DEMOGRAPHIC STATUS AND GENETIC VARIATION OF SANGAI (*Cervus eldi eldi*) IN KEIBUL LAMJAO NATIONAL PARK, MANIPUR

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By

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CERTIFICATE

This is to certify that the thesis of **Ms. Sangeeta Angom** entitled **"DEMOGRAPHIC STATUS AND GENETIC VARIATION OF SANGAI (*Cervus eldi eldi*) IN KEIBUL LAMJAO NATIONAL PARK, MANIPUR"** is an original piece of work submitted to the Saurashtra University, Rajkot (Gujarat), for the award of the Doctor of Philosophy in Wildlife Science.

Ms. Sangeeta Angom has put in more than six terms of research work embodied in this thesis under my guidance and supervision. The work presented in this thesis has not been submitted for any other university or institution.

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EXECUTIVE SUMMARY

The Eld's deer or the sangai (*Cervus eldi eldi*), popularly known as Manipur's brow antlered deer is one of the most endangered species of deer in India. It was once believed to be extinct; a small population of around 14 individuals was rediscovered in 1975 at the southeastern fringe of the Loktak Lake, Manipur. This area was protected and declared as Keibul Lamjao National Park (KLNP). Since then the population of sangai has increased considerably. The reported population in 2003 was around 180 individuals of different age and sex.

Small populations suffer higher rates of extinction because of demographic fluctuations due to random variations in birth and death rates, genetic problems related to loss of genetic variability, inbreeding, loss of heterozygosity and genetic drift. The environmental fluctuations due to variation in predation, competition, disease, food supply and natural catastrophes are some of the other reasons affecting population. These forms of stochasticity are believed to influence population persistence. A combination of demographic, genetic and ecological studies is necessary to get a better understanding of the population dynamics of a species of conservation significance.

The present study was intended to improve upon the existing ecological and genetic knowledge base of the severely fragmented and isolated population of sangai in the Keibul Lamjao National Park. The major objectives of the study were to estimate the abundance and demographic parameters of sangai and the associated cervids in the Park and to assess the genetic variability using mtDNA (cytochrome *b* and control region) genes

and polymorphic microsatellite markers so as to develop appropriate measures for the conservation of this endangered species. The thesis addresses the following five key questions; (a) What is the current trend in population of sangai and hog deer in the Keibul Lamjao National Park? (b) What is the current age structure and sex ratio of sangai and hog deer? (c) What is the genetic relationship of sangai with other subspecies of Eld's deer and also with related cervids? (d) What is the degree of genetic heterogeneity/ homogeneity in the wild and captive population of sangai? (e) Does the sympatric hog deer have the similar genetic status?

The demographic status of sangai and hog deer in Keibul Lamjao National Park was determined by conducting the population estimation exercise during 2006 - 2008 using point count method. Deers were counted from eighteen bamboo machans and four observation points. Morphological traits were used for individual identifications of different sex and age groups of sangai and hog deer. The counting of the animal was carried during 0600 - 0900 hours for five consecutive days. The data generated were analyzed using DISTANCE 5.0 and density was estimated.

The findings of the study revealed that the density of sangai in the Park was 4.05 ±0.62 (CV 15.38%), 4.09 ±0.75 (CV 18.48%) and 4.06 ±0.8 (CV 19.9%) individuals/km^2 with a minimum of 2.97, 2.83, 2.73 and a maximum of 5.51, 5.88, 6.01 individuals/km^2 at 95% confidence level during 2006, 2007 and 2008 respectively. The estimated population size of sangai was 90 ±13.8, 88 ±16.2 and 92 ±18.3 individuals during 2006, 2007 and 2008 respectively, with a minimum of 66, 61 and 62 and maximum of 123, 127 and 137 sangai at 95% confidence level for the entire study period.

The population structure of sangai based on the percentage sightings of different size classes seen during the population estimation exercise in 2006, 2007 and 2008 showed higher number of females as compared to males as well as juvenile and fawn. The adult sex ratio and the doe to fawn ratio of sangai was 47, 57, 58 males/100 females and 18, 13 and 18 fawns/100 females during 2006, 2007 and 2008 respectively. The overall observed male to female ratio was 54 ±3.57 males/100 females and fawn to female ratio was 16 ±1.7 fawns/100 females respectively for the study period. The population trend of sangai during 2006 - 2008 was more or less stable in the Park. Subsequent, analysis of the data generated by the Manipur Forest Department for the period of 1984 to 2003 revealed 5% exponential rate of increase in the population during this period (p <0.05, R^2 = 0.898) in contrast to 10% growth rate for the period of 1975 – 2003.

The density of hog deer in the Park was found to be 2.94 ±0.57 (CV 19.5%), 2.75 ±0.44 (CV 16.3%) and 2.51 ±0.40 (CV 16.2%) individuals/km^2 during 2006, 2007 and 2008 respectively with a minimum of 1.82 individuals/km^2 and maximum of 4.32 individuals/km^2 at 95% confidence level. The estimated population size of hog deer was 65 ±12.6, 61 ±9.9 and 57 ±9.2 individuals with a minimum of 44, 44, 41 and maximum of 96, 84 and 79 hog deer at 95% confidence level during 2006, 2007 and 2008 respectively.

The population structure of hog deer derived is based on the percentage sightings of different size classes of hog deer seen during the population estimation exercise in 2006, 2007 and 2008. It showed that in all the sampling years the number of adult female was higher followed by adult male. The overall population structure also indicates a higher number of

female as compared to male and juvenile. The adult sex ratio and doe to fawn ratio of hog deer were calculated based on the sightings during population estimation in the Park. The male to female ratio and doe to fawn ratio of hog deer in 2006-2008 was 34.2, 34.5, 39.9 males/100 females and 16.4, 17.2 and 15.8 fawns/100 females respectively. However, the analysis of sex ratio for the study period revealed that the male to female ratio was 36.2 ±1.9 males/100 females and doe to fawn ratio were 16.5 ±0.4 fawns/100 females respectively. The population trend of hog deer during 2006 to 2008 in the Park showed a declining trend of 8% per annum. (p>0.05, R^2 = 0.992).

The phylogenetic variation in the mtDNA Cytochrome *b* (150 to 471 bp) and control region (230 to 478 bp) genes were analyzed for wild and captive populations of sangai and hog deer to understand the genetic relationship and variability among these species using software like Clustal *X* 1.83 for alignment, BioEdit 7.0.9 for editing the sequences, and MEGA 5 for phylogenetic reconstruction. No haplotype variations were detected among the 16 individuals of sangai using mtDNA cyt *b* gene. However, two haplotypes were identified among the 17 individuals of sangai at nt282 position in mtDNA control region of Cerv.tPro / CervCRH gene. Both these variable positions were transition mutation (T→C). Incorporation of more sample size of blood and tissue from wild and captive population of sangai may reveal better understanding on its haplotype diversity and its phylogenetics.

The phylogenetic trees of Neighbor-Joining (NJ) and Minimum Evolution (ME) with D-loop R / Lo-F gene showed that the wild and captive populations of sangai forms a monophyletic clades showing first clade comprising of captive population of sangai from Nehru Zoological Garden,

Hyderabad (Hyderabad Zoo) and National Zoological Garden, Delhi (Delhi Zoo) and the second group forming one clade comprising of sangai from KLNP and Manipur Zoological Garden (Manipur Zoo). However, this result is based exclusively on a short fragment of 230 bp of the primer D-loop R / Lo-F gene. To compare and check the result, a larger fragment of 478 bp of control region Cerv.tPro / CervCRH gene as identified by Balakrishnan et al. (2003) was analyzed using Neighbour Joining (NJ) and Maximum Parsimony (MP) methods. The result showed that the population of sangai was grouped into a single clade consisting of both captive and wild with high bootstrap support (100%). This indicates that the captive and wild population of sangai may not be genetically divergent as evident earlier from the D-loop R / Lo-F gene study.

The population of *R. e. eldii* from Manipur showed closest relationship with *R. e. thamin* than to *R. e. siamensis* from Thailand. The sequences of *R. e. siamensis* from Thailand were interspersed with *R. e. hainanus* from Hainan Island, China. This shows that the status of *hainanus* needs a formal study to examine its accurate taxonomic position and relationship with other subspecies of Eld's deer. Most of the sources of the *siamensis* sequences are not known and only few sequences are available in GenBank and these sequences of *siamensis* showed variation among themselves. More studies using different classes of genetic markers and incorporation of more sample size from wild and captive population of all Eld's deer subspecies may reveal better understanding on its phylogenetics and for planning an effective conservation intervention.

The genetic relationship of sangai with related cervids using control region gene has close affinity with sambar (*Rusa unicolor*) and hog deer (*A.*

porcinus) which is closely related to chital (*A. axis*) and were grouped together as monophyly. On the other hand, swamp deer *R. duvaucelii* which recently revised from *Cervus* to *Rucervus* appear closely related to *Muntiacus muntjak* than to Eld's deer.

The divergences times of three subspecies of Eld's deer were calculated using MEGA 5 by referring to the age of the oldest known fossil records of Eld's deer at 18,000 and 8,000 years. The results were plotted using Neighbour joining (NJ) tree which showed that *R. e. eldii* was diverged earlier than *R. e. thamin* and *siamensis* / *hainanus i.e. R. e. eldii* > *R. e. thamin* > *R. e. hainanus* or *siamensis.* The range of divergence time between *R. e. eldii* and *R. e. thamin* was estimated at approximately 73,683 years, while that of *R. e. thamin* and *R. e. siamensis* at approximately 51,748 years and that of *R. e. siamensis* and *hainanus* at 18,000 years respectively.

The genetic diversity estimates of sangai and hog deer were determined using the CERVUS 3.0 and the deviations from the Hardy–Weinberg Equilibrium (HWE) was examined using the exact test of GENEPOP 1.2. The genetic diversity estimates of wild sangai revealed that the mean numbers of allele at 22 loci were 2.64 ±0.15, observed heterozygosity (H*o*) was 0.20±0.05, expected heterozygosity (H*e*) was 0.38 ±0.04 and polymorphic information content (*PIC*) was 0.32 ±0.03 respectively. The genetic variability of captive sangai at 22 loci showed that the mean numbers of allele were 2.09 ±0.19, observed heterozygosity (H*o*) was 0.08 ±0.02, expected heterozygosity (H*e*) was 0.25 ±0.04 and *PIC* was 0.20 ±0.03 respectively. However, the diversity estimates of hog deer revealed that the mean numbers of allele at 23 loci were 2.70 ±0.18, observed heterozygosity (H*o*) was 0.42 ±0.02, expected heterozygosity (H*e*) was 0.51 ±0.03 and *PIC*

was 0.43 ±0.03 respectively. The allelic diversity of sangai showed reduction of genetic diversity approximately 40% in wild indicative low genetic variability and a significant loss of genetic diversity in captive population due to inbreeding depression in captivity. The sympatric hog deer showed a moderate genetic variation of around 50% at KLNP.

The F_{ST} estimations within the wild samples of sangai and between captive populations were calculated with GENEPOP 1.2 software using pair wise analysis. Parameters comparisons between four data subsets *viz.* KLNP, Manipur Zoo, Delhi Zoo and Guwahati Zoo populations were made with the procedure ‘‘Comparison among groups of samples’’ of F_{ST}. The F_{ST} analysis showed that the genetic differentiation among wild population of KLNP and Manipur Zoo (0.265) were lowest. Probably the captive populations of Manipur were preserving some of the rare and significant alleles from wild population. The Delhi (0.2845) and Manipur Zoos (0.2829) individuals had similar genetic makeup since both the Zoos obtain their founders from the wild. However the Delhi and Guwahati Zoos population (0.5755) showed the highest levels of differentiation followed by Manipur and Guwahati Zoos population (0.5321). The F_{ST} values were very high for all the dataset, indicated high level of genetic differentiation in sangai populations, probably reflective of inherent skewness due to kinship patterns as almost all captive individuals are descendent from few wild founders.

Evidence for a genetic bottleneck was evaluated using software BOTTLENECK 1.2.02 which assumed that a signature of a severe reduction in effective size of a population was an excess of H_E relative to H*eq*. For bottleneck estimation of sangai in wild population, the allele frequency distribution showed a recent bottleneck provoking a shifted mode in wild

population of sangai. Bottleneck events were detected in the ancestors of captive samples of sangai. The observed allelic frequency distribution showed a shifted mode characteristics due to existence of bottlenecks in the founder events. Although the hog deer population has declined in the recent years the population has been retaining some of its rare alleles and 50% genetic diversity in the wild. The observed allelic distribution revealed the hog deer population did not encounter a genetic bottleneck in the recent past. The allele frequency distribution was approximately L-shaped (as expected under mutation-drift equilibrium).

The findings of this study indicate that the population of sangai in the Park is more or less stable. The population growth rate of sangai during 1975 - 2003 was 10% per annum and during 1984 - 2003 it was 5% per annum. This indicates that the population growth rate was higher during 1975 - 2003 and now it has reached an asymptote. There could be several factors that are affecting the demographic structure of the sangai in the Park (a) deteriorating habitat condition *i.e.* thinning of *phumdi* (b) lack of connectivity for recolonization (c) poaching and incidental mortalities (d) increased probability of disease and mortalities. The sangai population being small and highly fragmented is subject to a higher chance of extinction because they are more vulnerable to inbreeding depression and genetic drift, resulting in stochastic variation in their gene pool, their demography and their environment. In addition, the long term viability of small populations can impact on population persistence *viz.* lower the fecundity and survival of inbred individuals within a population, will depress population growth rate, which in turn has contributed to accelerated rates of extinction and reduction in genetic load.

The evidences of poaching and mortality due to disease could be other factors affecting the population. Appropriate protection strategy and disease monitoring plan needs to be developed and implemented to reduce the mortality of sangai and hog deer due to these factors.

During the study, evidences of the presence of sangai and hog deer were found in adjacent areas. There is an urgent need for extension of the Park to these areas. The relocation and reintroduction efforts need to be carried out after careful ecological examination of the sites along with long term scientific research and monitoring. The species needs to be introduced in more or less similar habitat in wild through conservation breeding programme for rapid multiplication in order to sustain a viable population in wild. It is important to conduct regular monitoring of population that would provide valuable up-to-date information, to help identify the critical population and sites for prioritized conservation actions and to support and guide the protection of the species. It is necessary to study the demographic parameters, population dynamics, requirement of space and forage for sustained reproduction and social structure and behavior. For future reintroduction the founder population should be greater than the believed to be original 14 founders in the Park. The founder population should compose of a cohort of over 20 same-age individuals with 1:1 sex ratio. Genetic monitoring for both the source and reintroduced populations should be carried out in order to assess the effectiveness of conservation program.

During this study it was estimated that less than 100 mature sangai exist in the KLNP. As stated earlier the species is protected under the Schedule I of the Indian Wildlife (Protection) Act, 1972. The IUCN has listed

the Eld's deer as 'Endangered'. The resultant categorization as 'Endangered' for the species has compromised the status of some sub species *viz.* the sangai which is geographically isolated, distinct and have drastically low population numbers. Threats posing for this subspecies include habitat degradation, developmental process; human encroachments and low genetic diversity which have all lead sangai to a high degree of isolation. In the absence of adequate information and extinction proneness of some subspecies, there is a need to reconstruct the threat status of Eld's deer by upgrading it to 'Critically Endangered' under the IUCN Red List category. During the study, it was observed that the hog deer population in the Park is also showing a declining trend. This indicates that the major threats experienced by sangai and hog deer are similar, largely due to habitat degradation and poaching that needs to be addressed in an integrated manner.

Chapter I
INTRODUCTION

1.1 CONSERVATION OF SMALL POPULATIONS

Small populations suffer higher rates of extinction than larger populations (Brown 1971, Jones & Diamond 1976, Newmark 1987) as more variable populations of a given size (Thomas 1990, Reed & Frankham 2003). Small populations have greater susceptibility to a number of deleterious genetic effects, such as inbreeding depression, loss of evolutionary flexibility and outbreeding depression, leading to a decline in the population size and a greater probability of extinction (Primack 1993). The extinction of a species is directly related to a reduction in the genetic variability which is a crucial factor both for the short term fitness of individuals and long term survival of the species. Reduction of genetic variability can reduce the ability of a species to cope with adverse environmental conditions, cause a reduced population density and, in some cases, lead to extinction of the species. For conservation of genetic diversity in small populations, it is very important to have an adequate knowledge regarding the loss of genetic variability over time (Soulé 1980).

A combination of demographic, genetic and ecological studies is necessary to obtain a better understanding of the population dynamics of species. The key component is the effective population size, based on the number of individuals that actually produce offspring and hence contribute to the gene pool (Primack 1993). The calculated effective population size is

often much lower than simply the number of individuals alive. The population may exhibit large fluctuations over time because many individuals may not be reproducing, there may be an unequal sex ratio, and there may be variations among individuals in the number of offspring produced. A population may occasionally be severely reduced in size due to some environmental or demographic events that kill all but a few individuals (Barrett & Kohn 1991). This phenomenon is referred to as a genetic bottleneck (Carson 1983). When a population is greatly reduced in size, rare alleles in the population are lost if no individuals possessing these alleles survive (Carson 1983). With fewer alleles present and the heterozygosity declining, the overall fitness of individuals in the population declines (Primack 1993).

The population sizes of most threatened species are small. The effects of inbreeding are of concern with such small population sizes (Soulé 1980). Genetic variability is thought to be essential for the long-term persistence and adaptability of populations, and thus the management of captive and wild populations of endangered species should be so as to minimize the loss of genetic variability. Both morphological and molecular techniques can be used to compare variability between populations and to follow the decline of variability in small populations (Wayne 1991). Molecular genetic markers are powerful tools in identifying the genetic uniqueness of an individual's population or species (Avise 1994, Linda & Paul 1995). DNA based studies have been of great interest in the conservation biology of endangered species and in the population genetics of cervids (Avise 1994). Molecular genetic markers such as mitochondrial DNA (cytochrome *b* and control region) and nuclear microsatellites are used in estimating the genetic diversity and

effective population size, identifying the genetic uniqueness of an individual's population or species, understanding gene flow patterns among populations, determining parentage, linkage mapping and determining relationships among individuals in a population (Primack 1993, Avise 2004).

The study species, Eld's deer (*Rucervus eldii*) (McClelland 1842), is a highly endangered Southeast Asian cervid. It has been extirpated from much of its historical range due to anthropogenic pressures such as hunting and habitat degradation. It now persists only in small and fragmented populations from Manipur in North-east India to Myanmar, Cambodia, Laos, Vietnam and Hainan Island in China (Wemmer 1998, McShea et al. 1999, Grubb 2005). The historical range included four major components. One of these components was the Manipur region, which was inhabited by *Rucervus eldii eldii*, locally called the sangai. Now, the sangai is restricted to the southeastern part of Loktak Lake (Gee 1960, Ranjitsinh 1975) of Keibul Lamjao National Park, between latitudes 24°25′ and 24°42′N and longitudes 93°46′ and 93°55′E. So far, an assessment of the demographic status of the sangai and the genetic variability of the wild population has not been carried out. The present study was aimed at assessing the demographic and genetic status of sangai to develop appropriate measures for conserving the severely fragmented, declining small population of this endangered species**.**

1.2 ELD'S DEER: A PROFILE

The Eld's deer belongs to Mammalian Order Artiodactyla, Family Cervidae. Of the seventeen species of cervids occurring in Southern Asia and far East, seven species *viz.* muntjac (*Muntiacus muntjak*), sambar (*Rusa*

unicolor), chital (*Axis axis*), hog deer (*A. porcinus*), swamp deer (*Rucervus duvaucelii*), hangul or Kashmir stag (*Cervus elaphus hanglu*) and the Eld's deer (*Rucervus eldii*) occur in the Indian subcontinent (Whitehead 1993, Menon 2003, Hussain et al. 2006). Among these, the Eld's deer or the brow-antlered deer (*Rucervus eldii*) has a much localized distribution occurring only in the State of Manipur (Whitehead 1972, Hussain et al. 2006). The Eld's deer in India is also referred to as Manipur's brow-antlered deer or sangai with correct zoological name as *Rucervus eldii eldii*, McClelland, 1842 following Wilson & Reeder (2005) and Timmins & Duckworth (2008). Henceforth in this thesis the Eld's deer will be referred to as sangai or Manipur's brow antlered deer (*Rucervus eldii eldii*). The Eld's deer is divided into three sub species *viz.* the sangai, *R. e. eldii*; the thamin or Myanmar brow-antlered deer (*R. e. thamin*) and the Siamese brow-antlered deer (*R. e. siamensis*) (Whitehead 1972, Balakrishnan et al. 2003). Some authors have identified a fourth subspecies; Hainan's Eld's deer (*R. e. hainanus*) which occurs in Hainan Island, South China (Wilson & Reeder 2005, Zeng et al. 2005). Eld's deer is a medium sized deer with distinctive antlers, measuring 150–170 cm in length. The long brow tine and the main beam form a continuous curve at a right angle to the closely set pedicels. The height and weight of a fully grown stag may be approximately 120–130 cm at the shoulder and 95–150 kg respectively. The female is smaller and weighs less than its male counterpart. The length of the body from the base of the ear to the tail is about 145–155 cm in both sexes. The tail is short, and the rump patch is not pronounced.

(a)

(b)

Plate 1. The sangai (*Rucervus eldii eldii*) in Manipur Zoo (a) a group with adult male having spiked antlers (b) adult male having multiple spiked antlers.

As the name Manipur brow-antlered deer suggests, the forward protruding beam appears to emerge from the eyebrow (Plate 1). The two antlers are asymmetrical, forming the shape of a half moon, which is unique among cervids (Shamungou 1997). The beams are unbranched initially. The curvature of the beams increases until they become forked. The sexes are moderately dimorphic in terms of body size and weight. The body of the sangai is covered with coarse hair. The colour of the hair particularly that of the male, varies with the season. In winter, the coat is dark brown, whereas in summer it is reddish-brown. The sangai possesses a modified foot, with splaying hooves and cornified skin on the back of its digits (Geist 1998). These modifications have been viewed as adaptations that may enable the deer to walk more easily on moist ground (Whitehead 1972).

1.2.1 Systematics

Eld's deer was first described in 1839 from Manipur Valley, India, by Captain Guthrie, who named it *Cervus frontalis*. Subsequently, it was renamed *C. eldi eldi* by McClelland (1841) after its discoverer, Captain Percy Eld. Later, McClelland (1842) renamed it *Cervus* (*Rusa*) *frontalis.* Thomas, in 1918, placed it under the genus *Rucervus*, thereby aligning it with the swamp deer (*R. duvaucelii*), which has a close taxonomic relationship with the extinct Shomburgk's deer, *R. schomburgki* (Corbet & Hill 1992). Historically, three subspecies were recognized (Figure 1.1), namely *R .e. eldii*, *R. e. thamin* and *R. e. siamensis* (Gee 1960, McShea et al. 2001, Balakrishnan et al. 2003, Johnson et al. 2004, Tordoff et al. 2005). A recently recognized fourth subspecies, found in Hainan Island off southern China, was named *Cervus*

eldii hainanus by Xu et al. (1983). The Manipur Eld's deer was formerly considered to be a local race of the *thamin* deer of Myanmar (Lydekker & Dollman 1985). The *thamin* deer found in the upper and central parts of Myanmar were named *R. thamin brucei* and *R. e. thamin* respectively, by Lydekker & Dollman (1985). These names were assigned according to the morphology of the antlers, which are slightly palmate. The *thamin* deer of westernmost and upper Thailand, near the Myanmar border was described as *Panolia platyceros* by Gray (Lydekker & Dollman 1985). Later, it was considered to represent a race of *R. eldii* and subsequently recognized as being a distinct species called *R. platyceros* (Thomas 1918).

Pocock (1943) revived the generic name *Panolia* for *Rucervus* and preferred to combine all three subspecies into a single species. The study conducted by Pitra et al. (2004) wherein they analysed the mtDNA (cyt *b*) of several deer taxa demonstrated that placement of Eld's deer in *Cervus* had been, in fact, phylogenetically more appropriate. Subsequently, Groves (2006) opined that Eld's deer did not belong to the genus *Rucervus* and pointed out that, under the phylogenetic species concept, the three taxa are extremely different. The differences between *R. e. siamensis* and the other two subspecies, *R. e. eldii* and *R. e. thamin* are prominent. Groves (2006) suggested that the taxon *siamensis* should be recognized as being specifically distinct from *R. eldii* and urged that a formal study be carried out. However, Grubb (2005) revived Thomas (1918) assignment of the species to *Rucervus*. Subsequently, Wilson & Reeder (2005) accepted this and used the name *R. eldii* for Eld's deer, and this name was adopted by the IUCN Species Survival Commission (Timmins & Duckworth 2008). The species name is

often misspelled *eldi*, but the correct original spelling, which must be used, is *eldii* (Timmins & Duckworth 2008).

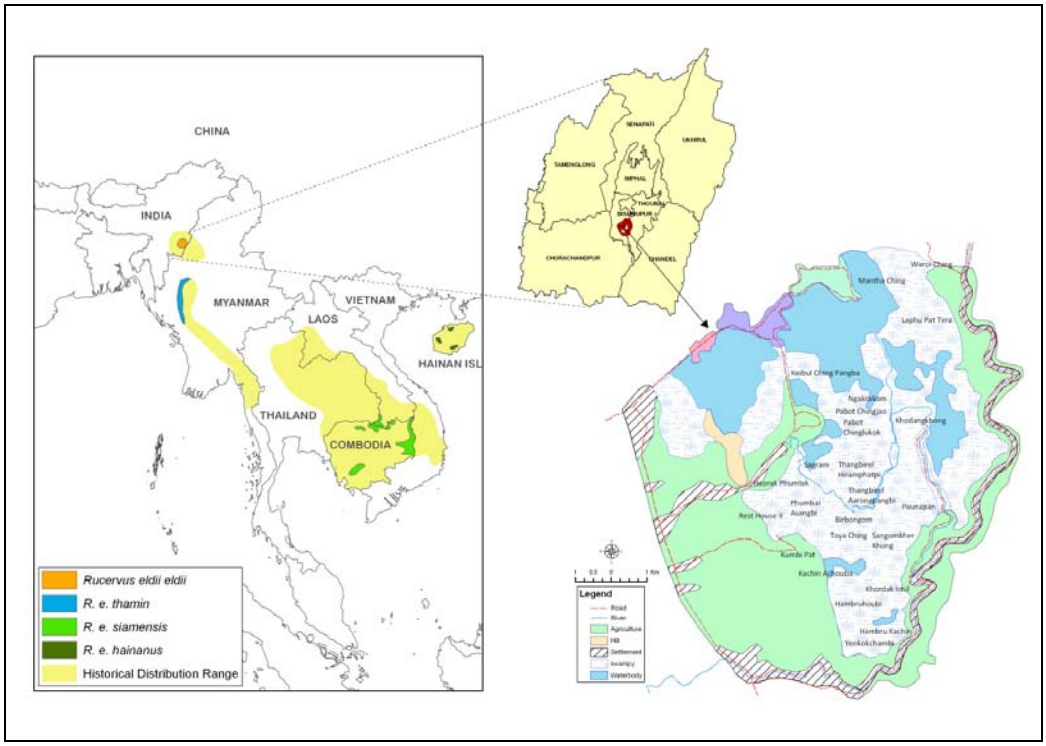


Figure 1.1 Present and historical distribution of Eld's deer in Southeast Asia and location of its occurrence in India.

1.2.2 Conservation status

Eld's deer is listed as "Endangered" in the IUCN Red List (Timmins & Duckworth 2008) based on estimated rates of decline which, averaged across the species, exceed 50% in three generations (Timmins & Duckworth 2008), and in Appendix I of the Convention on International Trade in Endangered Species (CITES). In India, the species is listed in Schedule I of the Indian Wildlife (Protection) Act, 1972. After its rediscovery on the southern fringes of Loktak Lake, in Manipur, the area was declared as a protected area (1954)

and subsequently as a National Park (1977). The State government of Manipur recognized it as the State animal in 1989. With concerted efforts, its population is showing an increasing trend (Hussain et al. 2006).

