.
* NCBI accession numbers of the sequences generated in this study
† NCBI accession showing best BLAST hit with the corresponding sequences generated in our study from known animal sources
# Number in the parenthesis indicates the number of individuals tested, where N>1
‡ The lower bits score of these animal species was due to following reasons:
High levels of heteroplasmy in *Elephas maximus* cytochrome b sequence possibly due to co-amplification of nuclear pseudogene copies (Reviewed by Bensasson *et al*[90])
Presence of ambiguous nucleotides in *Gallus gallus* cytochrome b sequence available for comparison in NCBI database
The sequences of *Ploceus benghalensis, Naja naja* and *Lepidochelys sp.* are novel. These sequences were not available in NCBI database for comparison and further delineation of exact identity; therefore the closest family, genus or species was picked up by BLAST.

signatures generated from these animals establishing their identity to the levels of family, Genus and species. The international patent for these primers has already been filed (International publication number under PCT: WO 02/077278 A1) and the approach is being used to resolve the forensic investigation of the cases forwarded by various crime investigation agencies and wildlife curators.

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