In Myanmar, the Shwesettaw and Chatthin wildlife sanctuaries were established in 1986 for conserving *R. e. thamin*, although a significant number of animals are found outside these areas (McShea et al. 2005, Tordoff et al. 2005). The species receives nationwide protection from hunting under the 1936 Burmese Wildlife Protection Act. It is one of 15 species listed by the Wild Animals Preservation and Protection Act, 1992, as national reserved species. In spite of such efforts, the species is showing a continuous declining trend, largely due to hunting and deterioration of its habitat (McShea et al. 2001). The Royal Government of Thailand designated it as a national reserved species, and it is protected by the Thai Wildlife Law since 1960 (Blower 1983). In the Lao PDR, it is listed as Threatened–Vulnerable and At Risk (Timmins & Duckworth 2008). In Chonnabuly district, where it occurs, an area of 93,000 ha extent in Savannakhet was formally designated as a protected area. In Cambodia, where it was previously thought to be extinct but was recently rediscovered in the northwestern part of the country (Owen 2009), it is listed as a protected species. One or two small and isolated populations have also been identified in the Lao PDR in 2002 (Johnson et al. 2004). In Vietnam, It is listed as Endangered in the Red Data Book and is also listed in Group IB of the government's directives, which strictly bans its hunting and use (Dang & Nguyen undated). It is also regarded as a Rank I key species afforded national protection under the Wild Animal Protection Law of China (Liu 1998).

1.3 REVIEW OF LITERATURE

1.3.1 Demographic status of Eld's deer

Eld's deer, once distributed throughout much of Southeast Asia, from Manipur in Northeast India to Indochina and southern China, is now confined to small, fragmented patches (Wemmer 1998, McShea et al. 1999, Grubb 2005). Its historical range has been broken into four major parts, one of which is the Manipur region of India, inhabited by *R. e. eldii*, found in Keibul Lamjao National Park (Gee 1960, Ranjitsinh 1975). Myanmar's Eld's deer, or *R. e. thamin* (Thomas 1918), is found in the central plains of Myanmar and westernmost Thailand and along the eastern Downa Range and the upper Tenasserim Range in Thailand, near the Myanmar border (Bhumpakphan et al. 2004). The captive *thamin* population at Khao Khiew Open Zoo in Chon Buri, Thailand, originated from 11 founders in 1983. This captive population has increased from 98 individuals in 1996 to 200 *thamin* in 2003 (Singhasenee 1996). *R. e. siamensis* occurs in one or two small localized populations in the Lao PDR (Johnson et al. 2004) and scattered small subpopulations in the northern and eastern lowlands of Cambodia (Tordoff et al. 2005). The subspecies *siamensis* of eastern Thailand is possibly extinct in the wild (Khan et al. 1992). A captive population of *R. e. siamensis* maintained at the Paris Zoo since 1937 was founded with only five individuals and has never been supplemented with animals from other populations (Thevenon et al. 2000). The fourth population, of *R. e. hainanus* (Thomas 1918), consisting of populations in Hainan Island and mainland southern China, appears to have been made up of disjunct outliers of *R. e. siamensis*, separated from the

main range by mountainous terrain in the Lao PDR and Vietnam (Xu et al. 1983, Timmins & Duckworth 2008).

Thailand is the geographical centre of the distribution range of Eld's deer (Ginsburg et al. 1982). The oldest record in Thailand is the one reported by Ginsburg et al. (1982): fossil remains of teeth belonging to many carnivores, primates, ungulates and Eld's deer, from 18000 to 8,000 years old, were found within Quaternary reddish clay deposits (from the later part of the Middle Pleistocene) at Wiman Nakin Limestone Cave in Kon San district, Chaiyaphum Province, in northeastern Thailand (Tougard et al. 1996). A fossil form of Eld's deer, from ca. 3,000 years BP was found in Java, Indonesia (Corbet & Hill 1992).

Before the second world war, *R. e. siamensis* occurred throughout the upper northern part of Thailand; presently, it is found in the lowland forest of Dong Khanthung, Champasak Province (Round 1998), and in Chonbuly district, Savannakhet Province (Vongkhamheng & Phirasak 2002). Recent sightings of the subspecies have been concentrated at the trans-boundary area of the Phanom Dong Rek Range. In 1995, a small herd was seen at the Lao PDR–Thailand trans-boundary area, in Yot Dom Wildlife Sanctuary in Ubon Ratchathani Province and at the border. A herd was also reported from near Chong Pong Daeng Border Pass of Phu Jong–Na Yoi National Park in Ubon Ratchathani Province, Thailand (Kotmongkhon 1997).

In the Lao PDR, historically, *siamensis* ranged across the Mekong lowlands in dry dipterocarp forests from Vientiane to Champasak Province on the Cambodian border (Arlyne et al. 2003). At present, 5,566 km^2 of suitable

Eld's deer habitat remains, and it is fragmented into 51 patches with a mean patch size of 109 km^2 (McShea et al. 2001). Round (1998) found deer signs (n = 6) in one of these patches near the villages of Kadan and Kadian and estimated that 10–12 deer remained in the area. The isolated populations of *R. e. siamensis* in southern Laos (Round 1998) and Ang Trapeang Thmor (ATT) Reservoir in northwestern Cambodia (Owen 2009) inhabit marshy areas similar to those in Thailand described by Lekagul & McNeely (1977). Lekagul & McNeely (1988) suggest that the predisposition of stags to wallow in mud indicates that the current distribution of Eld's deer is a result of agricultural expansion and hunting and that the historic distribution included moister dipterocarp forests and grasslands.

The sangai, once thought to be extinct, was rediscovered in 1953 by Edward Pritchard Gee on the southern fringe of Loktak Lake (Gee 1960). In 1959, six heads were counted, leading to the beginning of intensive conservation efforts. Subsequently, Gee (1961) estimated the total population of sangai in the Park at 100–112 individuals. The first aerial census carried out in the Park, in 1975, recorded the presence of only 14 individuals (Ranjitsinh 1975). The first ground census, conducted in 1984, provided an estimate of 51 sangai, including 20 stags, 25 hinds and 6 fawns (Shamungou 1997). The population of sangai had increased to around 180 individuals by 2003, consisting of 65 stags, 74 hinds and 41 fawns (Singsit 2003, Hussain et al. 2006).

R. e. thamin has the largest population; however, it is showing a declining trend. The *R. e. thamin* conservation programme was relatively

successful, with the animals breeding in Zoos and being reintroduced into the wild later at two wildlife sanctuaries, the Shwesettaw and Chatthin wildlife sanctuaries. Census conducted in the past indicated that there were around 2,200 individuals in Chatthin Wildlife Sanctuary (Salter & Sayer 1986). The *R. e. thamin* population in Shwesettaw Wildlife Sanctuary is estimated to have a minimum of 240 animals (FAO 1982). Regular transect surveys conducted in Chatthin Wildlife Sanctuary between 1983 and 1996 indicated a population decline of more than 40% (McShea et al. 2001), with the population estimated at about 500 *R. e. thamin* individuals.

The current status of *R. e. siamensis* in Indo-China is largely unknown, although it is thought that small scattered herds may still remain there (McShea et al.1999). In 2002, a second population of *siamensis* was reported by the Department of Forestry from a 200 km^2 area in Chonbuly district (McShea 2002). Deer track and sign surveys in June 2002 found 2.61 signs / km, and one individual was observed. It was estimated that potentially 20–30 deer were present (Vongkhamheng & Phirasak 2002, Arlyne et al. 2003). The Wildlife Conservation Society has recorded sightings of *R. e. siamensis* made during community patrols in ATT since 2005. However, no systematic survey of these deer has been conducted. The seasonal nature of dipterocarp forests and the availability of permanent water sources may be significant for the density of animals that can be supported (Timmins & Duckworth 2008). McShea et al. (2001) identified four factors explaining the presence of the deer in Cambodia's Northern Plains, including the extent of the wetlands.

In Vietnam, small herds of *R. e. siamensis* have been reported from the A Yun Pa and Chu Prong areas of Gia Lai Province, Yok Don National Park of

Dak Lak Province and Yok Don NP Chu Mom Ray Nature Reserve of Kon Tum Province. However, their exact status is not known (Timmins & Duckworth 2008). So far, 19 protected areas have been established or proposed in the distribution range of Eld's deer in Vietnam. The animal has been recorded in five of these protected areas recently (Dang & Nguyen, undated).

The population size of *R. e. hainanus* in the central and western regions of Hainan Island was estimated at more than 500 individuals in the 1950s (Liu 1998). However, following severe exploitation, commercial hunting and a rapid increase in the human population and expansion of agricultural lands, the extent of the habitats continually reduced (Song 1993). This subspecies suffered a major range contraction and was considered almost extinct by the early 1970s (Song 1996, Liu 1998). The Datian Nature Reserve, where the last 26 survivors were found, was established in 1976 (Song & Li 1992). Subsequently, the relict population has recovered slowly, although it has continued to experience sporadic poaching and habitat degradation (Song & Li 1992, 1995; Song 1996). The population was once on the verge of extinction, with only two isolated groups with a total of 46 deer were recorded in 1976 in Datian Nature Reserve (DNR) and Dongfang and Bangxi Nature Reserve Baisha (BNR) (Song 1996). After the deer at BNR were finally wiped out by poachers in 1981, DNR and its vicinity became a unique site, harbouring the last group of Hainan's Eld's deer (Yu et al. 1984). The population at DNR increased to 75 individuals in 1983 and to 375 in 1993, with an average annual growth rate of 17.46% since 1983 (Zeng et al. 2005). Unfortunately, the population of Hainan declined to 342 in 1994 due to a food

shortage caused by a serious drought that year and by the extremely high density of 87 deer / km^2 within an enclosure with an area of only 3 km^2. Since DNR was entirely enclosed, the deer population has increased again, with an annual growth rate of 16.70% on average between 1994 and 2000. By 2000, the population size had reached 864 individuals (Yuan et al. 2001). It was estimated that there were over 1,000 deer at DNR in 2003 (Zeng et al. 2005). In 2002, after a reintroduction, the population of Hainan's Eld's deer at BNR increased to around 115 animals (Zeng et al. 2005).

1.3.2 Conservation genetics of Eld's deer

Very few investigations have been conducted on the genetics of Eld's deer in the wild based on karyotype analysis, mitochondrial DNA genes and microsatellite loci. Thevenon et al. (2000) studied the karyotype identity of two Eld's deer subspecies, *R. e. siamensis* and *R. e. thamin*, and their findings showed no chromosomal differences at the subspecies level. The findings suggested that, at least from a karyotypic perspective, no obvious differences delimit the two subspecies, and hybridization between *R. e. siamensis* and *R. e. thamin* is not likely to lead to impaired fertility in hybrid animals. On similar lines, Tanomtong et al. (2008) studied the cytogenetics of *R. e. siamensis* of Thailand and *R. e. thamin* of Myanmar and showed that these two subspecies exhibit the same karyotype, with a diploid number of 2n = 58 (fundamental number, NF = 70) for females and 2n = 58 (fundamental number, NF = 71) for males.

Pitra et al. (2004) used mitochondrial cytochrome *b* sequences to assess the phylogenetic pattern and timing of radiation of Old World deer in

33 Cervinae taxa. The major findings were that the genera/subgenera *Axis, Rucervus* and *Rusa* and the species *C. elaphus* are non-monophyletic and that *Elaphurus davidianus* belongs to genus *Cervus*. Of the species referred to *Rucervus*, two (*R. duvaucelii and R. schomburgki*) form a clade which is only remotely related to other Cervini but may be distantly linked to *A. axis*, while the third (*R. eldii*) is closely related to *Cervus* and linked to *E. davidianus*. A recent phylogenetic study placed Eld's deer as the sister taxon to another swamp adapted species, Pére David's deer (*E. davidianus*), and these two species constituted the basal lineage in the *Cervus* clade (Randi et al. 2001). Balakrishnan et al. (2003) analysed the variation in the mitochondrial DNA (control region) of the three subspecies of Eld's deer and showed that the ecologically divergent *R. e. eldii* is related more closely to *thamin* than to *siamensis*. The study showed that *R. e. thamin* and *R. e. siamensis* are distinct subspecies, but *R. e. hainanus* is characterized by a unique and relatively divergent mtDNA haplotype, suggesting a duration of historical isolation. A strong degree of phylogeographic structure both between subspecies and among populations within subspecies is also indicated, suggesting that the dispersal of individuals between populations has been very limited historically. The haplotype diversity was relatively high for *thamin* and *siamensis*, indicating that the recent population decline has not yet eroded the genetic diversity, whereas no haplotype variation was found within the *R. e. eldii* and *R. e. hainanus* populations, which are known to have suffered population bottlenecks. Pang et al. (2003) found no genetic variability using mtDNA control region genes in *R. e. hainanus*, which has suffered recent population contractions. Their study employed a simulation approach

to test the likelihood of various bottleneck scenarios and showed, in the context of what is known about the recent demographic history of this population, that there are credible scenarios for a bottleneck driven by hunting pressures in the 1960s.

Guha & Kashyap (2005) examined the mitochondrial 16S rRNA gene for identification of blackbuck, goral, nilgai, hog deer, chital, sambar and Myanmar's Eld's deer. The heminested PCR assays designed by them were successfully validated for sensitivity and specificity and provide a reproducible and rugged method allowing analysis of low copy number DNA recovered from decomposed or highly processed tissues under a wide range of conditions. Guha et al. (2007) analysed two mitochondrial genes, 16S rRNA and cytochrome b, to resolve the phylogenetic position of the pecoran species, i.e., species of the families Bovidae, Cervidae and Moschidae, endemic to the Indian subcontinent. The results established the basal position of the family Tragulidae and the monophyly of the infra-order Pecora within the suborder Ruminantia and demonstrated that the families Bovidae, Cervidae and Moschidae are allied to the musk deer, *Moschus chrysogaster*, showing that this species is more closely related to bovids than to cervids.

Franklin's (1980) experience with captive animals suggests that isolated populations should have at least 50 breeding individuals and preferably 500 individuals to maintain genetic variability. As a result of more recent work, Lande (1995) concluded that "effective population size" will need to be on the order of 5,000, rather than 500, to ensure long-term viability. Song (1996) used the program VORTEX to determine the essential

requirements for the long term conservation of two isolated deer populations of Hainan's Eld's deer. The results indicated that both groups are susceptible to extinction, given demographic challenges or environmental variations. At the same time, separating the groups reduced the population sizes, which could have led to further losses in genetic variability. Since 1995, the two isolated groups have been merged into an intact population again.

Ali et al. (1998) analysed the genome of the central Indian swamp deer (*Rucervus duvaucelii branderi*) found in Kanha National Park to provide a genetic basis for their extinction using the evolutionarily conserved repeat sequence motifs $(GATA)_{3.75}$, TA $(GATA)_{4}$, $(GACA)_{3.75}$ and $(TGG)_{6}$ and used a set of mouse β-actin primers to uncover the sequence variation within and between related species by employing the hybridization and AP-PCR amplification techniques. From the analysis of a very limited number of *Cervus* DNA samples, a high level of genetic homogeneity was observed. This may be a prime reason for the extinction of this species. This study has implications in the context of the conservation of the endangered *R. duvaucelii branderi*.

Molecular phylogenetic studies based on mitochondrial DNA (Miyamoto et al. 1990, Randi et al. 2001, Cook et al. 1999, Polziehn & Strobeck 2002, Ludt et al. 2004) and nuclear DNA (Comincini et al. 1996) sequence comparisons have contributed considerably to resolving evolutionary relationships among deer species at the family level (Cervidae), but these studies have not fully resolved the phylogeny of the Cervinae because they lacked material from many of the extant Old World deer

species. The maternal ancestry of *E. davidianus* is proto-*R. eldii*, from its stems. This unites Pére David's deer (*E. davidianus*) with the tropical Southeast Asian Eld's deer (*R. e. thamin* and *R. e. hainanus*) which have in the past almost invariably been associated with *R. duvaucelii* in a genus or subgenus *Rucervus* (Ellerman & Morrison-Scott 1951). Its paternal ancestor would then be the species ancestral to the rest of the genus *Cervus*. Nagata et al. (1999), using the D-loop, and Cook et al. (1999), using cyt *b*, studied the sika population from China and showed that the northern Japanese sika and the southern form actually form three equal branches. The northern form of the Japanese sika is much larger than the southern form their sizes barely overlap.

Randi et al. (2001) used complete mitochondrial control region sequences to infer phylogenetic relationships in 25 Cervinae taxa. *Cervus* splits into clades that are partially discordant with the current species delimitations. The nominate *C. elaphus* includes two divergent clades that must be referred to as the species *elaphus* (European elaphoid deer) and *canadensis* (Eurasian and North American wapitoid deer). *C. nippon* splits into Japanese and continental-plus-Taiwan sika. Père David's deer is nested within *Cervus*, suggesting that *Elaphurus* should be merged with *Cervus*. The study found a deep and consistent split between Japanese and mainland/Taiwan sika.

Molecular studies employing microsatellite DNA loci were limited to *R. e. hainanus.* Zhang et al. (2005) characterized 10 polymorphic microsatellite markers for Hainan's Eld's deer. Their results showed that these markers

should be suitable for conducting population genetic studies on Eld's deer and possibly other ungulates. Zhang et al. (2008a) assessed the genetic variability in the one source (Datian Reserve) and two introduced populations of *R. e. hainanus* (Bangxi and Ganshiling Reserve). They found that the genetic variability was low in each of the three populations and suggested that founder effects and genetic drift had affected the two translocated populations. They recommended that the three populations be managed as a meta-population for conservation. Zhang et al. (2005, 2008b) studied the isolation and characterization polymorphic microsatellite markers for *R. e. hainanus* and showed that these markers provide a useful tool for conducting population genetic studies on Eld's deer and possibly other ungulates.

According to Weber & May (1989), microsatellites, or simple tandem repeat markers, have become a ubiquitous source of polymorphic markers for genetic analysis. In recent years, microsatellites have become the markers of choice in many population genetic studies owing to their abundance and high heterozygosity in most eukaryote genomes (Queller et al. 1993, Jarne & Lagoda, 1996). Tate et al. (1998) identified species specific genetic markers and compared Pére David's deer (*E. davidianus*), the sister taxon of Eld's deer, red deer (*C. elaphus scollicus*) and North American wapiti (*C. e. manatobensis*). The analyses have identified over 300 genetic markers which distinguish Pére David's deer sires distinct from farmed red deer and over 100 markers which distinguish wapiti sires differ from red deer.

Zeng et al. (2007) studied the genetic diversity in Pére David's deer and the genetic consequences of population relocations in China using

mtDNA D-loop and polymorphic microsatellite loci. Their study revealed that the genetic diversity was extremely low in Pére David's deer populations in China. Their results suggest that effective management of a species of low genetic diversity such as Pére David's deer should consider the genetic background of each founder to make sure genetic variations are preserved in both the source population and relocated population. Frantz et al. (2008) investigated the fine scale genetic structure of the red deer, *C. elaphus* population using 14 microsatellite loci and attempted to find evidence for a change in its genetic structure over time. The results point towards a pattern of fine scale spatial structure amongst female red deer, *C. elaphus* but not amongst males, as would be expected for a typical mammalian system with male biased dispersal and female philopatry.

Brinkman et al. (2010) used microsatellite markers and attempted to solve problems associated with recovering DNA from faeces by investigating the influence of factors such as season, diet, collection method, preservation method, extraction protocol and time on Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) in a temperate rainforest environment. The objective of their study was to determine the length of time after which the DNA in a faecal pellet became too degraded for individual identification. Faecal pellets were extracted from the rectums of recently killed deer and placed in an environment protected from rainfall and an environment exposed to rainfall. Eighty percent of the samples in the protected environment and 22% of the samples in the exposed environment were successfully genotyped, with no samples being successfully genotyped in the exposed environment after 7

days. The study showed that rainfall significantly increases degradation rates of DNA from ungulate pellets.

The present study was intended to estimate the abundance and demographic parameters of the sangai population. The study undertook to compare the genetic variability of sangai within the species, between wild and captive populations and in comparison with related cervids. The information obtained will contribute to our understanding of the genetic sustainability of sangai and to designing a conservation strategy for the species in the wild.

1.4 OBJECTIVES OF THE STUDY

The following were the objectives of the present study.

1) To estimate the abundance and demographic parameters of sangai and hog deer in KLNP, Manipur

a) the population growth rate in the Park.

b) the male to female ratio and adult to juvenile ratio.

c) the status of the population of hog deer in the Park.

2) To assess the genetic variability using the mtDNA (Cyt *b* and control region) genes and polymorphic microsatellite markers

a) within the wild sangai population.

b) between wild and captive sangai populations.

c) between sangai and related cervids.

1.5 KEY RESEARCH QUESTIONS

Keeping in view the background provided in the foregoing sections, the following research questions were posed while formulating the present study.

1. A previous population analysis had shown that the exponential rate of increase in the sangai population between 1975 and 2003 was 10% per annum. What is the current trend in the population, the age structure and the sex ratio of the sangai and hog deer in Keibul Lamjao National Park?
2. As both sangai and the hog deer occurs in flood plains area and sympatrically in Keibul Lamjao National Park how are they genetically related with each other and with other cervids of Northeast India?
3. As we know, the sangai population had 14 founder individuals in 1975. There is apprehension that this sangai population might be going through a genetic bottleneck and are susceptible to inbreeding depression. What is the degree of genetic variability in the wild and captive population of sangai and the sympatric hog deer which is facing population decline in most of its distribution range?

1.6 SIGNIFICANCE OF THE STUDY

Studies on the ecological and genetic profiles of Eld's deer are still in their infancy compared with the detailed work and conservation efforts carried out with other cervids elsewhere in the world. There is lack of information on the overall abundance and distribution of various subspecies. There is need for a regular monitoring programme in order to obtain the latest information for

identifying critical habitats for prioritized conservation actions and for supporting and guiding the protection of the species. There still remain many knowledge gaps in the systematic and genetic status of Eld's deer. Taking into account its decreasing population trend, a continued ecological monitoring programme combined with long term scientific research on the genetic population structure and diversity and the relatedness and kinships of the current populations of sangai is clearly needed. Previous population estimates were done using aerial and ground census techniques. However, the data were not analysed to derive the confidence intervals. Very few studies on genetic aspects have been conducted on sangai in captivity. This study is the first attempt to examine and develop reliable and replicable monitoring protocols for abundance estimation in the wild. This study has been undertaken to provide an overview of the current demographic status and baseline information on conservation genetics aspects and to suggest future studies to fill in the existing research gaps to assist in developing a conservation and management strategy for this species.

1.7 ORGANIZATION OF THE THESIS

This thesis is organized in seven chapters. The sections of Chapter 1 deal with the study background, a review of the literature, the objectives, the key research questions and the significance of the study. Chapter 2 describes the study area in detail, providing a biogeographic and historical background. Chapter 3 deals with the research design and the broad methods adopted, with further details being provided in the relevant technical chapters. Chapter 4 deals with the population ecology. Chapters 5 and 6 investigate the

phylogeny and genetics structure of the sangai and associated cervids. Chapter 7 synthesizes the findings of the study, highlights the conservation implications of the study and gives pointers for future work. Each chapter has an introduction, methods, results and discussion section. A reference section with a detailed list of literature consulted is provided at the end of the chapters.

Chapter 2
STUDY AREA

2.1 INTRODUCTION

Eld's deer, once distributed across much of Southeast Asia, is essentially restricted to the Irrawaddy and lower Mekong valleys between Thailand and Cambodia, with an isolated small population in the west in Manipur and lower to the south in the east in Hainan Island, southern China (McShea et al. 2001). Eld's deer is believed to have originated via a land bridge from the Southeast Asian mainland and arrived to the Island of Javan and Hainan during the end of Pleistocene and early Holocene (18,000-8,500 years BP) when the sea level went down below 85 m from the present mean sea level (Ginsburg et al. 1982, Bhumpakphan et al. 2004). Although the primary forest type of most Eld's deer populations is dry dipterocarp forest (McShea et al. 2005), the fringe populations of this species occupy wetter ecosystems. Hainan's population is found in this tropical moist island in shrub forest (Zeng et al. 2005). Isolated populations of *R. e. siamensis*, both in southern Laos (Round 1998) and in Ang Trapeang Thmor Reservoir (ATT), in northwestern Cambodia, inhabit marshy areas, in conditions similar to those described by Lekagul & McNeely (1977) in relation to extirpated deer in Thailand. The last remaining population of *R. e. eldii* inhabits floating mats of dense vegetation within a small (<22 km^2) region in Manipur, India.

Eld's deer show variability in their habitat preferences. *R. e. eldii* inhabits low lying swamps (Lekagul & McNeely 1977) and is especially adapted to the unique *phumdi* habitat. It has divided hooves, and its pasterns are greatly elongated, unlike those of other deer species (Gee 1960). Therefore, the animal is especially adapted to walking conveniently over the quaking surface of Keibul Lamjao National Park (KLNP) (Singh 1992). The Eld's deer of Manipur is present only in the state of Manipur within a habitat of extent 40 km^2, making it endemic to KLNP. The sangai naturally occurs in low densities by virtue of the limited availability of its habitat, which is characterized by the swampy flood plains of KLNP, Manipur.

In contrast, *R. e. thamin* and *R. e. siamensis* are found mostly in dry deciduous dipterocarp forests with an open under storey (Salter & Sayer 1986). Evidence from Thailand and Cambodia indicates that *R. e. siamensis* is primarily associated with dry dipterocarp forests, open canopy woodlands characterized by deciduous trees and a grassland under storey (Koy et al. 2005), which are found mostly in the monsoon areas of the Mekong plains, and favours open canopies with a grass under storey or grassland patches having hydrological origins. However, Lekagul & McNeely (1977) proposed that Eld's deer originally inhabited swampy areas but were forced recently into drier habitats due to pressures imposed by hunting and the expansion of agricultural areas. There is, however, no evidence to suggest that Eld's deer is wetland associated, while a long term research programme on *R. e. thamin* in Myanmar concluded that Eld's deer are not dependent on water sources (McShea et al. 1999).

R. e. hainanus inhabits tropical plains and hills less than 200 m high, predominately scrubland and grassland with sparse trees (Xu et al. 1983). Historical records by the Annals of Hainan Island (Qionghai or Qiongzhou) include Chengmai, Qiongshan, Lehui, Dingan, Yazhou, Lingshui, Wanzhou) and Lingao as well as Qiongzhong according to the local people (Xu & Liu 1974, Yu et al. 1984, Yuan et al. 1993, Yuan et al. 2001). The species has disappeared from these sites since the 1950s. In the early 1950s, the species was found in only 20 districts in six counties, and its distribution range was estimated at 200-300 km^2 (Yu et al. 1984).

2.2 HISTORY OF SANGAI CONSERVATION

The sangai was once found in all parts of the state, but after rampant hunting and a reduction in its population size, it was declared extinct by the Government of Manipur in 1951. Before the rebellion of 1891 against British rule, the sangai was protected by the order of the royal family of the Meitei clan, and any man who was proved to have killed a sangai was imposed a fine or heavy punishment, which could even result in the chopping off of his hands. The Manipur State Durbar accepted the Draft Game Rules for Manipur State in 1916, but it was Captain Harvey, the president of the then Manipur State Durbar, who promulgated in 1931 the first Game Rules of Manipur, under which the deer was fully protected. In the past, the name “sangai” was given locally when people use to hunt with a permit issued under the Manipur Durbar Act. It is derived from “sa”, meaning “animal” in the local language, and “ngai”, meaning “wait” the animal that waited and ran from hunters

(Shamungou 1997). The animal used to run for 3 seconds, look behind repeatedly by turning its head and run again.

In 1953, the sangai was rediscovered by noted naturalist E.P. Gee, the then Honorary Secretary, Eastern Region, Indian Board for Wildlife. A total of 6 heads of sangai were counted in 1959. Due to the persistent efforts of E.P. Gee, the sangai was declared a protected animal, and its habitat, Keibul Lamjao, was declared a protected sanctuary in 1954, covering an area of about 52 km^2. In 1959 the total area was reduced to about 27 km^2 and subsequently increased to about 40 km^2, in 1965. The sanctuary was officially gazetted in 1966, and in 1967 KLNP came into being under the Wildlife (Protection) Act, 1972. With a view to ensuring protection for the animal, Keibul Lamjao was declared protected in 1965, a reserved forest in 1974 and finally a national Park in 1977 (Singh 1992). The Park received national and international attention when Loktak Lake was declared a site of the Ramsar Convention on 23 March 1990. This faunal significance led the state government of Manipur to declare the sangai the state animal in 1989 (Trisal & Manihar 2004).

2.3 PHYSICAL ATTRIBUTES

2.3.1 Loktak Lake

Loktak Lake (24°25′ to 24°42′ N and 93°46′ to 93°55′ E) is the largest natural freshwater Lake in northeastern India, covering 61% of the total extent identified as wetlands in Manipur (Trisal & Manihar 2004). The Lake can be broadly divided into northern, central and southern zones. The three zones

are characteristically different in terms of biodiversity and pressure of human activities. The southern zone of the Lake includes KLNP, Ungamel and Kumbipats. The largest island of Loktak Lake is KLNP. The Lake harbours a rich biodiversity and is of great cultural importance to the people of Manipur. It also plays an important role in providing ecological and economic security to a large population living in and around the Lake and depending upon its resources for sustenance. The Loktak Lake (Plate 2) is also called the "Floating Lake" due to the *phumdis* (a Manipuri word meaning floating mats of soil and vegetation) on it. These are heterogeneous masses of soil vegetation in organic matter which occur in all sizes and range in thickness from a few centimetres to about 2.5 m. They float on the Lake with about one-fifth of their thickness above water and the rest below the surface.

The Lake is oval in shape, with a maximum length and width of 32 km and 13 km, respectively, the long axis running north to south. The Lake covers an area of 289 km^2. It is located at 768 m above msl, and its depth varies from 0.5 to 4.58 m, with an average depth of 2.7 m. During the dry season, when the rafts sink, deer find shelter at the Lakeshore (Singh 1992).

The Lake comprises several smaller Lakes or *pats*. Until 1983 the area used to experience great changes in the water level annually, and so the several Lakes that were separated during the low water phase (65 km^2 extent at 765 m above msl) merged into one Lake only at the time of high flooding (495 km^2 at 780 m above msl). The Lake is under stress mainly due to anthropogenic pressures. Deforestation and shifting cultivation in the catchment area have promoted soil erosion, resulting in increased siltation of

the Lake. The problem has been aggravated further due to the prolific growth of the floating *phumdis*. Besides nutrients from the catchment area, pesticides used in the agricultural fields and domestic sewage from Imphal city are carried by the Nambul River, which finally discharges these into the Lake. In addition to the above threats, encroachments through construction of fishponds, roads and settlements have gradually led to a degradation of the Lake ecosystem (Trisal & Manihar 2004).

The basic problems of the Lake can be traced to loss of vegetal cover in the catchments area and construction of the Ithai Barrage in the southern part of the Lake. The permanent flooding of the Lake, with relatively small changes in the water level, caused by the withdrawal of water for hydel power generation and irrigation, has been attributed to the construction of a multi-purpose project, in 1983. This has brought about drastic changes in the hydrological regime and converted a natural wetland with a fluctuating water level into a reservoir with a more or less constant water level (Trisal & Manihar 2004).

2.3.2 Keibul Lamjao National Park (KLNP)

KLNP is the last natural refuge of the sangai. It is situated near Moirang in Bishnupur district, about 40 km south of Imphal. The Park is demarcated from the Lake by a discontinuous hill range (Thanga Hills). The eastern part, which constitutes one-third of the Park, consists entirely of marshy land, and in the western part, there are three hillocks *viz.* Pabot Chingjao, Pabot Chinglukok and Toya (Plate 3) (Singh 1991).

Plate 2. Views of Loktak Lake, Manipur with floating *phumdis.*

Plate 3. Views of Keibul Lamjao National Park, Manipur.

The habitat consists of woodlands on the hillocks, grasslands in the floating *phumdis* and elevated strips of land. Based on the thickness of the *phumdis*, the Park is classified into three zones, namely the western zone, eastern zone and northern zone. The western zone forms the main habitat of the sangai, and the *phumdis* in it are 3 m or more thick. The eastern zone is mainly covered by thin *phumdis* intermixed with dense vegetation whereas the northern zone comprises open water of moderate depth covered with thin *phumdis*. Between two stretches of *phumdis* is an elevated portion, called Thangbirelyangbi that gets exposed during the lean season and is an important place for the breeding of the sangai.

The Pabot and Toya hills, which are located in the northern and southern parts of this zone, are extremely important as shelters and resting places. Sagram, Keibul and Chingmei are the main villages on the border of the western zone. The eastern zone extends from Nongmaikhong village to the end of the Khordak channel in the east and to Pabot and Toya in the west. It is mainly covered by *phumdis* intermixed with a thick growth of plant species such as *Saccharum* spp*., Zizania* spp. and *Phragmites* spp*.* The northern zone extends from Keibul hill to Chingthi hill in the northern side and from Komlakhong village to Laphupat Tera in the eastern side. This is an area of open water. It is relatively deep and covered with thin *phumdis* (Trisal & Manihar 2004).

The total area of the Park is 40 km². Of this, 26 km² is covered by a thick and almost contiguous mat of *phumdis*, and the remaining 14 km² consists of open water, drylands, uplands and sporadic hillocks (Singh 1992).

2.4 EDAPHIC CHARACTERS

The Park is a low lying lacustrine swamp with a thick floating mass of vegetation and soil, the *phumdis*. The soil in and around the Park is alluvial, underlined by argillaceous rock of the Disang series from the Cretaceous to Eocene period. The soil is generally ferruginous clay to clayey loam. The geological formation of the area is of a sedimentary type. The soil in the *phumdis* is slightly acidic, with the pH value ranging from 5.2 to 6.0 approximately (Singh 1991).

2.5 ECOLOGICAL ATTRIBUTES

2.5.1 Climate

The Park is characterized by low temperatures and heavy dew at night from November to February. Frost occurs in winter early in the morning and during the night, being common in December and January. The temperature ranges from a maximum of 34.4°C to a minimum of 1.7°C (Singh 1992). The annual rainfall varies between 1000 and 3500 mm, and the average rainfall is ~1500 mm. The area is most humid in August when the relative humidity is as high as 81%. The relative humidity is least in March, 49% (Singh 1992).

The Park experiences a moderate climate throughout the year. Summers prevail from March till May, and the monsoons formally arrive in June and drench the state with heavy showers of rain up to September. The months of October and November are, more or less, dry. Winter extends from December to February, when the temperature usually drops down to 0°C. The

southwest monsoon chiefly determines the weather and rainfall throughout the state. The state has a tropical to temperate climate, depending upon the elevation (Singh 1992, Shamungou 1997).

2.5.2 Floral diversity

More than 100 species of grasses and sedges have been recorded on the *phumdis*, of which *Zizania latifolia, Phragmites karka, Saccharum munja, Narenga porphyrochroma, Learsia hexandra, Carex* spp., *Oryza perensis* and *Capillipedium* spp. constitute the major food items of the sangai (Shamungou 1985). The dominant plant species are *Pinus kesiya*, *Bombax malabricum, Bauhinia variegata, Ficus glomerata, Phyllanthus emblica, Lantana camara, Xanthium* spp., *Chrysopogon* spp., etc. in the hills (Singh 1992).

2.5.3 Faunal diversity

The KLNP fauna includes 22 species of mammal, 81 species of bird and 25 species of reptile (Singh1991). Some important mammalian species that dwell in the Park along with the sangai are the hog deer (*Axis porcinus*), wild boar (*Sus scrofa*), large Indian civet (*Viverra zibetha*), small Indian civet (*Viverricula indica*), jungle cat (*Felis chaus*) and otter (*Lutra lutra*). The Park is also a unique wintering ground for various migratory waterfowl, the spot-bill duck (*Anas poecilorhyncha*), gadwall (*A. strepera*), shoveller (*A. clypeata*) and common teal (*A. crecca*), and the permanent home for many resident birds (Shamungou 1997).

The Loktak Lake is also the breeding ground of a number of riverine fishes such as the mrigal (*Cirrhinus mrigala*), reba (*Cirrhinus reba*), parmoun barmsky (*Crossocheilus burmanicus*), flying barb (*Esomus danricus*), swamp barb (*Puntius chola*) and orange-fin labeo (*Labeo calbas*) and continues to be a vital fisheries resource. It also supports a significant population of reptiles such as the viper (*Vipera russellii*), krait (*Bungarus caeruleus*), cobra (*Ophiophagus hannah*) and python (*Python morulus*) (Singh 1992).

Chapter 3
BROAD METHODOLOGY

3.1 INTRODUCTION

The term distance sampling refers to a suite of methods that are used to estimate the absolute densities of biological populations based on accurate measurements of the distances of objects from a line or point. Distance sampling is an extension of plot sampling, where it is assumed that all objects within sample plots are counted (Buckland et al. 1993). Distance sampling has advantages including the following: The absolute density of a population may be estimated, even when not every individual is detected. The density of a population can be calculated from data collected by two different observers even if one of these observers misses a number of objects. Only a relatively small proportion of individuals need to be detected within a sample area, possibly as small as 10.30%. The size of the sample area can be unknown. Central to the concept of distance sampling is the detection function. This is the probability of detecting an object, given that it is at any distance y from a random line or point. This distance y refers to either the perpendicular distance, in the case of line transects, or the sighting (radial) distance, in the case of point transects. Generally the detection function decreases with increasing distance (Buckland et al. 1993).

Distance sampling methods estimate a probability of detection, rely on few assumptions and can be used with both line and point transects. The assumptions for distance sampling can be reasonably valid with training, effective field techniques and appropriate field design. Possibly the greatest advantage of these methods is the small number of detections which are likely to be necessary for fitting the detection function (Barraclough 2000). In 'distance sampling', distances of detected animals from a line or point are modeled, to estimate detectability and hence abundance (Burnham et al. 1980). The method has been used successfully in a very diverse array of taxa, including trees, shrubs and herbs; insects; amphibians; reptiles; birds; fish; and marine and land mammals (Buckland et al. 2001). Point counts are used to sample bird populations for estimating densities in local areas, determining trends in populations over regional areas, assessing habitat preferences and other scientific and population monitoring purposes (Buckland et al. 1993). The present study aims to use the standardized census technique of point count to determine the population densities of sangai and hog deer to provide an overview of the current demographic status in the Park.

A combination of demographic, ecological and genetic studies is necessary to obtain a better understanding of the population dynamics of species. Population declines may be caused by a range of environmental and ecological factors, including overexploitation, pollution, the impacts of introduced species, as well as by stochastic events of a demographic, environmental or genetic nature (Brook et al. 2002). A molecular marker addresses many applications of genetic markers (polymorphic proteins and

DNA) from the perspectives of population biology, behavioural ecology, organismal evolution and phylogeny (Avise 2004). Molecular conservation genetics has received considerable attention in the last decade and holds great promise as a tool to aid conservation biologists in implementing programmes intended to preserve genetic diversity in both captive and natural populations (Philip & Philip 1992).

Most molecular based studies begin with extraction of DNA from a particular organism, followed by amplification (i.e. generation of many copies) of particular segments of DNA using the Polymerase Chain Reaction (PCR). The utility of PCR lies in the fact that only minute quantities of DNA are needed. There are many different types of DNA markers used in molecular ecology, including: mitochondrial DNA (mtDNA), which has several features rendering it particularly suitable for the analysis of phylogenetic relationships: high copy number, apparent lack of recombination, partially high substitution rate and maternal mode of inheritance (Arnason et al. 2002). and microsatellites (MSATs, highly repetitive sequences of DNA that mutate rapidly and are often used to identify individuals), minisatellites (similar to microsatellites but with longer repetitive sequences) and restriction fragment length polymorphisms (RFLPs, specific sites of DNA that can be cut by enzymes, yielding different sized fragments of DNA in different species, populations, and DNA sequence data). The markers generated by these methods are also visualized in different ways. Traditionally, MSATs and RFLPs were visualized as discrete bands revealed by agarose gel electrophoresis. The nucleotides constituting DNA sequences, however,

require finer levels of resolution, often achieved using polyacrylamide gels and autoradiography (Allan & Max 2010).

The present study was carried out in Keibul Lamjao National Park in Manipur, India. The objective of the study was to estimate the abundance and demographic parameters of the population of sangai. The study also undertook a comparative study of the genetic variability of sangai within the species, between wild and captive populations and among closely related cervids. This study describes two molecular methods commonly used in molecular ecological studies: mtDNA (cyt *b* and control region) and microsatellites analysis. An elaborate study design was drawn up to achieve the research goals in this context. This chapter provides an overview of the approaches used.

3.2 STUDY DESIGN, DATA AND SAMPLE COLLECTIONS

The fieldwork for the present study commenced in October 2005 and continued up to March 2008. In this period, approximately three years, field data collection relevant to different objectives of the study was undertaken. In the initial phase of the study, a detailed survey of the literature published on the classification and nomenclature of Eld's deer between 1841 and 2010 was carried out. The past distribution and population status were determined based on the reports published. The latest information available from genetic studies was reviewed.

A reconnaissance survey was completed between June and July in 2005. A base camp was established near the Park. After that, necessary permits were obtained from the Manipur Forest Department to enter the Park areas, Manipur Zoological Garden and Second Home of sangai near the Zoo itself**.** Special permits were obtained from the forest department for collecting samples from both wild and captive populations. The entire Park area had been previously divided into 24 counting blocks, each sufficiently large to encompass the range of a male or female sangai. The blocks were demarcated such that they had a perimeter of natural or man-made barriers so that any substantial movement in or out was prevented. The animals were searched for in each block. Line transects were laid in the Park according to the *phumdi* type and the presence of open water, excluding areas adjoining villages, to examine the distribution patterns of sangai and sympatric ungulates. This was done to collect information on the distribution of and habitat use by sangai, hog deer and wild boar and differentiate high, medium and low dung densities areas. Trial boat transect surveys were carried out in different study sites to identify specific locations in the Park that were suitable for constructing machans (temporary bamboo watch towers) for monitoring the population. This exercise not only provided baseline information for the construction of machans but also helped classify the habitat as thick and thin *phumdis*. No machan was constructed in those blocks in which no faecal pellets were found.

Forty observers, including members of local NGOs and clubs and college students, were imparted training in Manipur Zoological Garden and inside the Park for three days for counting the animals. Identification of the

sangai and distinguishing the sympatric ungulates in the Park, *viz.* the hog deer and wild boar, were emphasized. The observers were trained in recording data and especially in differentiating between juvenile sangai and fawns as well as between female juvenile sangai and adult female hog deer and in proper identification of the age and sex. The observers were taught how to measure accurately the distance between the observer and the animal and the direction of movement of the animal.

The population estimates were carried out during February–April, after the grass had been cut and had dried, when the visibility of the animals in the Park was better. The population abundances of the sangai and hog deer was estimated using the point count method in Keibul Lamjao National Park, Manipur during 2006–2008. The distance between each machans were maintained by demarcating with a flag on a distance of 800 to 1000 m. Eighteen machans of 6-7 m height were constructed at specific sites in the Park after monitoring the faecal transects where direct and indirect evidences of sangai and hog deer was recorded and four observation points were used for the population estimation in the Park. Each machan could accommodate at least two people. Field survey data were collected between 0530 and 0900 hours and between 15:00 and 17:30 hours by two to three observers assigned to each point/machan so that more accurate figures were obtained. Later, the evening data were discarded because of the low number of sightings. Snapshots sightings were maintained at every 5 minutes interval by the observers assigned to each point. The distances and angles to the centre of each cluster were recorded. On detecting an animal cluster, the species, cluster size, age and sex composition of the cluster, the observer-to-animal

radial sighting distance and sighting angle were recorded. The counting of the species was carried out only half an hour after arrival at the machan so that the disturbance period was eliminated and data collection continued for 3 hours in the morning. Parameters such as the GPS location of the machan, time and direction of movement, time of sighting, radial sighting distance, sighting angle, habitat type, weather and temperature were recorded.

Molecular genetic studies require particularly careful planning because they are usually relatively expensive, and therefore an attempt was made to maximize the information obtained per sample. The cost of this study was calculated taking into account not only the cost of chemicals and other consumables but also the cost of time and the cost of collection of the right tissues from degraded and non-degraded samples, appropriate storage of samples, choosing the right DNA extraction protocols for various biological samples for screening and assessing the right markers (primers). Before the initiation of a full scale study, a pilot study was conducted in which samples were collected from wild animals and reference samples collected from captive animals.

The entire Park area was surveyed, and on the basis of the occurrence of faecal pellets, footprints, fixed trails and nests, seven specific areas were identified for collection of biological samples: Thangbirelyangbi, Sagramkha Mayaidak, Phumbai Asangbi and Khodangkhong, including three hillocks, *viz.* Pabot Chingjao, Pabot Chinglukok and Toya. These three hillocks are very important as they serve as a dwelling ground during floods and the rainy season. In addition, during December–February, when velvet peels from the

antlers, rub their velvety antlers on hardwood trees on the hillocks to make the antlers sharp and pointed. Therefore, the study was designed for collection of genetic samples such as tissues and hair from dead animals, peeled off velvet tissue and antlers, skin samples and fresh as well as old droppings from seven sampling points. The study was also designed to collect samples opportunistically from dead animals during pellet transects. The tissue samples, faecal pellets and decomposed tissues that were collected were preserved in 95% ethanol and stored along with the antlers and skulls at −20°C.

3.3 METHODS

3.3.1 Population estimation

The point count method was used to identify the abundance of sangai and hog deer in a 20 km^2 area of the Park excluding the water bodies and thin *phumdis*. Based on the occurrence of faecal pellets of sangai and hog deer, 22 machans of height 7 m were constructed for population monitoring. Faecal pellets of sangai and hog deer was recorded only in areas of extent 22.15 ±0.35 and 22.34 ±0.20 km^2, respectively, in the Park. Based on the distribution of faecal pellet groups, the different areas of the Park were classified as high, medium, low density areas. The high pellet density areas in the Park had n = 8 machans, while the medium and low pellet density areas had n = 7 machans each.

Population monitoring studies were conducted in the middle of March in three consecutive years, 2006 to 2008. To get more accurate estimates, the

counts were replicated on five continuous days. Counting of animals was carried out when the grass was dry, the conditions forced the animals to come out from thick bushes in search of food and the male categories were easily distinguishable by the antlers. The animals were classified as adult male, sub-adult male, adult female, sub-adult female, juvenile and fawn (Eisenberg & Lockhart 1972, Singh 1992). The counting team at each point (machan) consisted of two or three experienced observers equipped with binoculars, a rangefinder and a compass. The count was carried out under the assumption that no animal remains undetected between observation points**.**

To determine the accuracy of the point count technique, another method, the drive count, was used. The drive count was carried out after the completion of the point count, at 0900 hours, when the animals were hidden in the tall grass and thick bushes. During the drive counts, local people and forest department personnel were used to flush and count the animals at each point. The numbers obtained using the two methods were compared.

3.3.2 Mitochondrial DNA (cyt *b* and control region) analysis

The total genomic DNA was extracted using a DNeasy Blood & Tissue Kit and a Qiagen Stool Mini Kit (Qiagen) from tissue samples obtained from dead animals and from faecal pellets. The mtDNA fragments containing cyt *b* and control regions were sequenced after amplification with primers listed for cervids and related species in the literature and GenBank, National Centre for Biotechnology Information (NCBI). PCR was carried out in a thermal cycler. The PCR amplified products were separated on agarose gel by electrophoresis and purified using a QIAquick Gel Extraction Kit (Qiagen,

Valencia, California). Subsequently, the gel was stained with ethidium bromide for visualization and photographed in a UV light transilluminator. The selected PCR products were subjected to sequencing and were analysed.

3.3.3 Nuclear DNA (microsatellites) analysis

Total genomic DNA was isolated from tissues/hair/antlers/shed velvet/faecal pellets from live and museum specimens using the DNeasy Blood & Tissue Kit/QIAamp® DNA Micro Kit and Qiagen Stool Mini Kit (Qiagen). The isolated DNA was amplified using microsatellite loci listed for cervids and related species in the literature and GenBank, NCBI. PCR was carried in a thermal cycler. The PCR amplified products were separated on 2% agarose gel. Subsequently, the gel was stained with ethidium bromide for visualization of the microsatellite loci and allelic pattern. The selected PCR products were subjected to genotyping and were analysed.

3.4 DATA ANALYSIS

The densities were analysed according to point transect analysis guidelines (Buckland et al. 1993) and were computed using Distance 5.0 (Thomas et al. 1998). The probability of point detection was estimated using six models: the uniform cosine, uniform simple polynomial, half normal cosine, half normal Hermite polynomial, hazard rate cosine and hazard rate Hermite polynomial models. To determine the abundance, the density was calculated using the maximum peak sightings for 30 minutes to avoid duplicate counts. The population trend of sangai and hog deer was derive using regression analysis for the period 2006–2008. The population trend of sangai and hog

deer was derived using regression analysis. The density of population for each year was transformed to logarithmic values before regression, thus the population growth rate and R^2 value was derived.

For cladistic analysis, the published mtDNA control region sequences of Eld's deer were downloaded from the NCBI database. The sequences were aligned using Clustal X version 1.83 (Thompson et al. 1997) and checked visually. The aligned sequences were then edited manually to fit them to the same length using the software BioEdit version 7.0.9 (Hall 1999). The phylogenetic tree was derived using MEGA 5 (Tamura 2011), using the best fit model. The program MEGA 5 (Tamura et al. 2011) was used to calculate divergence times based on fossil records (Ginsburg et al. 1982). These dated fossil records have been treated as calibrations and used at the appropriate nodes to obtain a range of values for the respective divergence times.

Allele frequencies and heterozygosities (observed and experimental) were calculated, and various other parameters of genetic variation were tested and analysed using GeneMapper v.3.7 (Applied Biosystems). Allele identification, sizing and allele diversity estimates was calculated using CERVUS 3.0 (Kalinowski et al. 2007). Deviations from the Hardy–Weinberg equilibrium (HWE) were conducted using the exact test of GENEPOP 1.2 software (Raymond & Rousset 1995). The F_{ST} estimations within the wild samples of sangai and between captive populations were calculated using the GENEPOP 1.2 software (Raymond & Rousset 1995). Evidence of a recent genetic bottleneck was tested using the program BOTTLENECK 1.2.02 (Piry et al. 1999).

3.5 ORGANIZATION OF FIELDWORK

The fieldwork was carried out from one base camp located near Keibul Lamjao National Park, in Bishnupur district at Moirang village, Manipur. Census operations were carried out during March–April from 2006 to 2008 to monitor the populations of sangai and hog deer in the Park. One local man and two boatmen were hired to provide assistance during the entire period of the study. During the census operations, 40 observers, including members of local NGOs and clubs and college students, were hired. Training was imparted to them in the Manipur Zoological Garden and inside the Park for three days. Samples were collected for genetic studies during the extensive fieldwork period in 2006–2008. Fresh faecal pellets of both sangai and hog deer were collected and differentiated according to their morphometry and preserved in 95% ethanol. Dead tissue, carcases and shed antlers were also collected opportunistically, and tissues were preserved in 95% ethanol. All the samples were brought to the headquarters for further laboratory work and genetic analysis. The methods are described in detail in the section on study design and methods.

3.6 LIMITATION OF THE STUDY

This study was carried out in a unique ecosystem where methods such as direct counts from line transects and boat transects were impractical because of the shy behaviour of the animals, which were difficult to detect and moved away from the observers and quickly hide in tall grasses. Studies of radio collared animals would have been time consuming and expensive, and,

moreover,the results would not necessarily have been applicable in small areas with small populations. In order to overcome these problems, these methods were replaced with the point count method. Some limitations exist, such as visibility biases in tall grass resulting in probability of missing some animals, especially juveniles and fawns.

Genetic study has certain limitations like extraction of DNA from non invasive samples (faecal pellets) or from bones and decomposed samples resulting in low DNA quality (i.e. degraded DNA or presence of PCR inhibitors). The more obvious drawbacks are the risk of DNA contamination during the extraction and amplification process and difficulty in amplifying long sequences as most DNA of the sample becomes degraded resulting in short fragments. There are limitations however in the application of polymerase chain reaction and amplification process in degraded samples. Hence it is necessary to genotype samples in duplicates to obtain reliable genetic profiles. During the study these two problems were avoided by conforming to stringent guidelines to avoid contamination and by choosing PCR primers that amplify short DNA fragments (<200 – 300 base pairs) as well as large fragments (<400 – 500 base pairs). The phylogenetic analysis using short fragment had poor accuracy as compare to the larger fragment. Although phylogenetic trees produced on the basis of sequenced genes may provide evolutionary insight, they have some limitations. They do not necessarily accurately represent the species evolutionary history (Woese 2002). Also, there are problems in analysis based on a single type of character, such as a single gene or protein. As phylogenetic trees constructed from unrelated data source often differ from each other, therefore great care is needed in inferring

phylogenetic relationships among species. This is true of genetic material that is subject to lateral gene transfer and recombination, where different haplotype blocks can have different histories.

In general, the output tree of phylogenetic analysis is an estimate of the character's phylogeny (i.e. a gene tree) and not the phylogeny of the taxa (i.e. species tree) from which these characters were sampled, though ideally, both should be very close (Avise 2004). For this reason, serious phylogenetic studies generally use a combination of genes that come from different genomic sources (e.g., from mitochondrial vs. nuclear genomes), or genes that would be expected to evolve under different selective regimes, so that homoplasy (false homology) would be unlikely to result from natural selection (Primack 1993). Phylogenetic networks are used when bifurcating trees are not suitable, due to these complications which suggest a more reticulate evolutionary history of the organisms sampled (Huson & Bryant 2006). Therefore, in the present study some of the result may not be very conclusive because of difficulties faced while extracting DNA from faecal pellets and partially decomposed samples resulting low quality DNA. Besides limited number of sequences in the GenBank and in addition to these few sequences showed variations among themselves in drawing phylogenetic comparison.

Chapter 4

DEMOGRAPHIC STATUS OF SANGAI AND HOG DEER

4.1 INTRODUCTION

Conservation and management of endangered species in the wild requires adequate knowledge of their distribution and demography. Population estimation is one of the most important aspects of ecological studies as it plays a pivotal role in establishing priorities for species specific conservation and for delineating management practices (Carbone et al. 2001). The best way to study an animal population is with reference to its distribution and abundance (Greig 1983). In other words, density is an ideal instrument for studying animal population. The density estimates can be an operational term in ecology but only if they are defined with reference to its spatial scale (Smallwood & Schonewald 1996). Most modern methods of animal population estimation come under a general statistical framework that deals with two central concepts *viz.* spatial sampling and observability (Lancia et al. 1994, Thompson et al. 1998). Out of the two, spatial sampling concerns frequent inability of animal survey methods to cover entire area of interest (Smallwood & Schonewald 1996).

Distance sampling is a widely used group of closely related methods for estimating abundance of animal population. The main methods are line transects and point counts also called variable circular plots (Buckland et al. 2001). Distance sampling was developed to deal with the effect of distance on

visibility during ship-borne and airborne line transects surveys of cetaceans. The line transect theory has been extended with appropriate modifications for differences in geometry, to “point transects” which are sets of variables circular plot surveys (Ramsay & Scott 1979, Reynolds et al. 1980) with updated estimation methods (Buckland et al. 2001). Density estimates of large forest-dwelling species may be obtained from direct sightings or from counting signs of their presence and absence i.e. nest, dung/pellet, calls, footprints etc. along line transects (Anderson et al. 1979, Koster & Han 1988).

Considerable attention has been given to the use of point counts as a means of monitoring birds (Ralph & Scott 1981, Ralph et al. 1995). Based on empirical studies 50 to 70% of the total detections from long counts e.g. 20 minutes can be recorded in the first 3-5 min of a point count (Scott et al. 1981, Fuller & Langslow 1984, Gates 1995). The assumption is often made that monitoring for changes in populations does not require a complete census of all the individuals present and the statistical power to detect these changes often depends on rather large sample sizes (Johnson 1981, Thompson & Schwalbach 1995). Optimizing the number of detections unit time has been suggested as an approach for determining the appropriate duration for point counts used to monitor population trends (Verner 1988). However, Barker et al. 1993, points out that the optimal allocation of point count sampling efforts depends on the goals of the study *viz.* estimating population size, estimating population trends, the statistical test being used, the underlying population characteristics and the detection probabilities of the animals being counted. Detection rates vary among species (Mayfield 1981, Dawson et al. 1995), habitat types (Emlen 1971, Reynolds et al. 1980, Schiek 1997), seasons (Best

1981, Best & Petersen 1985) and times of the day (Robbins 1981, Lynch 1995).

The present study examines the abundance and demographic status of sangai with the objective to derive the population growth rate; sex ratio of male to female and adult to juvenile ratio. The study also assessed the population status of sympatric hog deer in the Park.

4.2 METHODS OF STUDY

The survey methodology was designed after referring to two standardized census techniques i.e. line transect and point count (Burnham et al. 1980, Buckland et al. 2001). Line transect was used to examine the distribution pattern of sangai and hog deer by recording the presence of faecal pellets. This method was expected to provide information on the distribution and habitat use by sangai and hog deer. The information collected from the transects can also help in segregating the habitat into thick and thin *phumdis*. The KLNP and the surrounding areas of the Park were stratified into 51 grids of 1 km x 1 km to examine the distribution pattern of sangai and hog deer. Depending on the open water, thin and thick *phumdis* 2 to 3 line transects of 500 m each (n = 66, 2006; n = 75, 2007; n = 72, 2008) were laid randomly on each of these grids. On each transect, plots of 50 m x 2 m (2006: n = 578, 2007: n = 750, 2008: n = 720) were laid to look for presence of faecal pellets. All the 24 blocks and adjacent areas were searched for the presence of faecal pellets. Three people were involved in the search operation.

During the survey, following parameters were recorded: habitat types, thickness of *phumdi*, water depth, distance from nearby village, GPS location, and presence of sangai / hog deer faecal pellets. The faecal pellets were aged on the basis of its colour and moisture contents: fresh (1-7 days), old (7-15 days) and very old (>15 days and indeterminate). During the exercise, the faecal pellets of sangai was recorded in 22.26, 21.5 and 22.7 km^2 during 2006, 2007 and 2008 respectively whereas in case of hog deer it was recorded in 22.26, 22.04 and 22.73 km^2 area of the Park during 2006, 2007 and 2008 respectively.

In KLNP, the population estimation of sangai was carried out in the second week of March to middle of April, for four to five consecutive days. Table 4.3 provides observation dates for the three consecutive years. The point count method (Buckland et al. 2001) was used to derive the abundance of sangai and hog deer in the Park. Eighteen machans (Figure 4.1) of 6-7 m height were constructed for this purpose and four observation points were used for the purpose in the areas where direct and indirect evidences of sangai and hog deer was recorded. The counting of the animal was carried during 0600-0900 hours.

At each machan 2-4 trained observers did the counts (Plate 4). Snapshot sightings were recorded at every 5 minutes interval. To get accurate estimate, the counts were replicated for 5 days. Animals were located with 8 x 40 optical binoculars and distance was measured using Laser Range Finder. Parameters like: GPS location of machan, time of

sighting and disappearance, sighting distance and angle, age and sex, habitat type, weather condition and temperature were recorded.

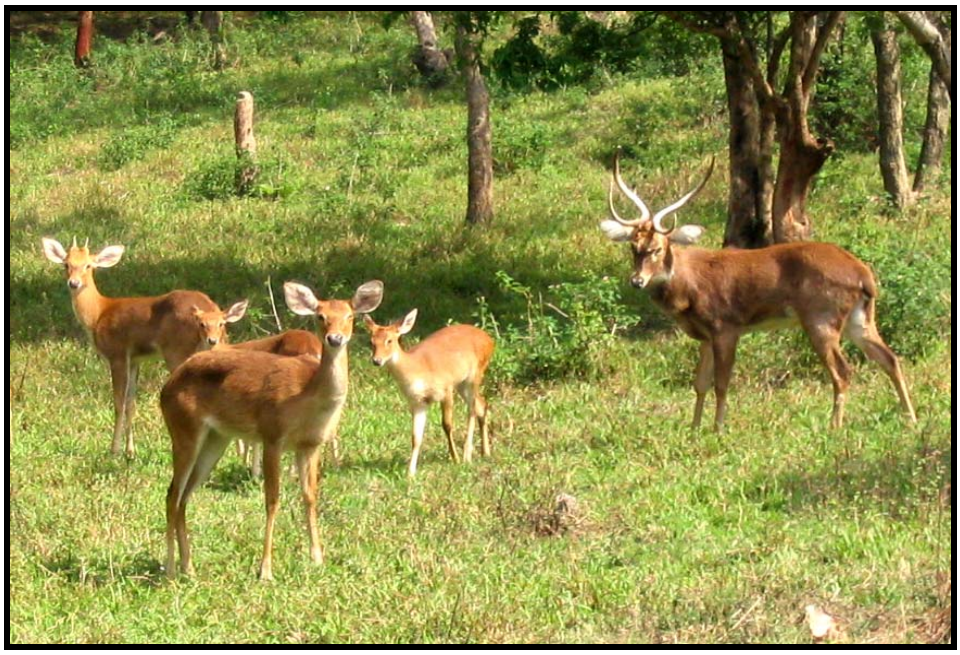
The locations of the machan were recorded with a hand-held GPS at the base of each machan. The time of sighting of animals were recorded every 5 minutes interval with the help of a stop watch. Subsequently, the time of disappearance of the animal were also recorded so that the animals sighted at the nearby machan (considering the time of reappearance to the next machan) were removed manually during analysis. Morphological traits were used for individual identifications of different sex and age groups of sangai *viz.* adult male were identified with multiple spiked antlers, sub adult male with single spiked antlers (Plate 5), whereas adult female (90 cm), Juveniles (60 cm) and fawns (45 cm) were identified with the help of their body size. Subsequently, same methods were used for individual identifications of different sex and age groups of hog deer.

To determine the accuracy of the point count, another method, the drive count, was used. The drive count was carried out after the completion of the point count, at 9:00 hours, when the animals were hidden in the tall grass and thick bushes. During the drive counts, local people and forest department personnel were used to flush and count the animals at each point. The numbers obtained using the two methods were compared manually (Table 4.1).

(a) Bamboo machan

(b) Permanent structure

Plate 4. Temporary machans and observation points used for population estimation.



(a)

(b)

Plate 5. Different size classes of (a) sangai and (b) hog deer

Table 4.1 Total number of sangai and hog deer seen at each point during drive count (2006-2008) in the Keibul Lamjao National Park.

Point	Location	Hog deer			Sangai		
		2006	2007	2008	2006	2007	2008
1	Keibulchingpangba	2	1	1	3	1	2
2	Ngakrakom	1	2	2	3	2	7
3	Pabot chinglukok	2	10	20	20	35	50
4	Pabot chingjao	5	2	32	30	12	48
5	Khodangkhong	2	5	7	12	20	5
6	Sagramkha Mayaidak Pangba	1	2	2	2	1	2
7	Rest house 2	2	10	2	2	3	6
8	Thanbirel Hiramphatpi	8	5	3	9	3	5
9	Sangomkherkhong	1	6	2	1	7	6
10	Haorak Phumlak	4	2	3	1	1	5
11	Phumbai asangbi	2	2	3	3	8	1
12	Kachin achouba	5	4	26	1	18	21
13	Thangbirel aarongpangbi	3	1	4	4	6	4
14	Toya	30	10	2	6	38	5
15	Hambruhoubi	3	1	3	6	4	12
16	Khordak Ichil	5	2	4	45	15	2
17	Yenkokchambi	25	3	2	9	10	4
18	Paunapan	8	11	2	4	11	18
19	Birbongom	4	2	0	19	2	2
20	Hambrukachin	0	3	3	14	11	4
21	Thamnahoubi Mapa	0	1	2	0	21	3
22	Chingjaokha	0	1	2	0	4	4

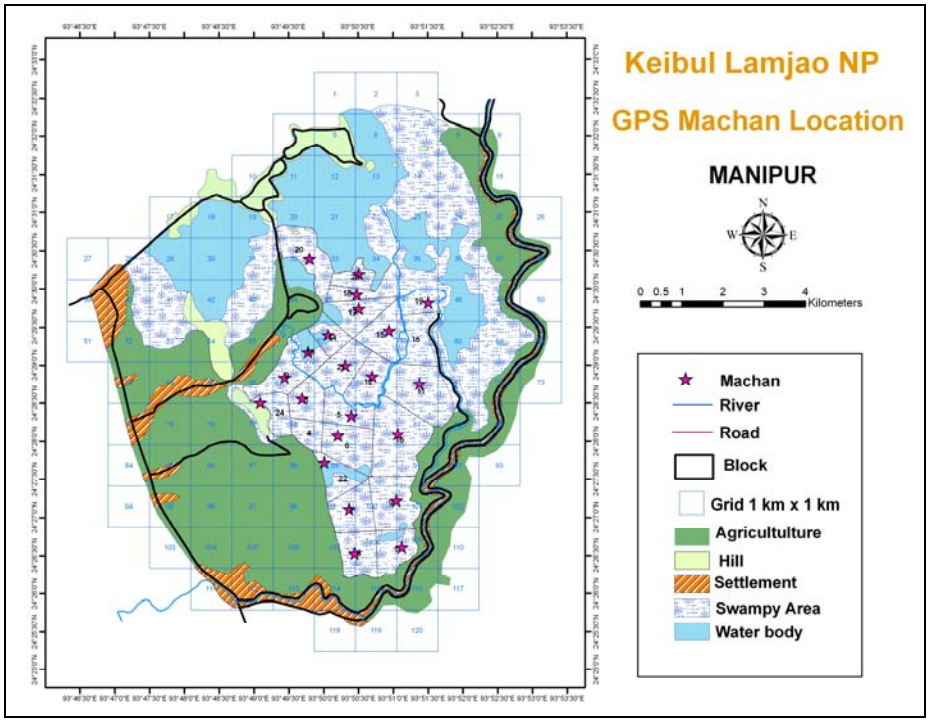


Figure 4.1 Machan Locations in Keibul Lamjao National Park.

4.3 DATA ANALYSIS

To derive the abundance, Distance 5.0 software was used (Buckland et al. 2000). Density of sangai and hog deer was calculated using maximum peak sightings for 30 minutes to avoid the duplicate counts. Model selections were made once the data set was prepared and proper truncation was performed. Several robust models were considered based on Akaike's Information Criterion (AIC) value. The criteria that models for the detection function should satisfy (Akaike 1973) conditions such as on robustness of the function (shape, estimator efficiency) (Galela & Roscom 2004). The probability of point detection was estimated using eight models *viz.* Uniform cosine, Uniform simple polynomial, Half normal cosine, Half normal simple polynomial, Half-normal Hermite polynomial, Hazard-rate cosine, Hazard rate

Hermite polynomial, Hazard rate simple polynomial. The lowest AIC is an estimate of the best approximating model. Since the AIC value of the Uniform cosine was the lowest, it was chosen as the best model for the detection function.

The population trend of sangai and hog deer was derived using regression analysis. The density of population for each year was transformed to logarithmic values before regression, thus the population growth rate and R^2 value was derived.

4.4 RESULTS

4.4.1 Demographic status of sangai in KLNP

In 2006 during the population estimation, 324 sightings of sangai and hog deer was made of which 61% sightings were of sangai, 37% sightings were of hog deer and rest 2% were unidentified, whereas during 2007, a total of 348 sightings were made, of which 71% sightings were of sangai, 26.4% sightings were of hog deer and rest 2.5% were unidentified. During 2008 a total of 389 sightings of animals were made, of which 59% sightings were of sangai, 39.3% sightings were of hog deer and rest 1.8% were unidentified. Percentage sightings of sangai and hog deer during the population estimation excercise in 2006, 2007 and 2008 have been summarized in Table 4.2.

Table 4.2 Percentage sightings of sangai and hog deer during the population estimation exercise in 2006-2008 in the Keibul Lamjao National Park.

Years	sangai	%	hog deer	%	unidentified	%
2006	196	61	121	37.0	7	2.0
2007	247	71	92	26.4	9	2.5
2008	229	59	153	39.3	7	1.8
Mean	224 ±14.9	64 ±3.7	122 ±17.6	34.2 ±4.0	7.6 ±0.67	2.1 ±0.21

4.4.2 Date and location wise sighting of sangai

For population estimation the observation was made for five consecutive days for three years (2006-2008). In 2006 a maximum of 64 sightings were made on 7th April while 43 sightings were made on 10th April. In 2007, the maximum sighting (60 individuals) recorded on 18th March and 40 sightings were recorded on 16th March. In 2008 maximum 59 sightings on 17th March and 32 sightings on 15th March were recorded (Table 4.3).

Point (machan) wise analysis revealed maximum sightings of sangai in 2006 were at Toya (point 16) with 45 sightings, followed Pabot Chingjao (point 4) and Pabot Chinglukok (Point 3) with 30 and 29 sightings. In 2007 the maximum sightings were recorded at Toya (point 14) and Pabot Chinglukok (point 3) with 38 sightings followed by Khodangkhong (point 5) with 29 sightings. In 2008, the maximum sightings were seen at Pabot Chinglukok (point 3) with 55 sightings and Pabot Chingjao (point 4) with 48 sightings (Table 4.4). The reason for the highest number of sightings at Toya, Pabot Chingjao and Pabot Chinglukok were because of less anthropogenic disturbance and proximity to the pristine hillock habitat.

Table 4.3 Date wise sighting of sangai during population estimation (2006-2008) in the Keibul Lamjao National Park.

Days	2006 (7^{th}- 10^{th} April)	2007 (14^{th}-18^{th} March)	2008 (15^{th}- 19^{th} March)
1	36	24	21
2	32	19	41
3	27	17	36
4	26	26	33
5	no data	15	29

4.4.3 Frequency of sighting of sangai

For the three consecutive years, the maximum number of sightings of sangai in 2006 was recorded between 0600-0630 hours with 47 sightings of animals whereas in 2007 the maximum number of sightings was recorded in the same time frame of 0600-0630 hours with 69 sightings. However, in 2008 the maximum number of sightings was recorded a little early, in the time frame of 0530-0600 hours with 57 sightings (Figure 4.2). Based on these observations the population estimation in the year 2006 and 2007 was done using the data for the time period of 0600-0630 hours, whereas in 2008 it was 0530-0600 hours. In 2006, the maximum peak sighting distance of sangai was recorded between 250 m to 300 m with a sighting frequency of 35 animals. However, in 2007 the maximum peak sighting distance was recorded between 150 m to 200 m and in 2008 the maximum peak sighting distance was recorded between 200 m to 250 m with a sighting frequency of 35 deers. The sighting frequency decreased with the increase in sighting distance (Figure 4.3).

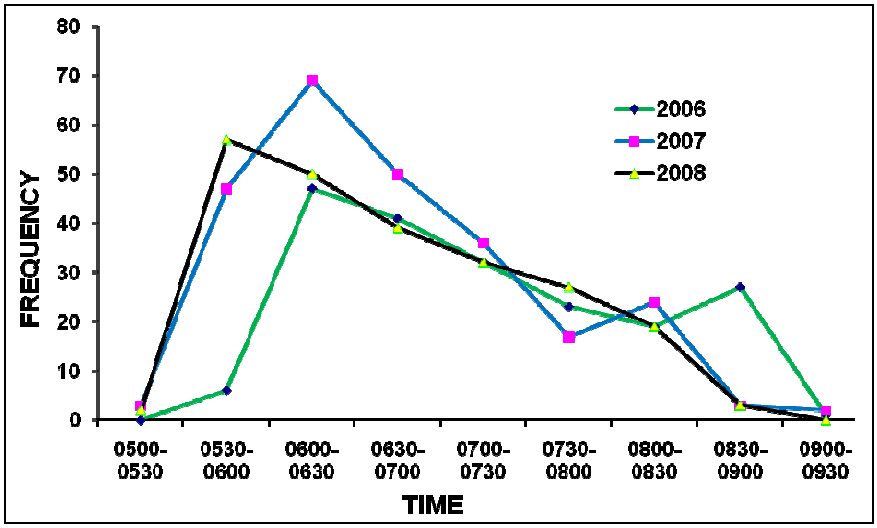


Figure 4.2 Frequency of sighting of sangai vs. time during population estimation (2006-2008) in the Keibul Lamjao National Park.

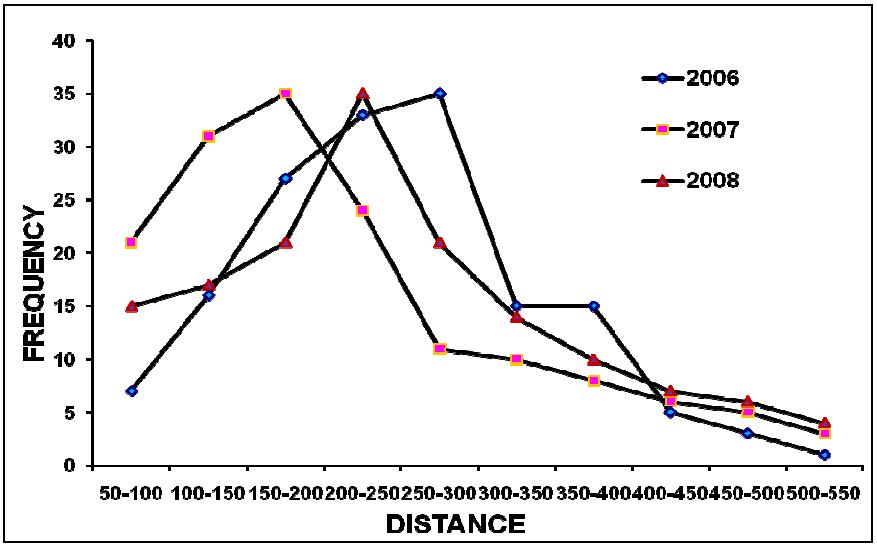


Figure 4.3 Frequency of sighting of sangai vs. distance during population estimation (2006-2008) in the Keibul Lamjao National Park.

Table 4.4 Total number of sangai seen at each machan/point during population estimation (2006-2008) in the Keibul Lamjao National Park.

Machans	Location	GPS Coordinates		Number		
		Latitude	Longitude	2006	2007	2008
1	Keibulchingpangba	24º 29.870'	93º 49.968'	3	3	4
2	Ngakrakom	24º 29.979'	93º 49.273'	3	2	6
3	Pabot chinglukok	24º 29.961'	93º 50.243'	29	38	55
4	Pabot chingjao	24º 29.739	93º 50.270'	30	12	48
5	Khodangkhong	24º 29.739'	93º 50.270'	12	29	5
6	Sagramkha Mayaidak Pangba	24º 29.841'	93º 49.407'	2	1	2
7	Rest house 2	24º 28.288'	93º 49.527'	2	3	6
8	Thanbirel Hiramphatpi	24º 28.796'	93º 49.861'	9	3	9
9	Sangomkherkhong	24º 28.451'	93º 51.694'	1	7	6
10	Haorak Phumlak	24º 28.818'	93º 48.975'	1	0	5
11	Phumbai asangbi	24º 28.237'	93º 49.639'	3	8	0
12	Kachin achouba	24º 28.237'	93º 49.639'	1	18	21
13	Thangbirel aarongpangbi	24º 28.499'	93º 50.187'	4	6	3
14	Toya	24º 28.031'	93º 50.117'	6	38	5
15	Hambruhoubi	24º 26.889'	93º 50.546'	6	5	12
16	Khordak Ichil	24º 27.358'	93º 50.596'	45	15	2
17	Yenkokchambi	24º 26.789'	93º 50.596'	9	10	5
18	Paunapan	24º 28.762'	93º 51.338'	6	11	18
19	Birbongom	24º 28.663'	93º 49.845'	19	2	2
20	Hambrukachin	24º 26.50.3'	93º 50.409'	12	11	4
21	Thamnahoubi Mapa	24º 28.582'	93º 50.955'	no data	21	3
22	Chingjaokha	24º 29.333'	93º 50.991'	no data	4	4

4.4.4 Model selection for estimating sangai density

Model selection was made once the data set was ready after truncation. Several robust models were considered based on AIC (Akaike's Information Criterion) value. The criteria for model selection for the detection function should satisfy (Akaike 1973) conditions (Galela & Roscom 2004) such as on robustness of the function (shape, estimator efficiency). The probability of detection was estimated using eight models *viz.* Uniform cosine, Uniform simple polynomial, Half normal cosine, Half normal simple polynomial, Half normal Hermite polynomial, Hazard rate cosine, Hazard rate Hermite polynomial, Hazard rate simple polynomial. The lowest AIC is an estimate of the best approximating model. Since the AIC value of the Uniform cosine was the lowest, it was chosen as the best model for the detection function to estimate population density and abundance of sangai population (Figure 4.4). The model with the lowest AIC is the uniform cosine (*). It yielded a density estimate (D) of 4.05 in 2006, 4.09 in 2007 and 4.06 in 2008 respectively. The abundance estimate (N) and chi square (p) value with the lowest AIC for three consecutive years yielded 90 (p = 0.96) in 2006, 88 (p = 0.99) in 2007 and 92 (p = 0.94) in 2008 respectively (Table 4.5).

4.4.5 Sangai density and the population size

The encounter rate of sangai was derived as 0.61 ±0.7 individuals/km^2 in 2006, 0.43 ±0.37 individuals/km^2 in 2007, and 0.39 ±0.3 individuals/km^2 in 2008 respectively. However, the estimated probabilities of detection under the curve were 0.29 ±0.20, 0.20 ±0.30 and 0.21 ±0.4 individuals/km^2 respectively.

Using Distance 5.0, an effective detection radius (EDR) was computed and the density of the animal was derived. Following the computation of the EDR the group density was estimated to be 2.90 ±0.4, 3.32 ±0.58 and 2.51 ±0.5 individuals/km^2 respectively during the three consecutive years from 2006 to 2008 respectively. The mean cluster size was during 2006, 2007 and 2008 as 1.4 ±0.75, 1.22 ±0.67 and 1.61 ±0.1 respectively (Table 4.6).

The density of sangai was generated using DISTANCE 5.0 software and was found to be 4.05 ±0.62 (CV 15.38%), 4.09 ±0.75 (CV 18.48%) and 4.06 ±0.8 (CV 19.9%) individuals/km^2 with a minimum of 2.97, 2.83, 2.73 and a maximum of 5.51, 5.88, 6.01 individuals/km^2 at 95% confidence level during 2006, 2007 and 2008 respectively. The estimated population size of sangai were 90, 88 and 92 individuals during 2006, 2007 and 2008 respectively, with a minimum of 66, 61 and 62 and maximum of 123, 127 and 137 sangai at 95% confidence level for the entire study period (Table 4.6).

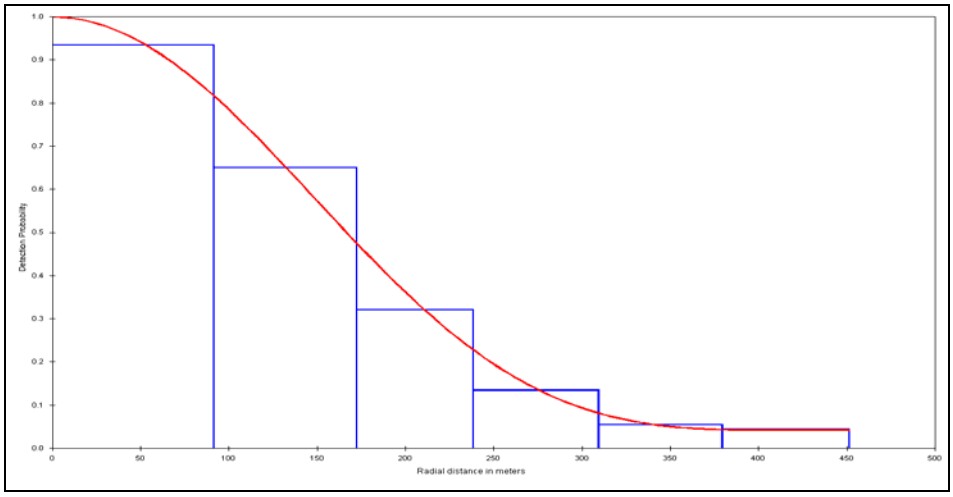


Figure 4.4 Detection probabilities of sangai vs. radial distance.

Table 4.5 Best model analysis of sangai during population estimation (2006-2008) in the Keibul Lamjao National Park.

Model	AIC			D			N			*p*		
	2006	**2007**	**2008**	**2006**	**2007**	**2008**	**2006**	**2007**	**2008**	**2006**	**2007**	**2008**
Uniform + simple polynomial	582	593	540	3.94	1.69	1.99	74	34	40	0.71	0.54	0.03
Uniform + cosine*	120	157	181	4.04	4.08	4.05	76	82	81	0.96	0.99	0.94
Half normal + cosine	583	576	521	4.73	3.96	4.06	89	80	81	0.43	0.57	0.86
Half normal + simple polynomial	583	577	522	4.73	3.24	3.49	89	65	70	0.23	0.66	0.43
Half- normal + Hermite polynomial	583	578	522	4.74	3.24	3.49	89	65	70	0.54	0.64	0.59
Hazard-rate + cosine	582	576	519	3.27	4.14	4.00	62	83	80	0.56	0.44	0.39
Hazard rate + Hermite polynomial	582	573	519	3.26	4.14	4.02	41	83	80	0.71	0.64	0.48
Hazard rate + simple polynomial	582	574	519	3.26	4.14	4.00	62	83	80	0.19	0.55	0.52

*** Uniform + cosine is the best fitted model based on lower Akaike's Information Criterion (AIC) value**

Table 4.6 Densities of sangai during population estimation (2006-2008) in the Keibul Lamjao National Park.

Parameter	Estimate/mean			% CV			95% CI					
							lower			upper		
	2006	2007	2008	2006	2007	2008	2006	2007	2008	2006	2007	2008
p	0.29 ±0.20	0.20 ±0.30	0.21 ±0.4	6.74	15.3	17.2	2.25	0.14	0.15	0.34	0.27	0.30
n/k	0.61 ±0.7	0.43 ±0.37	0.39 ±0.3	12.7	8.87	7.88	0.46	0.35	0.33	0.79	0.51	0.46
EDR	259 ±8.7	202 ±15.4	222 ±19.1	3.37	7.63	8.60	242	173	187.1	277	235	264
DS	2.90 ±0.4	3.32 ±0.58	2.51 ±0.5	14.4	17.6	18.9	2.16	2.34	1.72	3.89	4.72	3.65
ES	1.39 ±0.75	1.22 ±0.67	1.61 ±0.1	5.41	5.50	6.40	1.25	1.09	1.41	1.55	1.37	1.83
D	4.05 ±0.62	4.09 ±0.75	4.06 ±0.80	15.38	18.48	19.97	2.97	2.83	2.73	5.51	5.88	6.01
N	90 ±13.8	88 ±16.2	92 ±18.3	15.38	18.48	19.97	66	61	62	123	127	137

*** p- Probability of detection under the curve; n/k-Encounter rate; EDR- Effective detection radius; DS-Group density; ES Group size; D- Individual density; N- Population**

4.4.6 Population age structure of sangai

Following Christy et al. (2005) the population of sangai in different age class was derived based on the percentage sightings of different size classes of deer seen during the population estimation exercise in 2006, 2007 and 2008 and the estimated of total population size during the above mentioned years (Table 4.7). During the population estimation of sangai at KLNP, the age structure in 2006 was 25 % male, 52.6 % female, 12.8 % juvenile and 9.7 % fawn respectively where as in 2007 it was 25 % male, 52.6 % female, 12.8 % juvenile and 9.7 % fawn and in 2008 it was 25 % male, 52.6 % female, 12.8 % juvenile and 9.7 % fawn. The overall age structure of sangai during 2006-2008 in the Park was 29.1 ±2.1% male, 53.4 ±0.5% female, 8.9 ±2.0% juvenile and 8.7 ±0.9% fawn respectively (Figure 4.5). The population structure during 2006-2008 showed that in all the sampling years adult female population was highest followed by adult male (Figure 4.5). Overall population structure also indicates higher number of female as compared to male, juvenile and fawn. The overall juvenile age class during the three consecutive years showed a declining trend. The adult male and female showed a slight increase in number during 2006-2008 (Figure 4.6). The poplation of fawn showed a fluctuating number with a decreasing trend during 2006-2007 and marginal gain during 2007-2008.

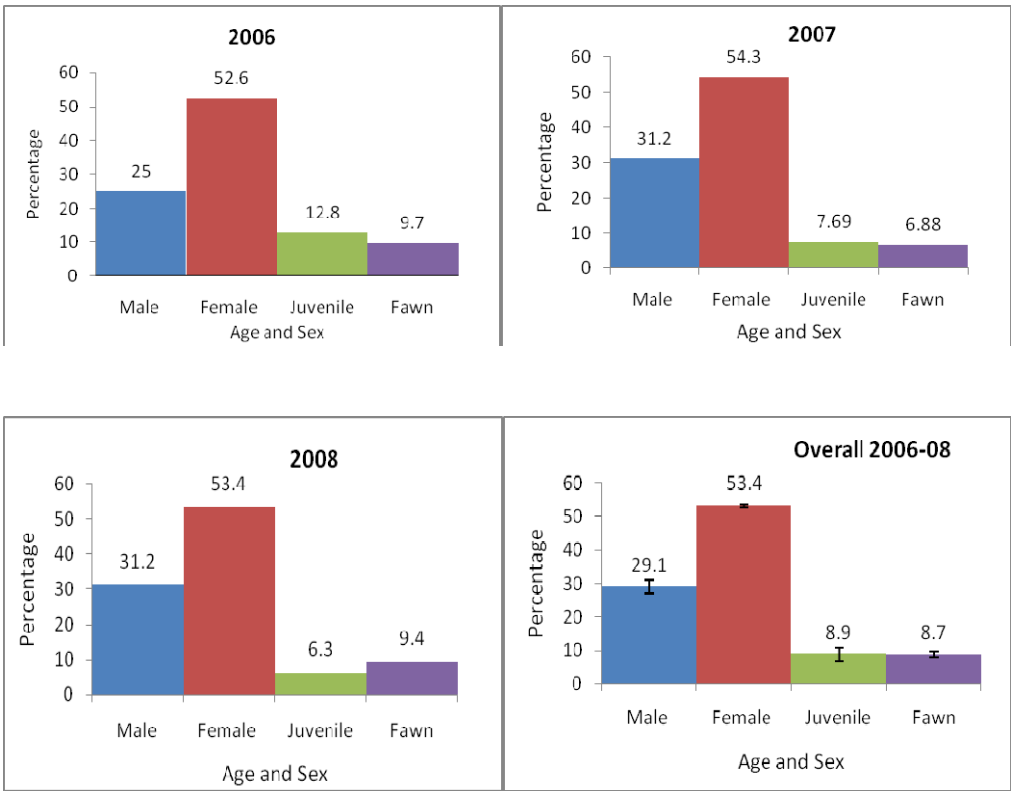


Figure 4.5 Age structure of sangai in the Keibul Lamjao National Park during 2006-2008.

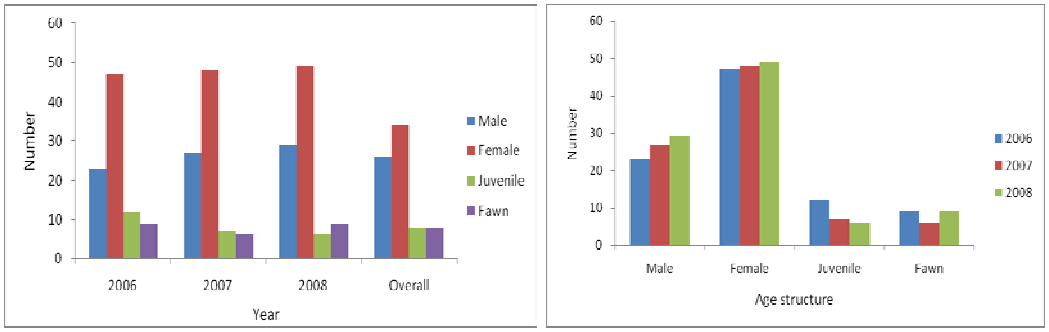


Figure 4.6 Trend in age structure of sangai during 2006-2008 in the Keibul Lamjao National Park.

Table 4.7 Estimated number of sangai in the various age and sex classes based on relative proportion seen during the population estimation exercise conducted in 2006 – 2008 in the Keibul Lamjao National Park and the estimated total population size.

YEAR	Adult male			Adult female			Juvenile			Fawn		
	Mean	95% lower CI	95% upper CI	Mean	95% lower CI	95% upper CI	Mean	95% lower CI	95% upper CI	Mean	95% lower CI	95% upper CI
2006	23	17	31	47	35	65	12	8	16	9	6	12
2007	27	19	40	48	33	69	7	5	10	6	4	9
2008	29	19	43	49	33	73	6	4	9	9	6	13
Mean	26	18	38	48	34	69	8	6	11	8	5	11
SEM	1.89	0.90	3.59	0.54	0.53	2.44	1.77	1.40	2.21	0.88	0.66	1.25

4.4.7 Sex ratio of sangai

The sex of sangai in the KLNP was determined by the presence or absence of antlers of male / female. The classification of age of fawn was done based on their body size. The adult sex ratio and doe to fawn ratio of sangai was estimated based on the sightings during population estimation in the Park during 2006-2008. The male to female ratio and doe to fawn ratio were calculated based on the point count estimate analysis that showed 47, 57, 58 males/100 females and 18, 13 and 18 fawns/100 females respectively (Table 4.8). However, the analysis of sex ratio for three consecutive years (2006-2008) was found that the observed male to female ratio was 54 ±3.57 males/100 females and fawn to female ratio was 16 ±1.7 fawns/100 females respectively (Figure 4.7).

Table 4.8 The sex ratio of male to female and doe to fawn ratio of sangai during population estimation (2006-2008) in the Park.

YEAR	Male/100 Female	Fawn/ 100 Female
2006	47.0	18.0
2007	57.0	12.7
2008	58.3	17.5
Mean	54.1 ±3.6	16.1 ±1.7

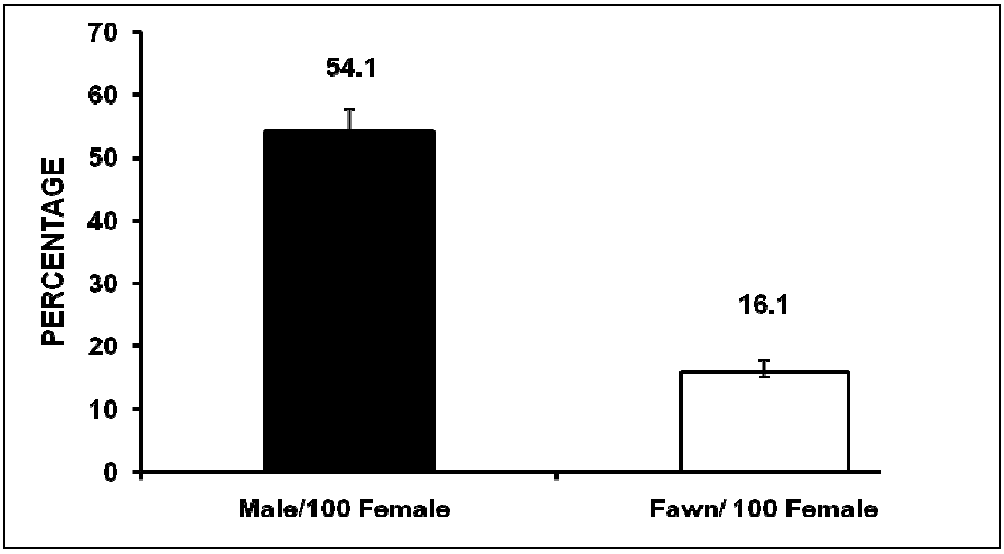


Figure 4.7 The male to female and doe to fawn ratio of sangai during population estimation (2006-2008) in the Keibul Lamjao National Park.

4.4.8 Population trend of sangai

The population assessment of sangai in the KLNP was conducted for the first time during 1975 in which presence of 14 sangai (6 stag, 5 hind, 3 fawn) was recorded. Subsequently, aerial census was conducted which revealed a steady increase in the number of sangai. In 1993; 98 sangai, 38 stag, 48 hind, 12 fawns was observed. Ground census in the Park is possible only during mid February to mid April when the vegetation is dried up and due to the reduction in the water level the *phumdi* settles in the Lake bed. The first ground census was conducted in 1984 and a total of 51 sangai (20 stag, 25 hind, and 6 fawns) in the Park was recorded. Since 1994 the ground census of sangai is being carried out regularly. The population of sangai has been consistently increasing over the years, the population in 2003 was estimated to be 180 animals (65 stags, 74 hinds and 41 fawns) (Singh 1992).

To determine the population trend of sangai in the Park, regression analysis was done using the previous population estimates done by the Forest Department during 1984-2003. The population number (N) was transformed to its logarithm value (Ln N) and then regressed against the years. The results showed that the change in population was significant ($p<0.05$, $R^2 = 0.898$) with 5% increase in population during 1984-2003 (Figure 4.8). The R^2 value indicates that 89% of the variation is explained by the equation: Log_{10} (Population) = 0.059 x Year + (- 114.1).

However, the population trend of sangai derived using above mentioned method for the three consecutive years (2006-2008) showed a more or less stable population trend in the Park. The results showed that the change in population was not significant ($p>0.05$, $R^2 = 0.244$) with 1% increase in population from 2006-2008 (Figure 4.9). The R^2 value indicates that only 24% of the variation is explained by the equation: Log_{10} (Population) = 0.011 x Year + (- 17.55).

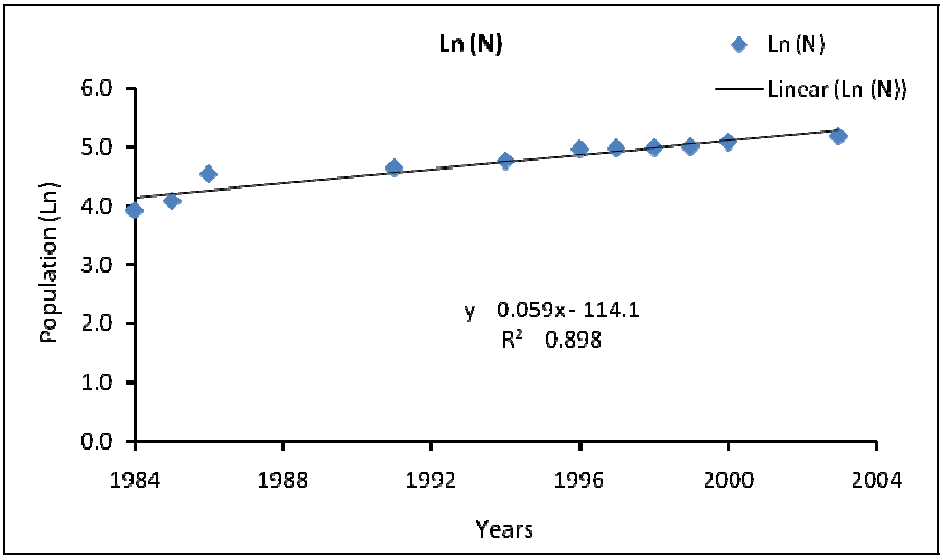


Figure 4.8 Population trend of sangai during 1984-2003 in the Park (Manipur Forest Department record).

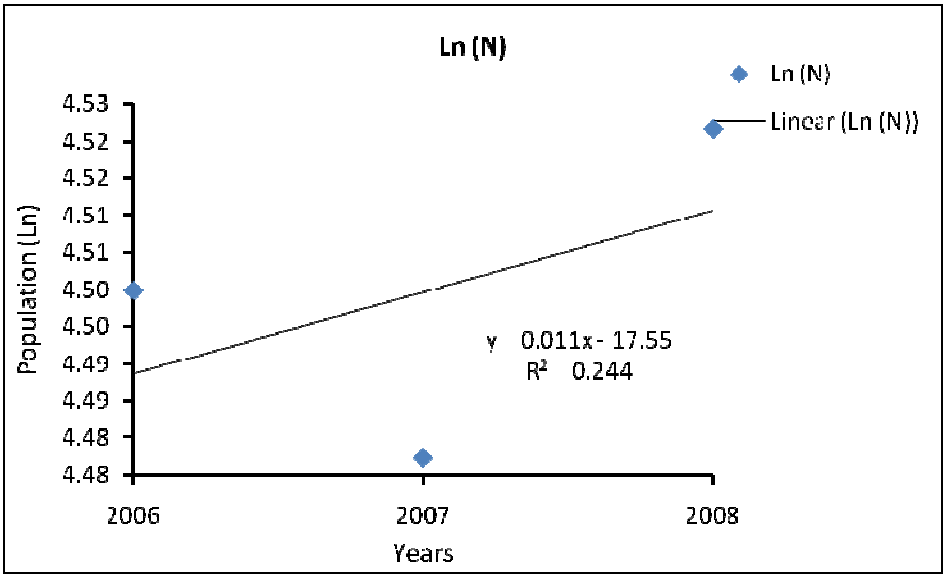


Figure 4.9 Population trend of sangai during population estimation (2006-2008) in the Park.

4.4.9 Demographic status of hog deer in KLNP

For hog deer, in 2006 a maximum of 36 and minimum of 26 sightings were made on 7th and 10th April, respectively. In 2007, the maximum number of sighting was on 17th March with 26 sightings and a minimum of 15 sightings were made on 18th March, whereas in 2008 a maximum of 41 sightings were made on 16th March and minimum count of 21 sightings on 15th March (Table 4.10). Maximum sightings in 2006 were made at point number 14 (Kachin Achouba) with 38 sightings followed by point number 17 (Hambruhoubi) with 21 sightings. In 2007 the maximum sightings were made at point number 14 (Toya) and point number 18 (Paunapan) with 15 sightings. However, in 2008, the maximum sightings were recorded at point number 4 (Pabot Chingjao) with 54 sightings followed by point number 12 (Thangbirel Aarongpangbi) and point number 3 (Pabot Chinglukok) with 31 and 22 sightings (Table 4.9). The reason for the large number of sightings of hog deer at Kachin Achouba, Hambruhoubi, Toya, Paunapan, Thangbirel Aarongpangbi, Pabot Chingjao and Pabot Chinglukok were because of the presence of thick *phumdi* (>3 m), hard ground, less anthropogenic disturbance and the proximity to the pristine hillock habitat.

Table 4.9 Total number of hog deer seen at each machan during population estimation (2006-2008) in the Keibul Lamjao National Park.

Machans	Location	GPS coordinates		Number		
		Latitude	Longitude	2006	2007	2008
1	Keibulchingpangba	24º 29.870'	93º 49.968'	1	2	3
2	Ngakrakom	24º 29.979'	93º 49.273'	1	2	2
3	Pabot chinglukok	24º 29.961'	93º 50.243'	2	9	22
4	Pabot chingjao	24º 29.739	93º 50.270'	7	2	51
5	Khodangkhong	24º 29.739'	93º 50.270'	2	4	7
6	Sagramkha Mayaidak Pangba	24º 29.841'	93º 49.407'	1	2	2
7	Rest house 2	24º 28.288'	93º 49.527'	1	8	3
8	Thanbirel Hiramphatpi	24º 28.796'	93º 49.861'	7	5	3
9	Sangomkherkhong	24º 28.451'	93º 51.694'	1	9	2
10	Haorak Phumlak	24º 28.818'	93º 48.975'	4	2	3
11	Phumbai asangbi	24º 28.237'	93º 49.639'	2	2	3
12	Kachin achouba	24º 28.237'	93º 49.639'	5	6	31
13	Thangbirel aarongpangbi	24º 28.499'	93º 50.187'	3	1	4
14	Toya	24º 28.031'	93º 50.117'	38	15	2
15	Hambruhoubi	24º 26.889'	93º 50.546'	3	1	3
16	Khordak Ichil	24º 27.358'	93º 50.596'	7	2	4
17	Yenkokchambi	24º 26.789'	93º 50.596'	21	3	2
18	Paunapan	24º 28.762'	93º 51.338'	11	15	2
19	Birbongom	24º 28.663'	93º 49.845'	4	2	0
20	Hambrukachin	24º 26.50.3'	93º 50.409'	0	6	3
21	Thamnahoubi M	24º 28.582'	93º 50.955'	no data	1	4
22	Chingjaokha	24º 29.333'	93º 50.991'	no data	2	4

Table 4.10 Date wise sighting of hog deer during population estimation (2006-2008) in the Park.

Days	2006 (7^{th}- 10^{th} April)	2007 (14^{th}-18^{th} March)	2008 (15^{th}- 19^{th} March)
1	36	24	21
2	32	19	41
3	27	17	36
4	26	26	33
5	no data	15	29

4.4.10 Frequency of sighting of hog deer

During the population estimation of hog deer the maximum number of sightings in 2006 was recorded between 0600-0630 hours with 31 sightings whereas, in 2007 the maximum number of sightings was recorded between 0700-0730 hours with 41 sightings. However, in 2008 the maximum number of sightings was recorded a little early during 0530-0600 hours with 49 sightings (Figure 4.9). The frequency of sighting vs the maximum peak sighting distance in 2006, 2007 and 2008 was recorded within the same distance frame of 150 m to 200 m with a sighting frequency of 23, 17 and 27 animals simultaneously (Figure 4.11).

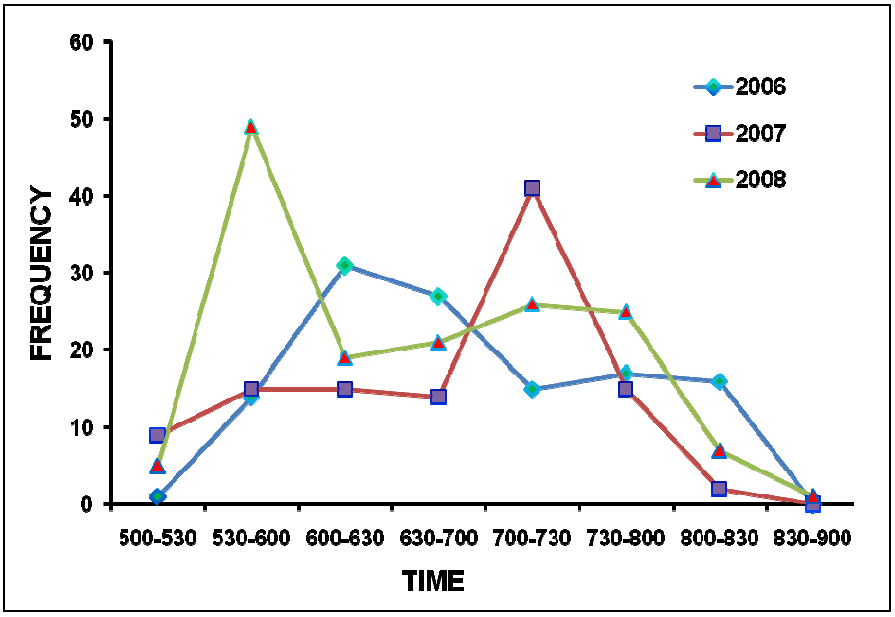


Figure 4.10 Frequency of sighting of hog deer vs. time during population estimation (2006-2008) in the Keibul Lamjao National Park.

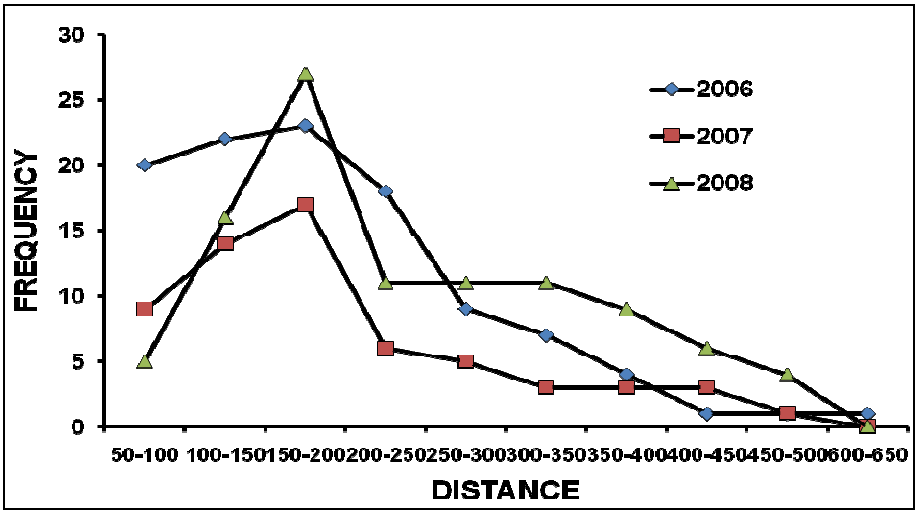


Figure 4.11 Frequency of sighting of hog deer vs. distance during population estimation (2006-2008) in the Keibul Lamjao National Park.

4.4.11 Model selection for estimating hog deer density

Best fitted Model were selected for hog deer based on AIC (Akaike's Information Criterion) value (Akaike 1973). The detection probability of each point was estimated using eight models *viz.* Uniform cosine, Uniform simple polynomial, Half normal cosine, Half normal simple polynomial, Half normal Hermite polynomial, Hazard rate cosine, Hazard rate Hermite polynomial, Hazard rate simple polynomial. The lowest AIC is an estimate of the best approximating model. Since the AIC value of the Uniform cosine is the lowest it was chosen as the best model for the detection function to estimate population density and abundance of hog deer population. The model with the lowest AIC is the uniform cosine (*) (Figure 4.12). It yielded a density estimate (D) of 2.94 in 2006, 2.75 in 2007 and 2.51 in 2008 respectively. The abundance estimate (N) and chi square (*p*) value with the lowest AIC for three consecutive years yielded 65 (*p* = 0.91) in 2006, 61 (*p* = 0.97) in 2007 and 57 (*p* = 0.96) in 2008 respectively (Table 4.11).

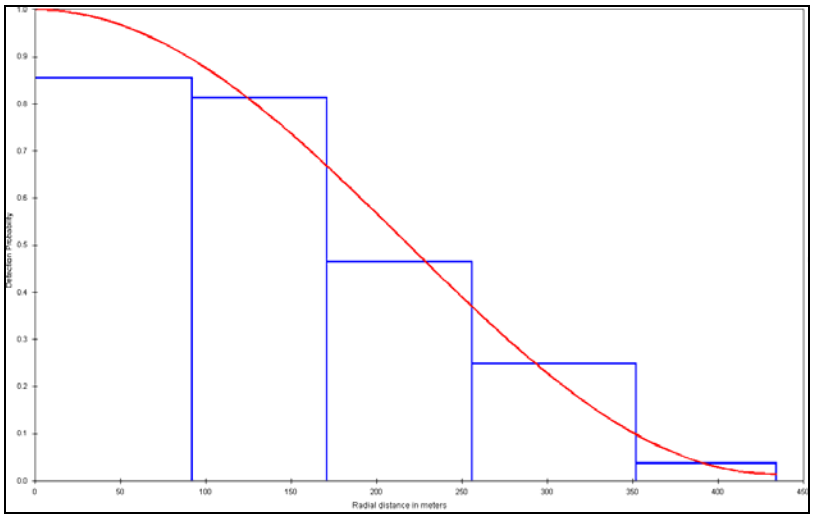


Figure 4.12 Detection probabilities of hog deer vs. radial distance.

Table 4.11 Best model analysis of hog deer during population estimation (2006-2008) in the Keibul Lamjao National Park.

Model	AIC			D			N			*P*		
	2006	2007	2008	2006	2007	2008	2006	2007	2008	2006	2007	2008
Uniform + simple polynomial	344	305	319	3.01	2.33	2.17	57	47	43	0.54	0.72	0.69
Uniform + cosine*	94	72	75	2.94	2.75	2.51	65	61	57	0.91	0.97	0.95
Half normal + cosine	345	301	313	2.87	2.41	2.53	54	49	51	0.34	0.44	0.21
Half normal + simple polynomial	346	301	313	2.88	2.42	2.54	54	49	51	0.53	0.38	0.66
Half- normal + Hermite polynomial	345	301	313	2.87	2.41	2.53	54	49	51	0.57	0.70	0.48
Hazard-rate + cosine	348	302	313	2.50	2.83	2.65	47	57	53	0.49	0.55	0.58
Hazard rate + Hermite polynomial	346	302	313	2.50	2.83	2.65	47	57	53	0.61	0.58	0.38
Hazard rate + simple polynomial	346	302	313	2.50	2.94	2.65	47	56	53	0.68	0.33	0.43

*** Uniform + cosine is the best fitted model based on lower Akaike's Information Criterion (AIC) value**

4.4.12 Hog deer density and the population size

Population estimation of hog deer was done using the data for the period of 0600-0630 hours in 2006, 0700-0730 hours in 2007 whereas 0530-0600 hours in 2008. The encounter rate of hog deer was estimated as 0.36 ±0.33 in 2006, 0.22 ±0.20 in 2007, and 0.24 ±0.20 in 2008 individuals/km^2 respectively. However, the estimated probabilities of detection under the curve were 0.38 ±0.58, 0.31 ±0.29 and 0.30 ±0.25 respectively. Using Distance 5.0, the effective detection radius (EDR) was computed and the density of the animal was derived. Following the computation of the EDR the group density was estimated to be 1.92 ±0.35, 1.19 ±0.17 and 1.25 ±0.17 respectively during the three consecutive years from 2006 to 2008. The mean cluster size was also calculated during 2006, 2007 and 2008 as 1.53 ±0.10, 2.29 ±0.18 and 2.02 ±0.18 respectively (Table 4.12).

The density of hog deer was found to be 2.94 ±0.57 (CV 19.5%), 2.75 ±0.44 (CV 16.3%) and 2.51 ±0.40 (CV 16.2%) individuals/km^2 during 2006, 2007 and 2008 respectively with a minimum of 1.82 individuals/km^2 and maximum of 4.32 individuals/km^2 at 95% confidence level. The population size of hog deer were 65 ±12.6, 61 ±9.9 and 57 ±9.2 individuals with a minimum of 44, 44, 41 and maximum of 96, 84 and 79 hog deer at 95% confidence level during 2006, 2007 and 2008 respectively.

4.4.13 Population age structure of hog deer

Using the similar methods as used for sangai (Section 4.4.6) the population of hog deer in different age class was derived based on the

percentage sightings of different size classes of hog deer seen during the population estimation exercise in 2006, 2007 and 2008 (Section 4.2) and the estimation of total population size for these years (Table 4.13). The population structure of hog deer during 2006-2008 showed that in all the sampling years adult female population was highest followed by adult male (Figure 4.13). Overall population structure also indicates a higher number of female as compared to male, juvenile and fawn. The overall adult male, adult female and juvenile showed flunctuating figures (Figure 4.13) where as the number of fawn during 2006-2008 showed a steady decline in number (Figure 4.14).

4.4.14 Sex ratio of hog deer

The adult sex ratio and doe to fawn ratio in hog deer were calculated based on the sightings during population estimation in the Park for the three consecutive years of 2006 to 2008. The male to female ratio and doe to fawn ratio of hog deer in 2006-2008 was 34.2, 34.5, 39.9 males/100 females and 16.4, 17.2 and 15.8 fawns/100 females respectively (Table 4.14). However the mean analysis of sex ratio for three consecutive years (2006-2008) was found that the observed male to female ratio was 36.2 ±1.9 males/100 females and doe to fawn ratio were 16.5 ±0.4 fawns/100 females respectively (Figure 4.15).

Table 4.12 Densities of hog deer during population estimation (2006-2008) in the Keibul Lamjao National Park.

*parameter	Estimate			% CV			95% CI					
							Minimum			Maximum		
	2006	2007	2008	2006	2007	2008	2006	2007	2008	2006	2007	2008
p	0.38 ±0.58	0.31 ±0.29	0.30 ±0.25	15.6	9.59	8.48	0.27	0.25	0.25	0.52	0.37	0.38
n/k	0.36 ±0.33	0.22 ±0.20	0.24 ±0.20	9.33	10.3	10.5	0.29	0.18	0.18	0.44	0.27	0.28
EDR	245 ±19.2	240 ±11.5	241 ±10.2	7.83	4.8	4.24	208.9	217.8	220.8	287.8	265.6	263
DS	1.92 ±0.35	1.19 ±0.17	1.25 ±0.17	18.2	14.1	13.5	1.33	0.90	0.95	2.76	1.59	1.63
ES	1.53 ±0.10	2.29 ±0.18	2.02 ±0.18	6.74	8.2	8.87	1.33	1.93	1.68	1.75	2.72	2.42
D	2.94 ±0.57	2.75 ±0.44	2.51 ±0.40	19.46	16.3	16.2	1.99	1.99	1.82	4.32	3.80	3.46
N	65 ±12.6	61 ±9.9	57 ±9.2	19.46	16.3	16.2	44	44	41	96	84	79

*** p- Probability of detection under the curve; n/k-Encounter rate; EDR- Effective detection radius; DS-Group density; ES Group size; D- Individual density; N- Population.**

Table 4.13 Estimated numbers of hog deer in the various age and sex classes based on relative proportion seen during the population estimation exercise conducted in 2006 – 2008 in the Keibul Lamjao National Park and the estimated total population size.

	Adult male			Adult female			Juvenile			Fawn		
YEAR	Mean	95% lower CI	95% upper CI	Mean	95% lower CI	95% upper CI	Mean	95% lower CI	95% upper CI	Mean	95% lower CI	95% upper CI
2006	13	9	20	39	27	58	6	4	9	11	7	16
2007	12	9	17	35	26	49	2	2	3	6	4	8
2008	13	9	18	40	28	55	5	3	6	5	4	7
MEAN	13	9	18	38	27	54	4	3	6	7	5	11
SEM	0.37	0.19	0.88	1.34	0.86	2.69	1.01	0.66	1.55	1.67	1.05	2.64

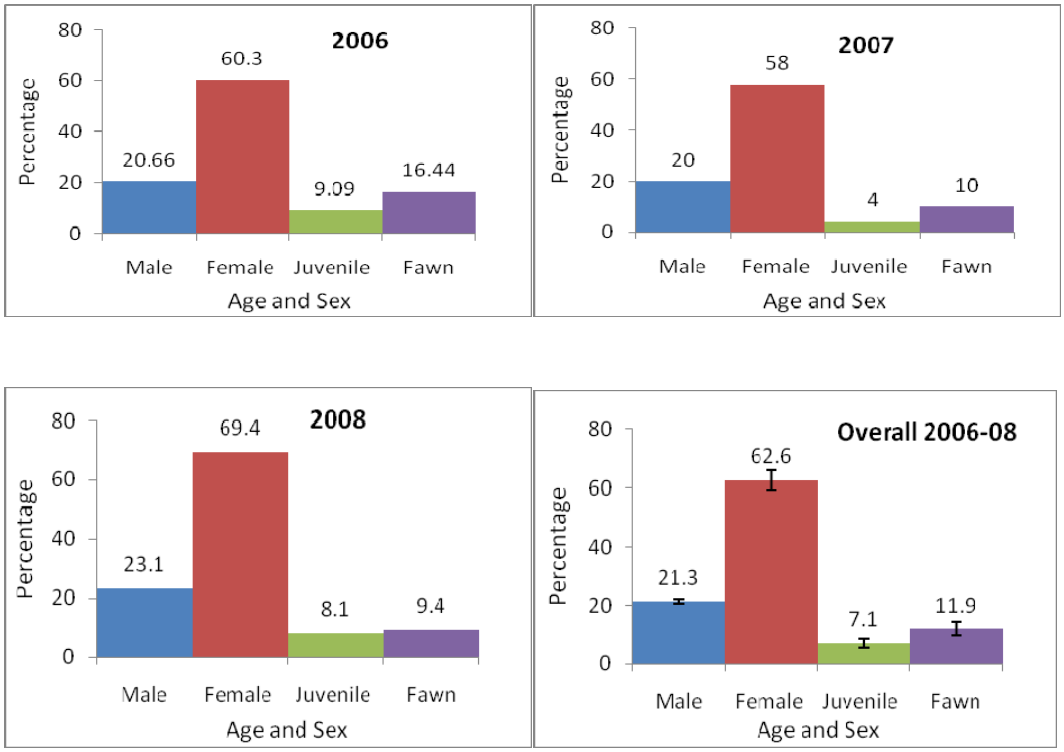


Figure 4.13 Age structure of hog deer during population estimation (2006-2008) in the Keibul Lamjao National Park.

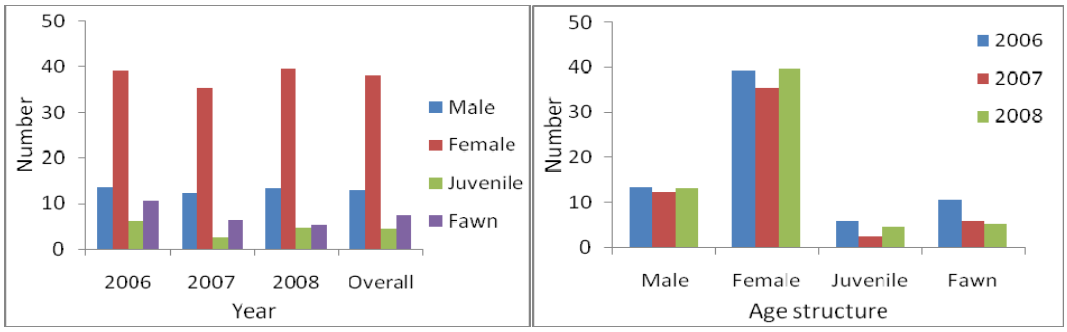


Figure 4.14 Trend in age structure of hog deer during population estimation 2006-2008 in the Keibul Lamjao National Park.

Table 4.14 Adult sex ratio and doe to fawn ratio of hog deer during population estimation 2006-08 in the Keibul Lamjao National Park.

YEAR	Male/100 Female	Fawn/ 100 Female
2006	34.24	16.43
2007	34.5	17.24
2008	39.9	15.8
Mean	36.2 ±1.9	16.5 ±0.4

4.4.15 Population trend of hog deer

The population trend of hog deer derived using similar method as used for sangai showed a declining trend in the Park. The results indicates that the change in population is significant ($p < 0.05$, $R^2 = 0.916$). The R^2 value indicates 91% variation in the observed trend is explained by the equation: Log_{10} (Population) = -0.250 x Year + 507.6. This equation indicates that the population of hog deer decreased -0.250 times with every t+1 year. The population estimation carried out during 2006 to 2008 in the Park showed a declining trend (Figure 4.16). The results showed that the change in population is not significant ($p > 0.05$, $R^2 = 0.992$) with *c* 8% decrease in population during 2006-2008 (Figure 4.17). The R^2 value indicates that 99% of the variation is explained by the equation: Log_{10} (Population) = -0.079 x Year + 159.6.

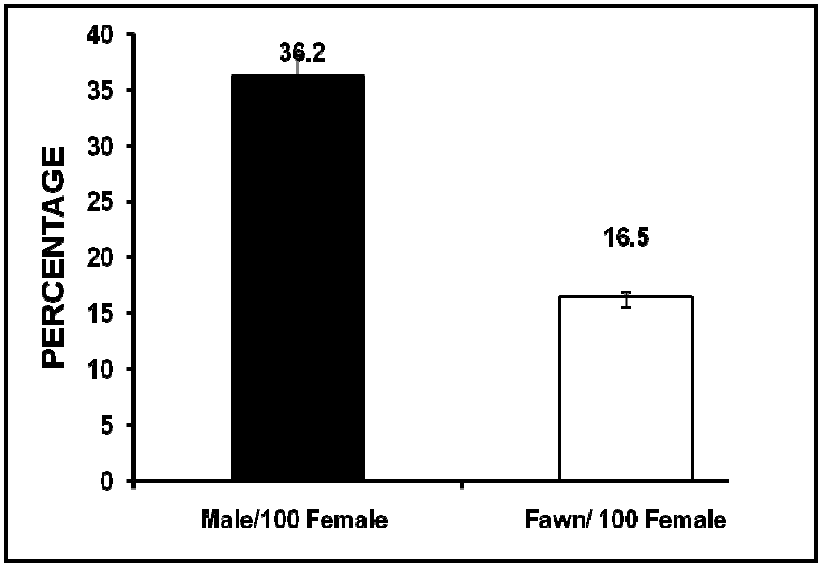


Figure 4.15 The male to female and doe to fawn ratio of hog deer during population estimation (2006-2008) in the Keibul Lamjao National Park.

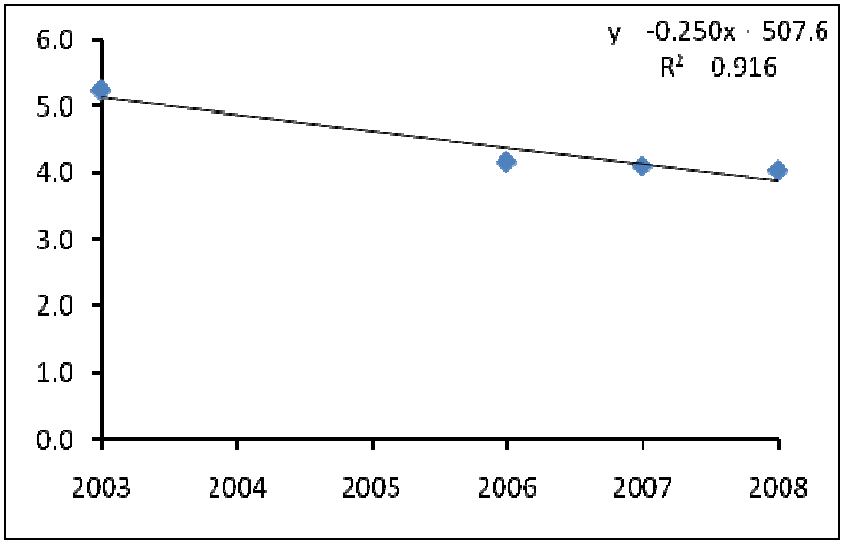


Figure 4.16 Population trend of hog deer during 2003-2008 in the Keibul Lamjao National Park (Data source: Manipur Forest Department and this study).

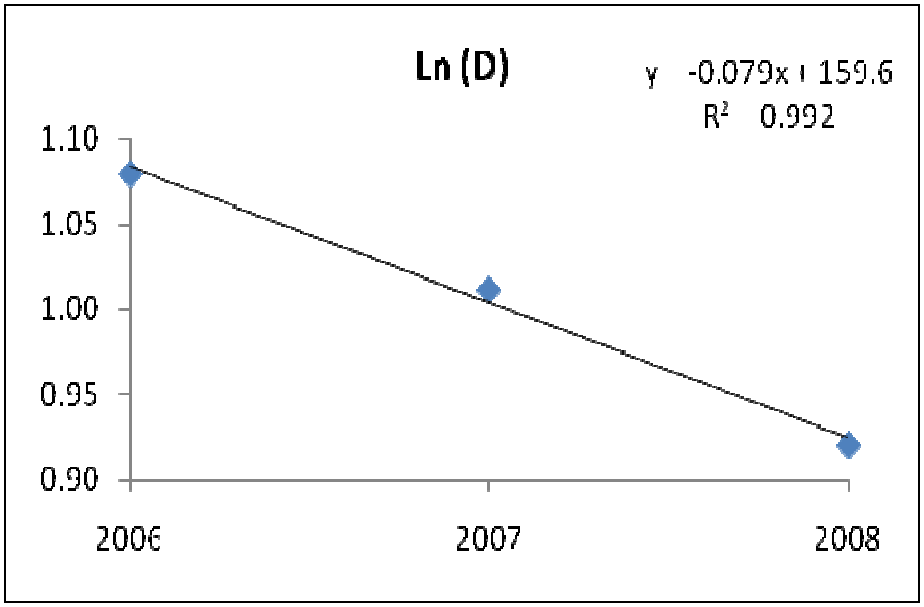


Figure 4.17 Population trend of hog deer during population estimation (2006-2008) in the Keibul Lamjao National Park (This study)

4.5 DISCUSSION

The sangai was once widely distributed in Manipur in Northeast India and is currently endemic to KLNP, Manipur (Hussain et al. 2006). It inhabits low-lying swamps (Lekagul & McNeely 1977) and is especially adapted to the floating mats of dense vegetation of *phumdi* habitat within a small region area in Manipur, India. The potential habitat of sangai has already been lost due to rapid rate of excessive *phumdi* deterioration, continuous thinning and fragmentation. Historical records shows that it was found in all parts of the state but due to rampant hunting and reduction in population size, due to rapid increase of anthropogenic pressure and habitat degradation due to construction of Ithai Barrage in 1983, it was declared extinct by the Government of Manipur

in 1951 (Singh 1992, Trisal & Manihar 2004). The two other cervids, the hangul *Cervus elaphus and R. duvaucelli* have also been seriously affected by hunting (Kurt 1978, Ranjithsinh 1978). Singh (1984) postulated that one reason for decline of swamp deer population was a high fawn mortality. Holloway (1973) and Singh (1984) recognized that poaching was the main cause for decline of swamp deer population at Sathiana. The major cause for decline in deer populations all over the world has been over exploitation by hunting (Holloway 1973).

Many studies of ungulates have been conducted using area sampling (Storm et al. 1992). Storm et al. (1992) used area sampling to estimate deer abundance using compartments bounded by roads, fences, or other obvious physical features. Similarly, Floyd et al. (1979) used area sampling to estimate deer abundance by searching plots bound by ridges or streams using overlapping circles. Elk population size was estimated and demographic statistics were determined using area sampling by dividing a survey area into land units in a study by Samuel et al. (1992).

Estimating the population size or density of an animal species in an area is fundamental to understanding its status and demography, and to plan for its management and conservation (Varman & Sukumar 1995). In spite of the development of sophisticated statistical methods of sampling animal populations (Burnham et al. 1980), their application to estimating densities in tropical forests is difficult mainly because of poor visibility and relatively low density of these populations resulting in inadequate sample sizes for statistically precise results. The methods of estimating size of ungulate

population fall into two categories: direct and indirect method. Population estimation using direct method such as line transects or more appropriately distance sampling (Burnham et al. 1980) have utilized to produce densities for different taxa in a variety of habitats. The line transect theory has been extended with appropriate modifications for differences in geometry, to “point transects” which are sets of variables circular plot surveys (Reynolds et al. 1980) with updated estimation methods (Buckland et al. 2001).

During the present study the line transects was not possible for population estimation because of the predominantly aquatic floating habitat conditions and elusive behavior of the species as they moved away from the observers and quickly hide amidst the tall grasses. In order to overcome these problems we replaced all the above mentioned methods with point count method. The technique of point count has potential for monitoring relative abundance which typically occupies habitats with dense ground cover making other methods *viz.* Track/trail transect counts, boat transects, spotlight counts difficult. However mark-recapture methods proved impractical because animals were difficult to detect as they moved away from the observer they quickly learned to avoid recapture. Studies of radio-collared animals are time-consuming and expensive and results are not necessarily applicable over small areas. To determine the accuracy of point count techniques another method *viz.* drive count was conducted after the completion of point count at 9:00 hrs when animals were hiding amongst the tall grasses and thick bushes. During the drive counts, local peoples and forest personal were used to flushed and count the animals in each point and match the counting number of this two methods.

No efforts were made to estimate the sangai population before the 1950's; the estimate of six individuals of sangai by noted naturalist E. P Gee in the early 1959, lead to the beginning of intensive conservation action that declared sangai as protected animal and its habitat, Keibul Lamjao was declared as a protected Sanctuary in 1954, covering an area of about 52 km^2. The first ground census conducted in 1984 was a simple ground survey analysis based on simultaneous observations and count from 8 check posts namely Toya hill, Pabot hills, Chingmai, Sagram, Keibul etc. estimated total population of 51 sangai (20 stags, 25 hinds, and 6 fawns) in the Park (Singh 1992, Hussain et al. 2006).

Point counts were carried out in the 22 km^2 area of the Park in the early hours between 0530 and 0900 hours in the morning and between 15:00 and 17:30 hours in the evening while 2-3 hours of evening. Counting of the species were carried out half an hour after arrival at the machan to eliminate the disturbance period and continue the estimate through a complete 3 hours during the process. The density estimates based on total number of sangai seen on each four to five consecutive dates and total number of sangai seen at each machan/point were compared between early and late hours, and the highest figure was selected for subsequent analysis. Later the evening data were discarded due to low number of sightings. It is reasoned that highest counts will result when feeding activity in the species is greatest, i.e. when most of the animal were feeding. Subsequently, comparison of drive count and point count techniques at KLNP to determine the accuracy of later, the population numbers obtained in the two methods were more or less same. Previous studies showed that Eld's deer form social groups only during their breeding

season during February to May (Aung et al. 2001). However, hog deer (Odden & Wegge 2007), can be found in social groups throughout the year. Singh (1992) reported that the census of the sangai in the KLNP could be possible only during a certain period of the year (from mid–February to mid-April). When the Park remained covered up with a luxuriant growth of vegetation and the deer are not visible at all from a distance rendering it impossible for counting. In other times of the year the vegetation of the Park dries up and the sangai are visible from a distance. Therefore, the population estimates were carried out during February– April.

The largest populations of Eld's deer still reside in Myanmar, but there has been a steady decline since the 1940s (Salter & Sayer 1986, McShea et al. 1999). The largest population remains in Chatthin Wildlife Sanctuary, but this population declined 40% between 1983 and 1995, and continued to decline through the most recent survey (Aung et al. 2004). McShea et al. (2005), identified 44,530 km^2 of potential habitat across Southeast Asia for Eld's deer (the Indian subcontinent and China were not included in the survey). The proportion of suitable forest was ≤1% of the remaining forest within each country, with the exception of Cambodia (13%). The suitable forest was not in large patches, with the mean patch size for each country at < 100 km^2 (a minimum sustainable size), with the exception of Cambodia (263 km^2) and Myanmar (147 km^2). The protected status of this potentially suitable forest was also low, at < 1%, with the exception of Cambodia (11.2%).

The study conducted by Zeng et al. (2005) in Hainan Eld's deer showed that with the rapid increase of human population and the fast expansion of

agriculture in the recent 350 years of Chinese history, the habitat of the deer has been greatly reduced. Habitat loss and hunting have driven the deer to the verge of extinction. The last group of 26 deer was protected at Datian Nature Reserve (DNR) which was founded in 1976. The habitat at present remains only 13.14 km^2 at DNR, just 4.38–6.57% of that of the 1950's. The deer population increased to over 1,000 individuals in 2003 under the protection of enclosure building along the boundary of DNR. Moreover, an off-site conservation approach for the deer has been launched since 1990. A total of 83 deer from DNR had been accumulatively introduced to establish off-site populations by the end of 2000. The off-site conserved populations had reached 263 by the end of 2002, of which 246 animals were born in captivity or semi-captivity. A new off-site population in the natural environment was founded by introducing 65 animals from DNR during July to December 2003. The deer population still however faces challenges of habitat limitation, decrease of genetic diversity, inbreeding and poaching. However, the current status of *R. e. siamensis* in Indo-China is largely unknown, although it is thought that small scattered herds may still remain there (McShea et al. 1999).

The hog deer population has undergone a severe decline in recent decades, and is now considered one of the most threatened large mammals in parts of its range (Meijaard & Groves 2004). This deer once occurred across a large part of Southeast Asia, from Pakistan to southern China, but now has a much more restricted distribution, existing only in highly fragmented and relict populations (Biswas 2004). The population declines in India and Nepal are likely to have been 30-40% in the last two decades whereas no recent information traced from Pakistan and Bhutan (Biswas & Mathur 2000, Biswas

2004). It has been reported as extinct from Sri Lanka, Bangladesh, China and Thailand (Biswas & Singh 2002). Schaller (1967) reported that the adult sex ratio of hog deer to be equal. However, subsequent studies showed a ratio ranging from 52 adult male: 100 adult female (Dhungel & Gara 1991) and 56 adult male: 100 adult females indicating a bias towards hinds (Biswas & Mathur 2000). The age structure of hog deer population at Jaldapara was observed to be 56 adult males: 100 adult females: 21 fawn: 17 sub-adult females: 8 sub-adult males. The adult: fawn ratio at Jaldapara was 100: 13 which is similar to that at Chitawan (Dhungel & Gara 1991). Although there has been no recent re-evaluation, and the relative historical distributions of these subspecies and nature of contact between them, if any, remain unclear (Maxwell et al. 2007).

The hog deer *A. porcinus* is listed as 'Endangered' and legally protected throughout its range, and receives protection from international trade under its listing on CITES, of which the past reduction of 50% or greater in three generations, through a combination of population trends across its range (Timmins & Duckworth 2008). The main threats identified were hunting, habitat loss and habitat degradation, with the species reported to be easier to hunt than other deer in the region, and its habitats having been largely lost to agriculture and urban development. The present distribution range of Indian subspecies inhabits low alluvial grasslands of the Indo-Gangetic plains of Pakistan, Nepal, Bangladesh, northern India where it ranged from Sind in the west through Punjab, UP, Uttarakhand, Bengal and Assam i.e. flood plains of Brahmaputra (Spillett 1966) to Manipur extending up to the flood plains of Irrawaddy River.

The hog deer *A. porcinus* population found in floating vegetations of Keibul Lamjao National Park, detailed information is lacking. The populations in KLNP, Manipur were estimated at 186 individuals (Singsit 2003). In the present study, the hog deer population in KLNP showed a declining trend since 2006 to 2008. Across the 3 consecutive years during 2006 to 2008, the hog deer densities were estimated at 2.93 with an upper confidence interval of 4.32 hog deer/km^2. In 2007 the density was found to be 2.75 with an upper confidence interval of 3.80 whereas in 2008 the density was estimated at 2.51 with an upper confidence interval of 3.46 hog deer/km^2.

Compared to other deer, muntjacs seem to be found wherever there is some forest cover without too much disturbance from humans (or feral dogs) (Eisenberg & Lockhart 1972, Seidensticker 1976, Barrette 1977, Mishra 1982, Chapman et al. 1993). On the similar lines, the sambar (Schaller 1967) has an exceedingly wide geographical distribution that includes India, Myanmar, Sri Lanka, extending through the Malay countries, and eastward to the Philippines and beyond (Prater 1971). Schaller (1967) estimated a sex ratio of 0.2 males : 1 female in Kanha. In Bandipur the average male : female ratio was 0.3 : 1, and the female: fawn ratio was 1 : 0.3 (Johnsingh 1983). The male : female ratio in Nagarahole (Karanth & Sunquist 1992) was 0.4 : 1. In Sariska the estimated average male: female ratio was 0.1: 1 and the average female: fawn ratio was 1 : 0.2 (Sankar 1994). In Gir, the average male: female ratio was 0.5 : 1, and the female: fawn was 1 : 0.1 (Khan et al. 1995). The relatively low male numbers may be either due to selective predation, or sambar stags may be more vulnerable to stress. In Kanha, sambar fawns were seen from April to December and the peak fawning period was in May and June (Schaller 1967).

However, in Sariska most of the sambar fawns were dropped between November and January (Sankar 1994). Varman & Sukumar (1993) studied the ecology of sambar in Mudumalai Sanctuary, Southern India. The result shows the average sambar densities was 8.0 animal / km², sex ratio showed a bias in favour of females, fawn to adult female ratio was more or less constant in different seasons and proportion of males in hard antlers was high during dry and second wet seasons.

By comparison, in the chital (*Axis axis*) for instance, invariably, the adult sex ratio of chital is biased towards females. Schaller (1967) reported sex ratio of 0.6 male : 1 female in Corbett National Park, 0.7 : 1 in Keoladeo Sanctuary, Bharatpur, and 0.7 : 1 in Kanha. In Bandipur, the average male: female ratio was 0.6: 1, and the female : fawn was 1: 0.4 (Johnsingh 1983). The male : female ratio in Nagarahole (Karanth & Sunquist 1992) was 0.7 : 1. In Sariska, the average male: female ratio was 0.4 : 1, and the female: fawn ratio was 1 : 0.2 (Sankar 1994). In Gir, the average male : female ratio was 0.4 : 1, and the female : fawn was 1 : 0.2 (Khan et al. 1995). De Silva & De Silva (1993) investigating the population of spotted deer in Ruhuna National Park, Sri Lanka, during the period of 1991 to 1992 found that the population density of the deer in the 140 km^2 was estimated at 10.3 individuals/km² but their distribution was patchy, the sex ratio (male to female) of adult was 0.48 whereas that of yearlings was 0.55.

Studies conducted by Schaller (1967) in 1965 estimated the barasingha *R. duvaucelii* population to be between 1400 and 1800 in India and approximately 1600 in Nepal. Qureshi et al. (1995) estimated 1500 - 2000

swamp deer in India by 1991. The present estimate of barasingha is 400 - 500 in Kaziranga National Park (Kaziranga Forest Department records) Manas National Park had approximately 50 individuals. The *R. branderi* survives only in Kanha (Qureshi et al. 1995). Schaller (1967) in 1965 observed fewer than 100 barasingha in Kanha which increased to 200 in 1974 (Martin 1977) and reached a maximum of 500 in 1988 (Kotwal 1987, Gopal 1995).

4.6 SUMMARY

The population estimation of sangai and hog deer in Keibul Lamjao National Park was carried out during 2006-2008 using point count method. Deers were counted from 22 temporary machans constructed for this purpose. GPS location of machan, time of sighting, time of disappearance, sighting distance and angle, age and sex, habitat type, weather condition and temperature were recorded. Morphological traits were used for individual identifications of different sex and age groups of sangai and hog deer. The presence of faecal pellets of sangai was recorded in 22.26, 21.5 and 22.7 km^2 and in hog deer it was recorded in 22.26, 22.04 and 22.73 km^2 area of the Park during 2006, 2007 and 2008 respectively.

The density of sangai was generated using DISTANCE 5.0 software and was found to be 4.05 ±0.62 (CV 15.38%), 4.09 ±0.75 (CV 18.48%) and 4.06 ±0.8 (CV 19.9%) individuals/km^2 with a minimum of 2.97, 2.83, 2.73 and a maximum of 5.51, 5.88, 6.01 individuals/km^2 at 95% confidence level during 2006, 2007 and 2008 respectively. The estimated population size of sangai were 90, 88 and 92 individuals during 2006, 2007 and 2008 respectively, with

a minimum of 66, 61 and 62 and maximum of 123, 127 and 137 individuals at 95% confidence level for the entire study period.

The population structure of sangai based on the percentage sightings of different size classes seen during the population estimation exercise in 2006, 2007 and 2008 showed that in all the sampling years adult female population was highest followed by adult male. The Overall population structure also indicates higher number of female as compared to male, juvenile and fawn. The overall juvenile age class during the three consecutive years showed a declining trend. The adult male and female showed a slight increase in number during 2006-2008. The poplation of fawn showed a fluctuating number with a decreasing trend during 2006-2007 and marginal gain during 2007-2008.

The adult sex ratio and doe to fawn ratio of sangai were calculated based on the point count estimate analysis that showed 47, 57, 58 males/100 females and 18, 13 and 18 fawns/100 females respectively. However, the analysis of sex ratio for three consecutive years (2006-2008) was found that the observed male to female ratio was 54 ±3.57 males/100 females and fawn to female ratio was 16 ±1.7 fawns/100 females respectively.

The population trend of sangai was determined in the Park with regression analysis using the previous population estimates done by the Forest Department during 1984-2003. The results showed that the change in population was significant (p<0.05, R^2 = 0.898) with 5% increase in population during 1984-2003. However, the population trend of sangai for the three consecutive years (2006-2008) showed a more or less stable population trend

in the Park. The results showed that the change in population was not significant ($p>0.05$, $R^2 = 0.244$) with 1% increase in population from 2006-2008.

The population estimation of hog deer was done using the data for the period of 0600-0630 hours in 2006, 0700-0730 hours in 2007 whereas 0530-0600 hours in 2008. The density of hog deer was found to be 2.94 ±0.57 (CV 19.5%), 2.75 ±0.44 (CV 16.3%) and 2.51 ±0.40 (CV 16.2%) individuals/km^2 during 2006, 2007 and 2008 respectively with a minimum of 1.82 individuals/km^2 and maximum of 4.32 individuals/km^2 at 95% confidence level. The population size of hog deer were 65 ±12.6, 61 ±9.9 and 57 ±9.2 individuals with a minimum of 44, 44, 41 and maximum of 96, 84 and 79 hog deers at 95% confidence level during 2006, 2007 and 2008 respectively.

The population structure of hog deer derived based on the percentage sightings of different size classes of hog deer seen during the population estimation exercise in 2006, 2007 and 2008 showed that in all the sampling years adult female population was highest followed by adult male. The Overall population structure also indicates a higher number of female as compared to male, juvenile and fawn. The overall adult male, adult female and juvenile showed flunctuating figures where as the number of fawn during 2006-2008 showed a steady decline in number.

The adult sex ratio and doe to fawn ratio in hog deer were calculated based on the sightings during population estimation in the Park for the three consecutive years of 2006 to 2008. The male to female ratio and doe to fawn ratio of hog deer in 2006-2008 was 34.2, 34.5, 39.9 males/100 females and 16.4, 17.2 and 15.8 fawns/100 females respectively. However the mean

analysis of sex ratio for three consecutive years (2006-2008) was found that the observed male to female ratio was 36.2 ±1.9 males/100 females and doe to fawn ratio were 16.5 ±0.4 fawns/100 females respectively. The population trend of hog deer derived using similar method as used for sangai showed a declining trend in the Park. The results indicates that the change in population is significant ($p < 0.05$, $R^2 = 0.916$). The population estimation carried out during 2006 to 2008 in the Park showed a declining trend. The results showed that the change in population is not significant ($p > 0.05$, $R^2 = 0.992$) with *c* 8% decrease in population during 2006-2008.

Chapter 5

PHYLOGENETIC STATUS OF SANGAI AND HOG DEER

5.1 INTRODUCTION

Evolution is regarded as a branching process, whereby populations are altered over time and may speciate into separate branches, hybridize together, or terminate by extinction (Quental & Marshall 2010). The problem posed by phylogenetics is that genetic data are mostly available for living taxa and fossil records (osteometric data) contains less data and more-ambiguous morphological characters (Sforza & Edwards 1967). A phylogenetic tree represents a hypothesis of the order in which evolutionary events are assumed to have occurred. Cladistics is the current method of choice to infer phylogenetic trees (Wiley et al. 1991).

The evolutionary connections between closely related organisms are represented graphically through phylogenetic trees. Due to the fact that evolution takes place over long periods of time that cannot be observed directly, biologists must reconstruct phylogenies by inferring the evolutionary relationships among present day organisms. Phylogenetic relationships in the past were reconstructed by looking at phenotypes, often anatomical characteristics. Today, molecular data, which includes protein and DNA sequences, are used to construct phylogenetic trees (Benjamin 2007).

The mitochondrial DNA (mtDNA) has simple genomic structure and it is one of the most widely used molecular markers for phylogenetic studies in

animals (Avise 2004) and to warranty the exact taxonomic position in generic as well as in species level. Molecular phylogenetics based on mtDNA (Miyamoto et al. 1990, Cronin 1991, Douzery & Randi 1997, Randi et al. 2001, Polziehn & Strobeck 2002, Li et al. 2003, Ludt et al. 2004), sequence comparisons has contributed considerably to resolve evolutionary relationships. The mtDNA is useful for studying the evolution of closely related species and many studies have focused on the mitochondrial D-loop region (also called the control region), the most variable part of mtDNA (Ishida et al. 1994) due to a higher substitution rate than in the rest of the mtDNA genome (Cann et al. 1984). However, the gene coding for cytochrome b (cyt *b*) as molecular marker to analyse phylogenetic relationships among deer is widely used because its tempo and mode of evolution is well understood, thought to be relatively constant and similar among large-bodied terrestrial mammals. The cyt *b* gene has been used in numerous studies of phylogenetic relationships among mammals and is the gene for which the most sequence information from different mammalian species is available (Johns & Avise 1998, Castresana 2001, Randi et al. 2001).

In vertebrates, the mtDNA is a small extra-chromosomal genome, ranging in size from 16 kb to 18 kb (Boore 1999). The typical mammalian mitochondrion contains 37 genes: 13 protein-coding genes, two ribosomal RNA genes, and 22 tRNA genes (Wolstenholme 1992, Boore 1999). The mtDNA codes for only a small proportion of the total DNA in an organism, and as a result its lineage may differ greatly from that of nuclear genes (Ferris et al. 1983). The mtDNA possesses two properties that make it particularly useful in the determination of genetic variation within and among animal and

plant populations (Wilson et al. 1985). First, the mtDNA is maternally inherited, thereby allowing the determination of maternal lineages of the population. Second, the rate of nucleotide substitution for mtDNA is generally five to ten times greater than in nuclear DNA. Because of this high rate of evolution, and because it is so highly polymorphic within species, mtDNA can be used to examine the geographic structure of populations of a given species or to investigate the differentiation of closely related species (Avise 2004). Very few studies have been conducted on the genetics of Eld's deer in the wild based on mtDNA genes.

The present study examines the phylogenetic status of Eld's deer and genetic relationships of sangai with their sister subspecies and related cervids, with the objective to estimate the genetic variability of sangai within the species, between wild and captive populations and among closely related cervids. The chapter in itself is an attempt to answer the following research questions:

As a population becomes smaller, it tends to lose genetic variability just by chance, a process known as genetic drift, leading to inbreeding depression and a lack of evolutionary flexibility (Primack 1993). The research question which was addressed in this chapter is what is the genetic relationship of sangai with other subspecies of Eld's deer and related cervids?

5.2 MtDNA (cytochrome *b* and control region genes) ANALYSIS

5.2.1 Collection of biological samples

A total number of 138 samples were collected for genetic analysis from both wild and captive populations of sangai and related cervids. All the tissue samples were preserved in 95% ethanol and stored at -20°C until DNA extraction. List of samples are given in Table 5.1. In total 85 samples of sangai were collected, of which 73 was from KLNP and 12 was from captive stock (6 from Manipur Zoo, 4 from Delhi Zoo and 5 from Guwahati Zoo). The samples consisted of muscle tissue, dried skin, ear pinna, hair, skull bones, antlers and faecal pellets which were found opportunistically during the field survey that was carried out between 2005 and 2008 (Plate 6). A total of 41 samples of hog deer were collected, of which 38 was from KLNP and 3 was from captive stock (two antlers were collected from Kaziranga National Park, Assam and a faecal pellet sample was also collected from Guwahati Zoo). Seven samples of swamp deer were also collected from Kaziranga National Park which comprised of five antlers and two tissue samples. One dried skin sample of barking deer from Arunachal Pradesh and one tissue sample of sambar was also collected from Punjab (provided by Dr. Sandeep Gupta, WII).

5.2.2 DNA extraction

A small portion of tissue (around 50-80 mg) was cut out carefully and chopped finely using separate sterile blades for every sample, taking care to

avoid contamination. The total genomic DNA from blood and tissue samples was extracted using DNeasy blood and tissue Kit (Qiagen) following the manufacturer's protocol, with slight modification. The genomic DNA from hair and antlers were extracted with QIA amp® DNA Micro kit (Qiagen) following the manufacturer's recommendation with the exception that 20 µl of 0.1% DTT (Dithiothretol) was added to the first incubation step in order to dissolve the hair and antlers to increase the DNA yield. The Qiagen Stool Mini kit was used for extraction of DNA from faecal pellets. All the extractions were verified with extraction negative controls.

Table 5.1 List of samples collected for genetic analysis.

Species	Sample description	Total	Source
Sangai	Muscle tissue	1	Keibul Lamjao National Park
Sangai	Skull	1	Keibul Lamjao National Park
Sangai	Feacal pellet	70	Keibul Lamjao National Park
Sangai	Antler	1	Keibul Lamjao National Park
Sangai	Dried skin	2	Manipur Zoo
Sangai	Ear pinna	1	Manipur Zoo
Sangai	Antler	3	Manipur Zoo
Sangai	Antler	4	Delhi Zoo
Sangai	Feacal pellet	2	Guwahati Zoo
Sangai	Antler	3	Guwahati Zoo
Hog deer	Muscle tissue	10	Keibul Lamjao National Park
Hog deer	Ear pinna	3	Keibul Lamjao National Park
Hog deer	Carcasses	12	Keibul Lamjao National Park
Hog deer	velvety antler	1	Keibul Lamjao National Park
Hog deer	Antler	1	Keibul Lamjao National Park
Hog deer	Blood	1	Keibul Lamjao National Park
Hog deer	Feacal pellet	10	Keibul Lamjao National Park
Hog deer	Antler	2	Kaziranga, Assam
Hog deer	Feacal pellet	1	Guwahati Zoo
Swamp deer	Antler	5	Kaziranga, Assam
Swamp deer	Muscle tissue	2	Kaziranga, Assam
Barking deer	Dried skin	1	Arunachal Pradesh
Sambar	Tissue	1	Punjab

Plate 6. Various types of samples for genetic study

5.2.3 PCR amplification and gel electrophoresis

The Polymerase Chain Reaction (PCR) was performed using the thermal cycler. PCR amplifications were carried out in 20 µl volumes containing 2 µl of genomic DNA, 2.0 mM $MgCl_{2,}$ PCR Buffer (1X), 1x BSA, 10mM each dNTPs mix, 2-4 pmol of each primer and 1U of Taq DNA polymerase. Two pair of cytochrome b (cyt *b*) and control region (D-loop) primers were used for amplification of mtDNA genes: cyt *b* primers: cytb42 (5'-GAACAACGCATTTATTGACCTC-3') / cytb607 (5' ACAGGATCCAATAACCCAACAG-3') (this study) and mcb398 (5'-TACCATGAGGACAAATATCATTCTG-3') / mcb869 (5'-CCTCCTAGTTTGTTAGGGATTGATCG-3') (Verma & Singh 2003) and control region primers: D-loop R (5'-GCATGGGGCATATAATATAATGTACTA-3') / Lo-F (5'-CCCAAAGCTGAAATTCTACTTAAACTA-3') (Galan et al. 2005) and Cerv.tPro (5'-CCACYATCAACACCCAAAGC-3') / CervCRH (5'-GCCCTGAARAAAGAACCAGATG-3') (Balakrishnan et al. 2003) were used for amplification. The PCR cycling parameters of cyt *b* gene: 5 min at 94˚C, 30 cycles of 40s at 94˚C, 40s at 55˚C, and 90s at 72˚C, terminated by 10 min at 72˚C and D-loop R and Lo-F gene: 5 min at 94˚C, 45 cycles of 35s at 94˚C, 45s at 53˚C, and 90s at 72˚C, terminated by 10 min at 72˚C respectively. The primer Cerv.tPro / CervCRH (Balakrishnan et al. 2003) has PCR cycling parameters: 1 min at 94˚C, 30 cycles of 20s at 94˚C, 20s. at 52˚C, and 60s at 72˚C, terminated by 10 min at 72˚C respectively. PCR was repeated three times to ensure quality control, such as avoiding random amplification errors. Negative control reactions were included in each PCR run.

The amplified PCR products were separated on 2% agarose gel by electrophoresis. Subsequently, the gel was stained with ethidium bromide for visualization and photograph in a UV light transilluminater.

5.2.4 DNA purification and sequencing

The PCR products were gel purified in 1% agarose, excised from the gel, and purified directly with a QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were separately sequenced using each PCR primers including cyt *b* and D-loop genes (for PCR product generated by this primer set, with Big Dye Terminator v 3.1 cycle sequencing kits, and were analyzed on an Applied Biosystems automated DNA Sequencer with a two step cycles: 1 min denaturation at 96˚C, followed by 28 cycles of 10s at 96˚C, 5s of annealing at 50˚C and 4 min extension at 60˚C.

5.3 DATA ANALYSIS

5.3.1 Species identification

The identification of both sangai and hog deer was confirmed through programme BLAST 1.4 (Altschul et al. 1990). The sequences were aligned applying the software BLAST 1.4 and searched a nucleotide sequences database using a nucleotide query. As a result, high scoring sequence pairs (HSP) were presented, consisting of the query sequence and databases entry in combination with a similarity value reporting the significance of the match (Smith et al. 1996).

5.3.2 Model selection for construction of cladogram

The sequences of mtDNA cyt *b* and control region genes were aligned using Clustal *X* version 1.83 (Thompson et al. 1997) and checked visually. The aligned sequences were then edited manually to fit them to the same length using the software BioEdit version 7.0.9 (Hall 1999). The phylogenetic trees were derived using MEGA 5 (Tamura et al. 2011) employing the best fit model of Tamura-3 parameter based on the lowest BIC scores (Bayesian Information Criterion). Model selections were made once the dataset has been prepared and performed proper Clustal X alignment with 1000 pseudo replicates. Several robust models were considered based on BIC scores (Bayesian Information Criterion). Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented. The Maximum Likelihood fits of 24 different nucleotide substitution models were used *viz.* GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor. All the 24 models were elaborately mentioned. The non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). The lowest BIC scores is an estimate of the best approximating model.

5.3.3 Phylogenetic analysis using cyt *b* & control region genes

The phylogenetic tree using cyt *b* was constructed using Neighbour-Joining (NJ) method with 18 sequences of which five sequences each of sangai from KLNP and Manipur Zoo and three sequences each of sangai from Delhi and Guwahati Zoo. Two sequences of genus Pere David's deer (*Elaphurus davidianus*) was chosen as outgroup. Since, the cyt *b* sequences of Eld's deer was limited in the GenBank, the genetic relationship within the subspecies of Eld's deer could not be drawn. However, few sequences of control region gene of Eld's deer were available in the GenBank based on which the genetic relationships were examined. The phylogenetic tree using control region gene were constructed using Neighbour-Joining (NJ) method with eight sequences of sangai of which each two sequences from KLNP, Manipur Zoo and Delhi Zoo, and two sequences of genus *E. davidianus* was chosen as outgroup. The robustness of the tree nodes was assessed by the bootstrap method after 1000 replication by adding sequences random, excluding nodes with <50% support.

5.3.4 Phylogenetic analysis of Eld's deer using control region genes

A total of 27 sequences, of which six sequences each of *hainanus* (accession numbers: AF359290.1, AF359286.1, AF359288.1, AF359289.1, AF359291.1, AF359287.1); *thamin* (accession numbers: AY137092.1, AY137088.1, AY137093.1, AY137090.1, AY137091.1, AY137089.1); and

three sequences of *siamensis* (accession numbers: AY137082.1, AY137081.1, AY137083.1); and seven sequences of sangai from GenBank (accession numbers: AY137120.1, AY137118.1, EU870591.1, AY137119.1, EU870590.1, AY137117.1, AY137121.1), two references samples of sangai from Manipur Zoo and two samples collected from KLNP were used for phylogenetic analysis. One sequence of *E. davidianus* were extracted from the GenBank (accession number: DQ295069.1) used as outgroup. The phylogenetic trees were constructed using Neighbour-Joining (NJ) and Minimum Evolution (ME) method with control region (D-loop R / Lo-F) gene (Galan et al. 2005) employing the best fit model of Tamura-3 parameter based on the lowest BIC scores (Bayesian Information Criterion). The robustness of the tree nodes was assessed by the bootstrap method after 1000 replication by adding sequences random.

The phylogenetic trees using Cerv.tPro / CervCRH gene were constructed using Neighbour-Joining (NJ) and Maximum Parsimony (MP) method employing the best fit model of Tamura-3 parameter based on the lowest BIC scores (Bayesian Information Criterion). A total of 44 sequences of which 17 sequences of sangai (one from KLNP, three from Manipur Zoo, four sequences from Delhi Zoo, three sequences from Guwahati Zoo and six sequences from GenBank, Accession number are mentioned on the branches), nine sequences of *thamin*, 7 sequences of *siamensis*, 10 sequences of *hainanus* and one sequence of *E. davidianus* were extracted from the GenBank (accession numbers are mentioned on the branches of the tree) for construction of cladogram.

5.3.5 Phylogenetic relationship of sangai with other cervids

A total of 22 sequences (five sequences from the GenBank and 17 from the present study) were used to investigate the relationship of sangai with related cervids. The scientific names and the GenBank accession numbers for the published sequences analyzed in the study are chital *Axis axis* (accession number: EU870593) and four sequences of barking deer *Muntiacus muntjak*, one from Arunachal Pradesh and three from GenBank accession numbers: EF523654.1, EF523653.1 and EF523652.1 were used as outgroup species. The cladistic analyses were done using two sequences each of sangai from KLNP and Manipur Zoo, two sequences of swamp deer *R. duvaucelii* (U1Z1 and U1Z2) from Kaziranga National Park, Assam, 10 sequences of hog deer (*A. porcinus*) from KLNP and one sequence of sambar *Rusa unicolor* (this study).The phylogenetic trees were constructed using Neighbour Joining (NJ) method based on Tamura 3 parameter (T92) model. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best.

5.3.6 Estimation of divergence times

The program MEGA 5 (Tamura et al. 2011) was used to calculate divergence times based on fossil records (Ginsburg et al. 1982). These dated fossil records have been treated as calibrations and used at the appropriate nodes to obtain a range of values for the respective divergence times. The fossil records used include: 18,000 to 8,500 years BP, as being the maximum and minimum age for the origin of Eld's deer based on fossils remains that

appeared during the end of Pleistocene and early Holocene in the Island of Java and Hainan (Ginsburg et al. 1982), that appeared during the later part of the middle Pleistocene in the Northeastern Thailand (Tougard et al. 1996 sited after Ginsburg et al. 1982).

The phylogenetic trees were constructed using Neighbour-Joining (NJ) method employing the best fit model of Tamura-3 parameter based on the lowest BIC scores (Bayesian Information Criterion). A total of 38 sequences of which 17 sequences of sangai (one from KLNP, three from Manipur Zoo, four sequences from Delhi Zoo, three sequences from Guwahati Zoo and six sequences from GenBank, Accession number are mentioned on the branches), eight sequences of *thamin*, two sequences of *siamensis*, 10 sequences of *hainanus* and one sequence of *E. davidianus* were extracted from the GenBank (accession numbers are mentioned on the branches of the tree) to calculate the time of divergence of Eld's deer.

5.4 RESULTS

5.4.1 Species identification

The BLAST findings of KLNP samples of sangai using cyt *b* gene showed a range of match identities of 94 to 96% and captive samples of different Zoos showed a range of match identities: Guwahati Zoo (95 to 96%), Manipur Zoo (96 to 99%) and Delhi Zoo (95 - 96%) respectively. The BLAST findings using control region gene showed a range of match identities of 99 to 100% in wild samples of sangai and 98% to 100 % in captive populations respectively. The BLAST findings of hog deer samples of KLNP using control region gene showed a range of match identities of 99 to 100% respectively.

5.4.2 Intraspecies comparision using mtDNA cyt *b* and control region genes

In this section intraspecies comparision of sangai from wild and captivity were compared. The fragment of mtDNA cytochrome *b* gene were amplified with primer cytb42 / cytb607 (this study) and mcb398 / mcb869 (Verma & Singh 2003) which includes 150 and 471bp. However the primer D-loop R / Lo-F (Galan et al. 2005) and Cerv.tPro / CervCRH (Balakrishnan et al. 2003) could successfully amplify the 5′ section of the hypervariable segment of mitochondrial control region gene of 230 and 478 bp. No haplotype variations were detected among the 16 individuals of sangai using mtDNA cyt *b* (mcb398 / mcb869) (Figure 5.1) and (cyt42 / 602) genes.

However, two haplotypes were identified among the 17 individuals of sangai at nt282 position in mtDNA control region of Cerv.tPro / CervCRH gene (Figure 5.2). Both these variable positions were transition mutation (T→C) Figure 5.1. First, a haplotype variation were found in 16 samples of sangai *viz.* KLNP (n=1; S8), Manipur Zoo (n=3; S9, MZ2 and MZ3), Delhi Zoo (n=5; DZS2, DZ2, DZ3, EU870590.1 and EU870591.1), Guwahati Zoo (n=3; GZ1, GZ2 and GZ3) Hyderabad Zoo (n=4; AY137120.1, AY137118.1, AY137119.1, AY137121.1 from Balakrishnan et al. 2003) whereas a second haplotype variation was found in one sample of Delhi Zoo (n=1; DZS1).

In case of D-loop R / Lo-F primer, no haplotype variation was identified because the region was not enough for any detection of variability (Figure

5.3). Incorporation of more sample size from blood and tissue from wild and captive population of sangai may reveal better understanding on its haplotype diversity and its phylogenetics.

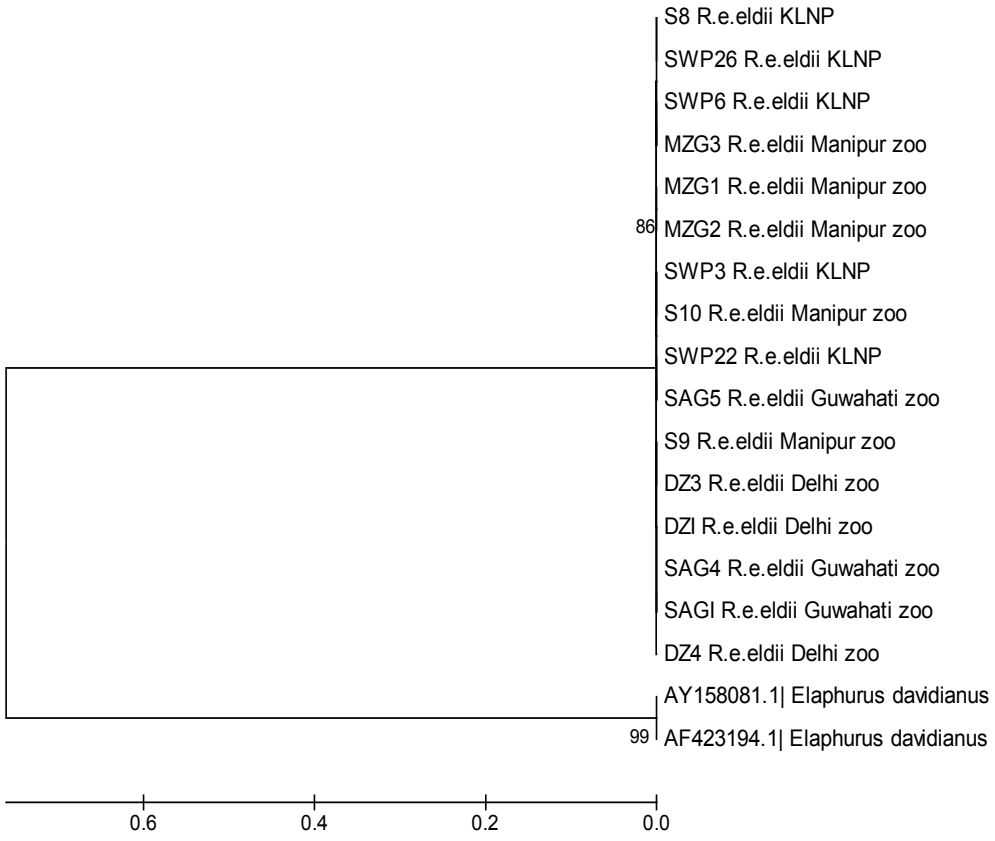


Figure 5.1 Cladogram of wild and captive population of sangai using mtDNA cyt *b* gene (mcb398 / mcb869, Verma & Singh 2003) with Neighbour joining method.

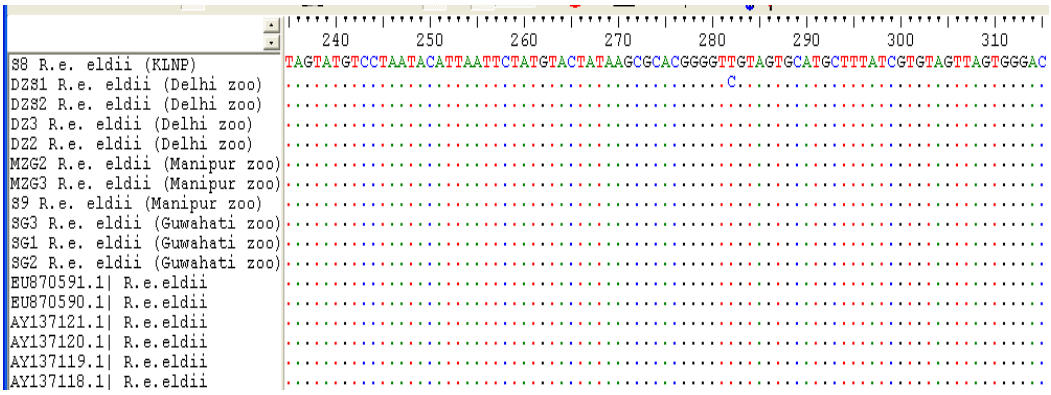


Figure 5.2 Variable positions in mtDNA control region of Cerv.tPro / CervCRH gene. Sequence identities are indicated by dots and variable positions detected at nt282 position (T→C).

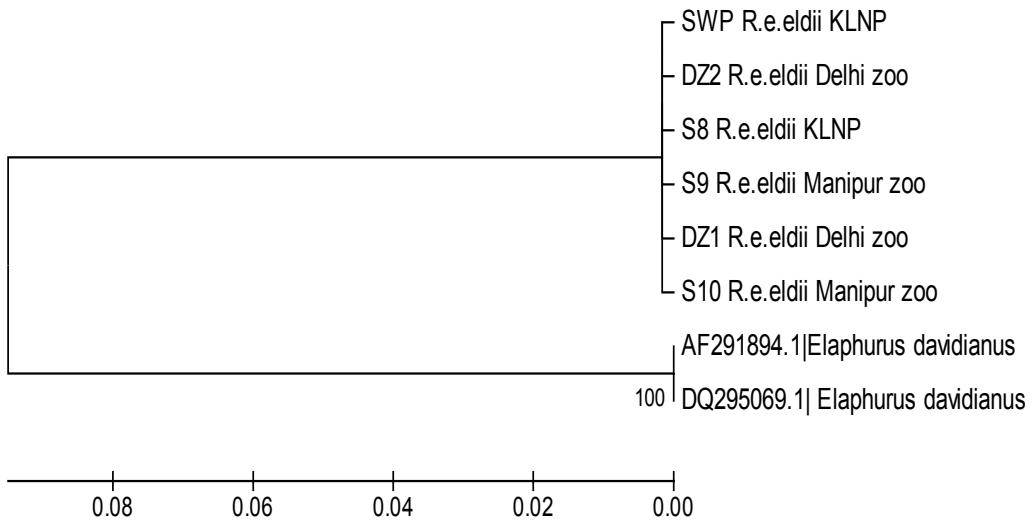


Figure 5.3 Cladogram of wild and captive population of sangai using control region (D-loop R / Lo-F, Galan et al. 2005) gene with Neighbour joining method.

5.4.3 Intraspecific relationship among Eld's deer

In this section intra specific relationship of different subspecies of Eld's deer was compared using control region gene (D-loop R / Lo-F and Cerv.tPro / CervCRH). The phylogenetic trees of Neighbor-Joining (NJ) and Minimum Evolution (ME) with D-loop R / Lo-F gene showed similar topologies (Figure 5.4 and 5.5). Since the BIC scores of the T92+G (Tamura 3-parameter) is the lowest (1805.8) it was chosen as the best model for constructing cladogram of Eld's deer (Table 5.2).

From the cladogram, it appears that Eld's deer forms a monophyletic clades showing first clade comprising of captive population of sangai from Nehru Zoological Garden (Hyderabad Zoo) and Delhi Zoo. The second clade represents sangai from KLNP and Manipur Zoo forming one group. The analysis indicates a split of two clades with approximately 50% bootstrap support (moderate) of sangai from wild and Manipur Zoo and the another clade from captive population of Hyderabad and Delhi Zoo which could reflect some degree of sequence variation within the sampled dataset themselves and also might be because of DNA sequence divergence from the database or could be due to wide differences in the base pair length that might be showing two clades and genetically divergent. However, this result is based exclusively on the primer D-loop R / Lo-F gene consisting of a short fragment of 230 bp of the hypervariable segment mitochondrial control region gene. The third clade constitutes the population of *thamin* from Myanmar, whereas the fourth group comprises of *hainanus* from Hainan Island, China and subspecies from Thailand, the *siamensis*, which is interspersed with

hainanus. This shows that the status of *hainanus* needs a formal study to examine its accurate taxonomic position and relationship among subspecies of Eld's deer. It appears that the accession number AY137115 and AY137116 which were labeled as *Cervus* (*Rucervus*) *eldii siamensis* in GenBank is actually originated from *Cervus* (*Rucervus*) *eldii hainanus*. Most of the sources of the *siamensis* sequences are not known and only few sequences are available in Genbank and the sequences of *siamensis* showed variation among themselves.

To compare and checked the result, a larger fragment of 478 bp of control region Cerv.tPro / CervCRH gene of Balakrishnan et al. (2003) were analysed with Neighbour Joining and Maximum Parsimony tree (Figure 5.6 and 5.7). The result showed the populations of sangai were grouped into a single clade consisting of both captive and wild with high bootstrap support (100%). This indicates that the captive and wild population of sangai is not genetically divergent base on the Cerv.tPro / CervCRH control region gene.

Table 5.2 Best model analysis of Eld's deer for phylogenetic analysis.

Model	Parameters	BIC	AICc	*lnL*
T92+G*****	58	1805.8	1458.3	-670.0
K2+G	57	1807.5	1465.9	-674.9
T92+G+I	59	1813.5	1460.0	-669.8
K2+G+I	58	1814.1	1466.6	-674.2
JC	55	1817.2	1487.6	-687.8
JC+G	56	1824.5	1488.9	-687.4
JC+I	56	1824.6	1489.0	-687.4
JC+G+I	57	1832.2	1490.6	-687.2
HKY+G	60	1834.8	1475.4	-676.5
HKY+G+I	61	1838.9	1473.6	-674.5
TN93+G	61	1841.4	1476.0	-675.8
TN93+G+I	62	1845.5	1474.2	-673.8
GTR	63	1853.0	1475.7	-673.5
GTR+I	64	1856.4	1473.2	-671.2
GTR+G	64	1861.0	1477.8	-673.5
GTR+G+I	65	1863.8	1474.7	-670.9
T92	57	1876.5	1534.9	-709.4
K2	56	1914.3	1578.7	-732.3
K2+I	57	1925.0	1583.4	-733.6
TN93	60	1960.2	1600.7	-739.2
T92+I	58	1961.8	1614.3	-748.0
TN93+I	61	1979.9	1614.5	-745.0
HKY	59	1986.0	1632.5	-756.1
HKY+I	60	1986.5	1627.1	-752.3

* T92+G is the best fitted model based on the lowest BIC scores (Bayesian Information Criterion). **Note** GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor; BIC scores (Bayesian Information Criterion), AICc value (Akaike Information Criterion, corrected) and Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented.

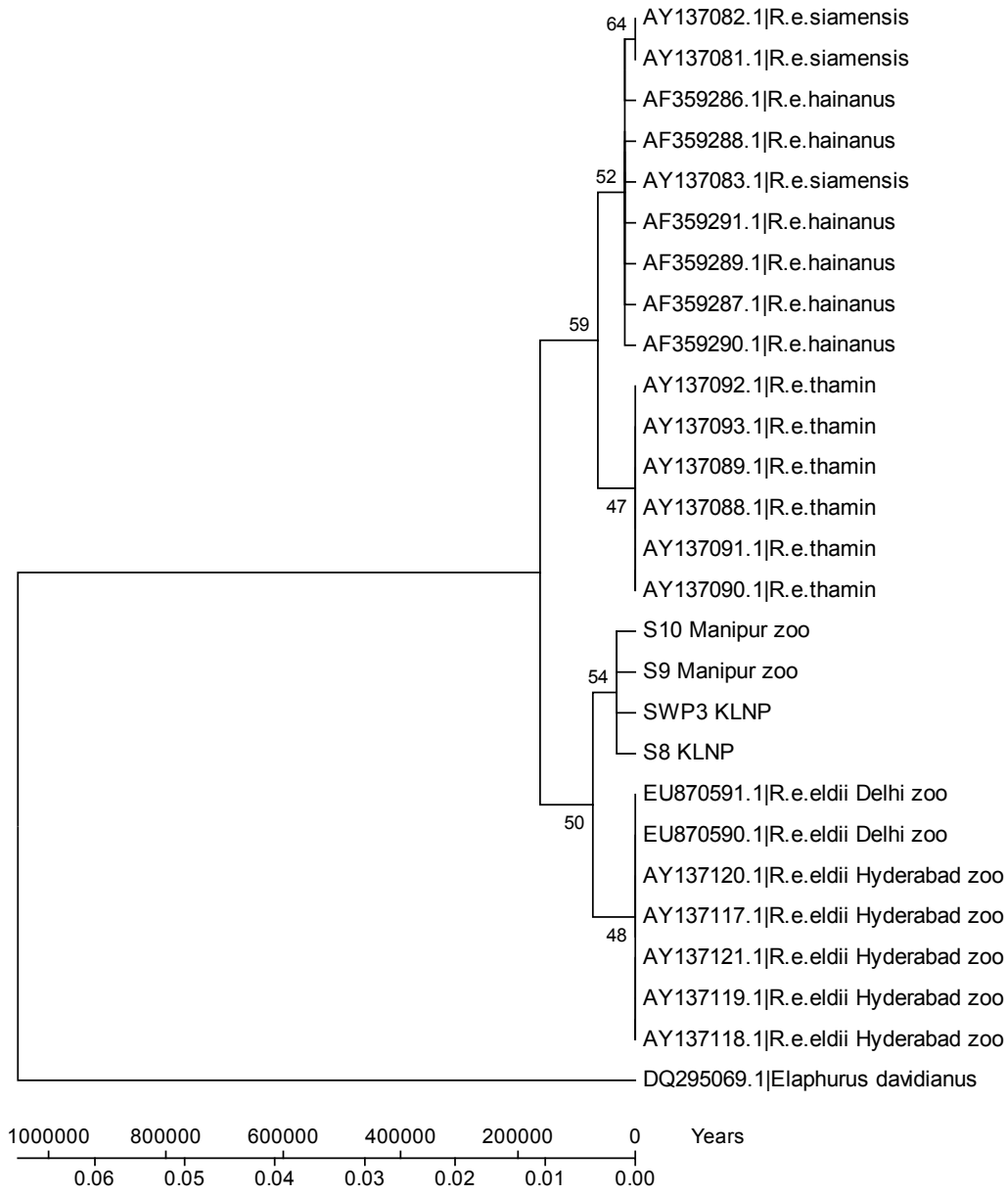


Figure 5.4 Cladogram of three subspecies of Eld's deer with Neighbour-Joining method, using Tamura 3 parameter (T92+G) model. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best.

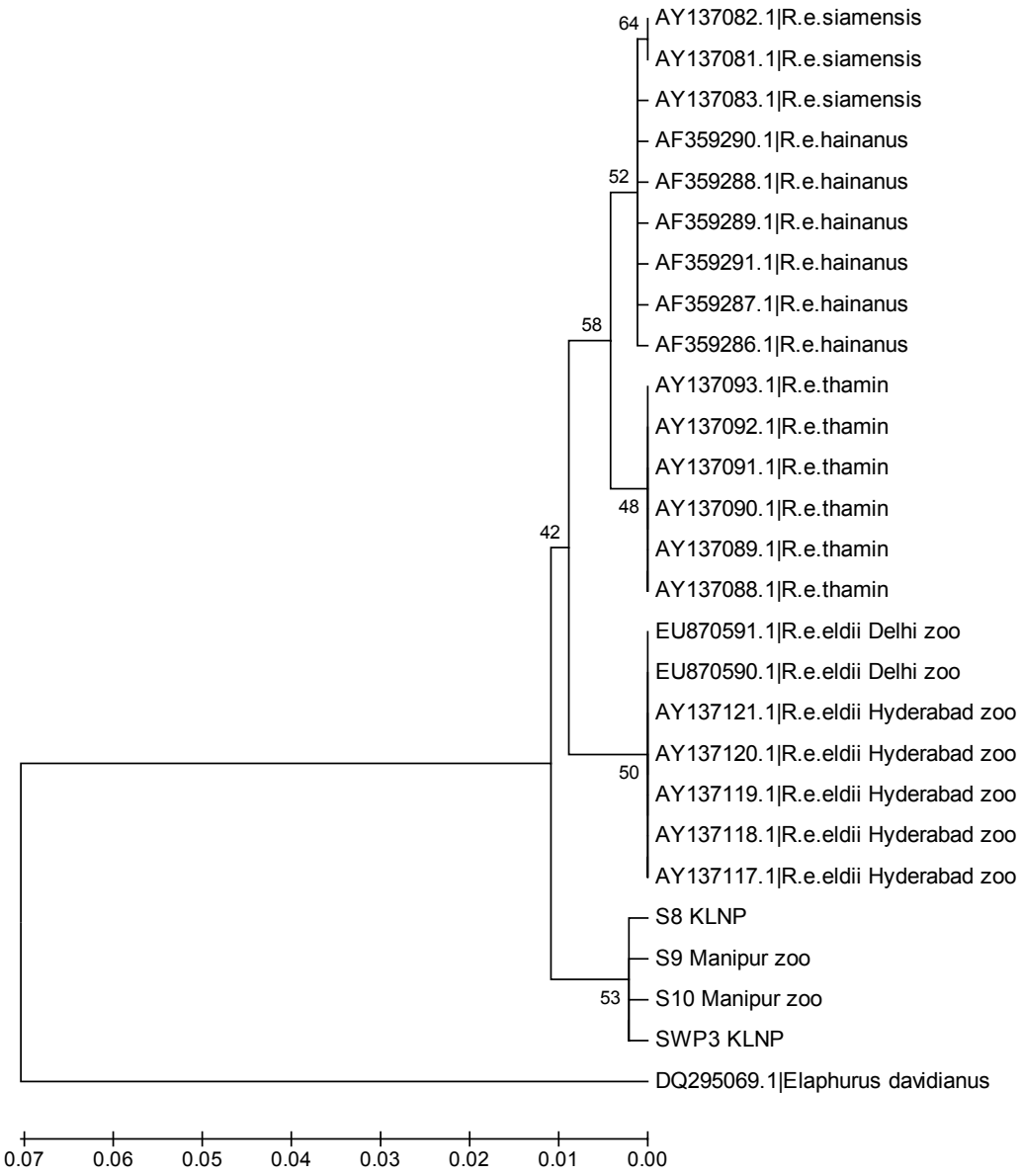


Figure 5.5 Cladogram of three subspecies of Eld's deer with Minimum Evolution (ME) method, using Tamura 3 parameter (T92+G) model. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best.

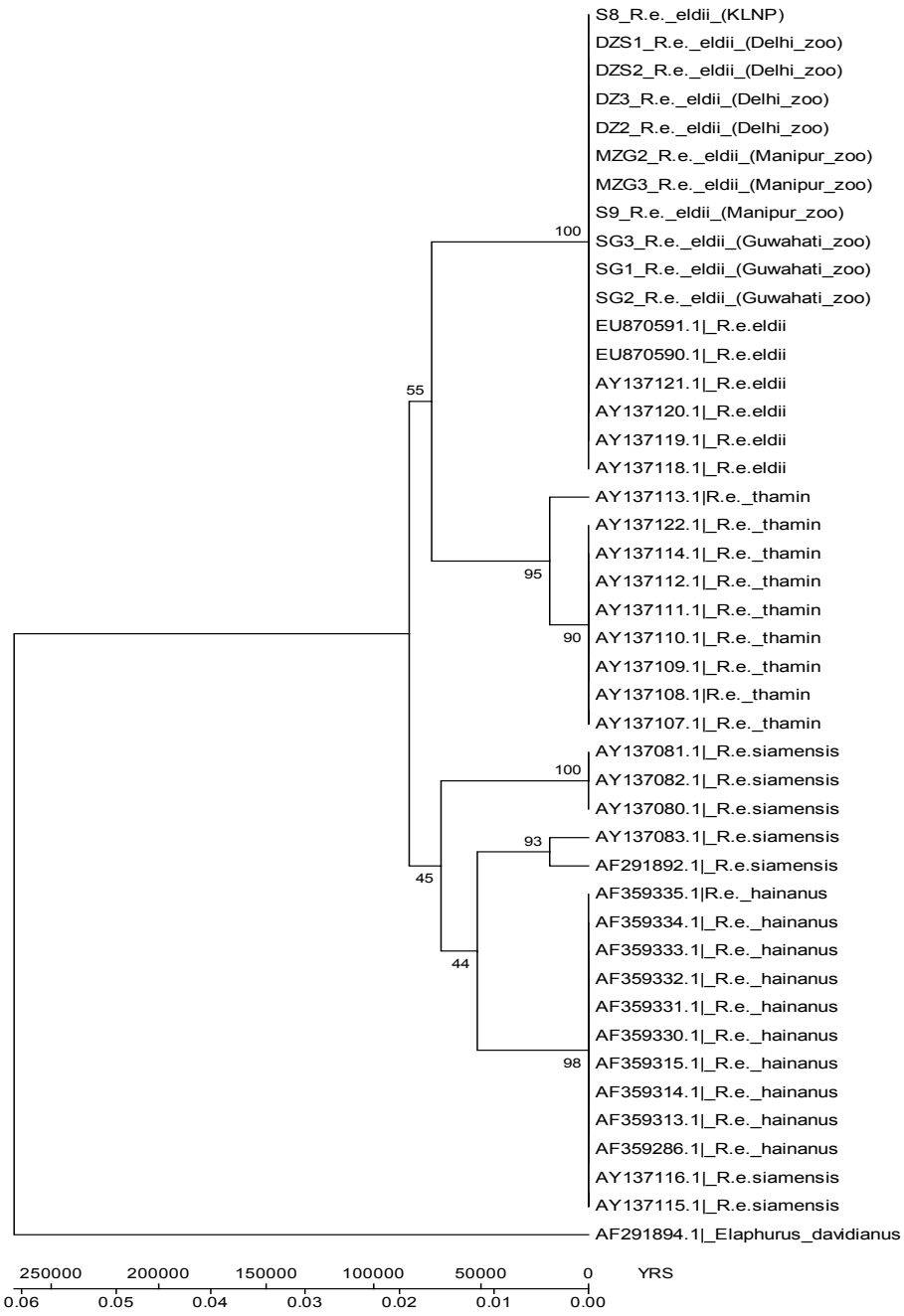


Figure 5.6 Cladogram of three subspecies of Eld's deer with Neighbour Joining (NJ) method, using Tamura 3 parameter (T92+G) model. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best.

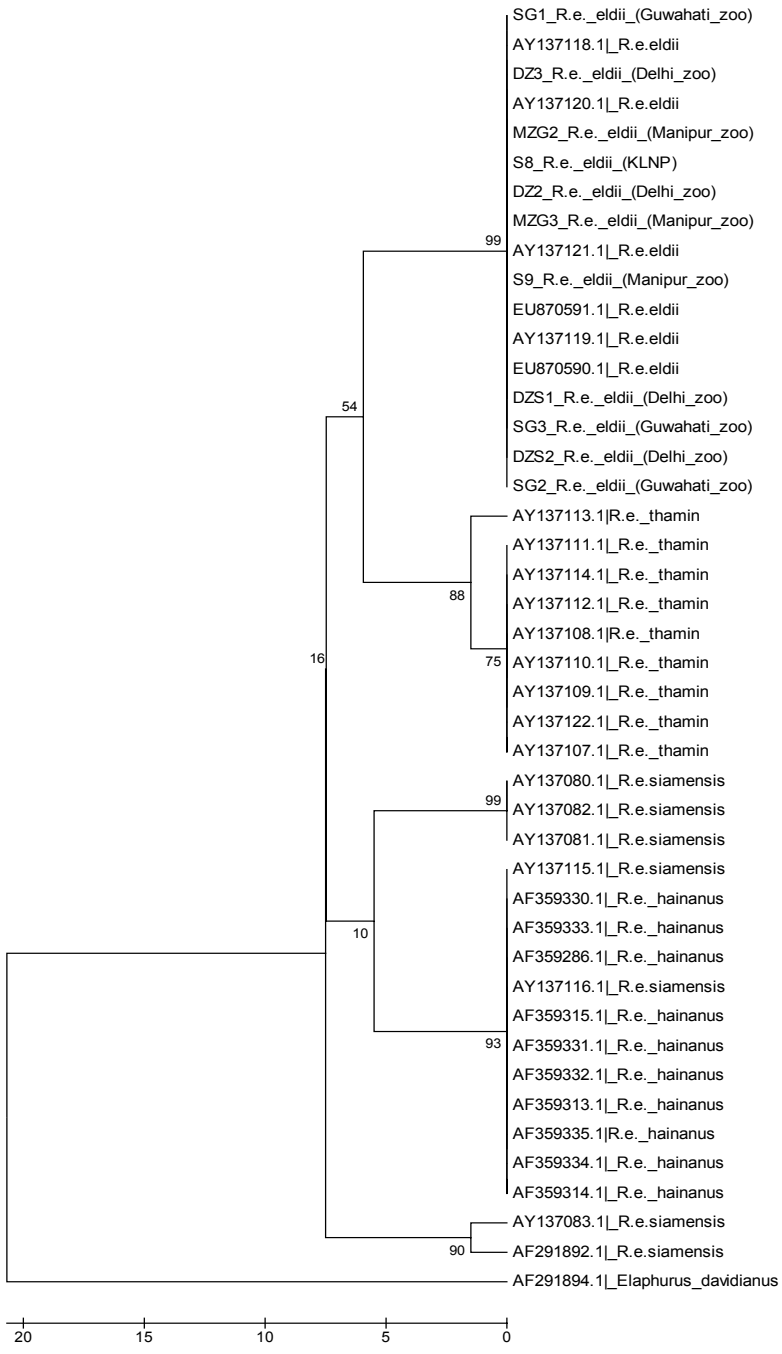


Figure 5.7 Cladogram of three subspecies of Eld's deer with Maximum Parsimony (MP) method, using Tamura 3 parameter (T92+G) model. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best.

5.4.4 Estimation of divergence time of different subspecies

The divergences times of three subspecies of Eld's deer were calibrated at 18,000 and 8,000 years, by referring to the age of the oldest known fossil records of Eld's deer (Ginsburg 1982). Using two fossil records as calibration points for nodes, a range of divergence times was obtained for each node in the Neighbour joining tree. The results showed that *R. e. eldii* was much diverge earlier than *R. e. thamin* and *siamensis* / *hainanus i.e. R. e. eldii* > *R. e. thamin* > *R. e. hainanus* or *siamensis.* The range of divergence time between *R. e. eldii* and *R. e. thamin* was estimated at approximately 73,683 years, while that of *R. e. thamin* and *R. e. siamensis* was approximately 51,748 years and that of *R. e. siamensis* and *hainanus* was 18,000 years (Figure 5.8). The results of the present study was supported by the Balakrisnan et al. (2003) that provide a smaller geographic distance between the historical ranges of *R. e. eldii* and *R. e. thamin* which indicates a more recent common ancestry. However, *R. e. siamansis* and *R. e. thamin* are more distantly related that corresponds to a historical range disjunction at the Dawna Ridge on the boundary between Myanmar and Thailand, which has probably presented a long-term barrier to gene flow between these populations (Balakrisnan et al. 2003).

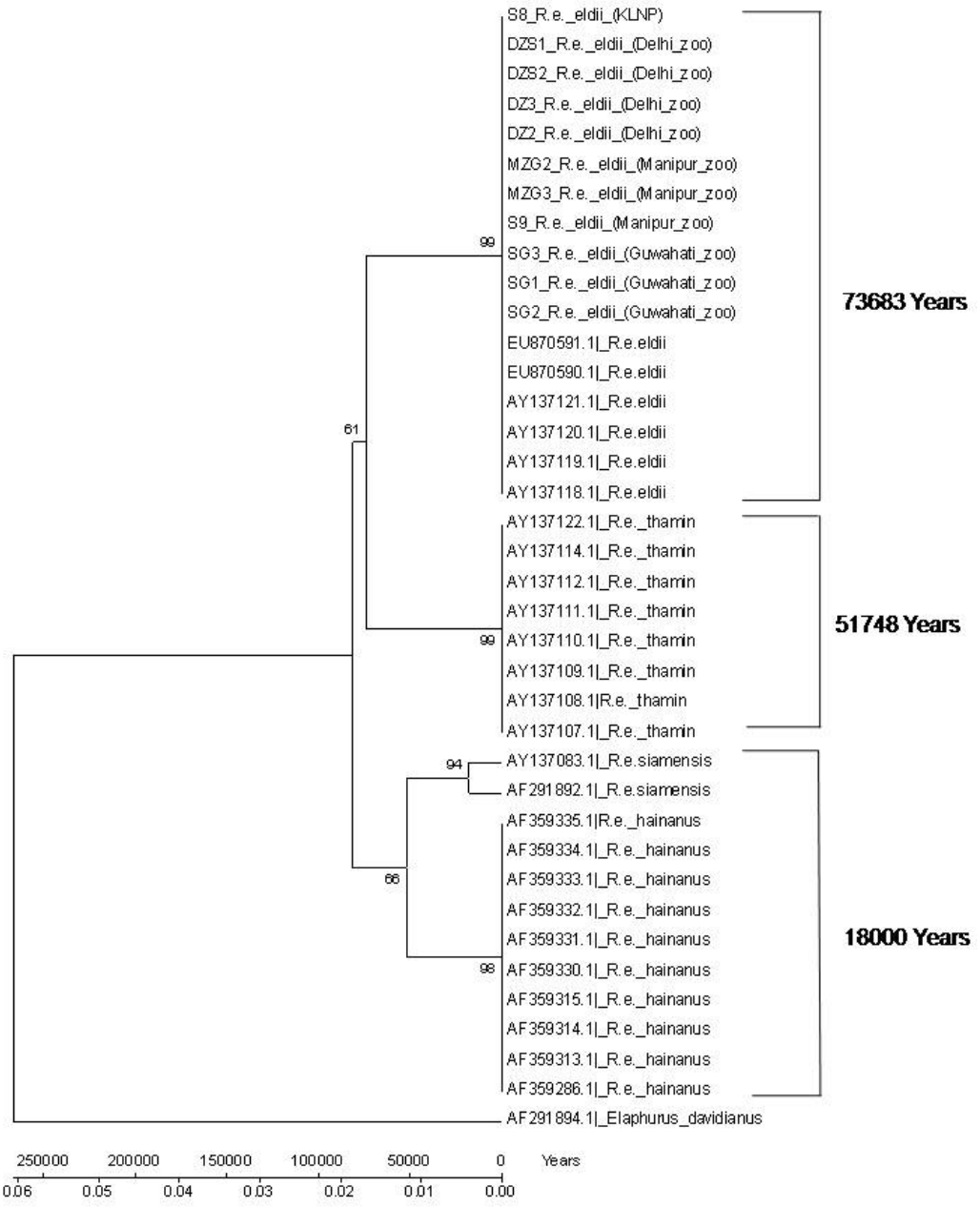


Figure 5.8 Estimation of time of divergence among three subspecies of Eld's deer with Neighbour-Joining (NJ) method, using Tamura 3 parameter (T92+G) model.

5.4.5 Interspecies relationship of sangai with related cervids

The family Cervidae includes 40 species of deer distributed throughout the northern hemisphere, as well as in South America and Southeast Asia (Gilbert 2006). Of the seventeen species of cervids occurring in Southern Asia and far East, seven species *viz.* muntjac, sambar, chital, hog deer, swamp deer, hangul or Kashmir stag (*Cervus elaphus hanglu*) and Manipur's Eld's deer occur in India (Whitehead 1993, Menon 2003, Hussain et al. 2006, Angom & Hussain unpublised). Among these, during this study the phylogenetic relationships among six species of deer from the subfamily Cervinae muntjac, sambar, chital, hog deer, swamp deer and Manipur's Eld's deer were investigated from the cladogram of Neighbour Joining (Figure 5.9), employing the lowest BIC (687.4) scores (Table 5.3) of the T92+G (Tamura 3-parameter), it was evident that the sangai has close affinity with sambar (*Rusa unicolor*) and hog deer (*A. porcinus*) which is closely related to chital (*A. axis*) were grouped together as monophyly. The study by Randi et al. (2001) pointed out that the hog deer (*A. porcinus*) is not closely related with sambar (*R. unicolor*) and also examination of the evolutionary relationships within Cervinae using the mtDNA control region marker revealed paraphyly of the genus *Cervus.* On the other hand, swamp deer *R. duvaucelii* which recently revised from *Cervus* to *Rucervus* (Wilson & Reeder 2005) appear closely related to *Muntiacus muntjak* than to Eld's deer. Eld's deer and swamp deer may seem functionally similar, but represent different lines of cervids that have each adapted to seasonally flooded dry forests (McShea et al. 2001).

Table 5.3 Best model analysis of phylogenetic construction of Eld's deer with related cervids.

Model	Parameters	BIC	AICc	*lnL*
T92*****	35	687.4	512.4	-220.1
T92+I	36	693.2	513.2	-219.4
T92+G	36	694.0	514.1	-219.9
T92+G+I	37	697.8	512.9	-218.2
HKY	37	703.1	518.2	-220.8
HKY+G	38	708.0	518.2	-219.8
TN93	38	708.3	518.5	-219.9
HKY+I	38	709.0	519.1	-220.2
K2	34	709.3	539.2	-234.6
TN93+G	39	709.7	515.0	-217.1
TN93+I	39	710.8	516.0	-217.6
HKY+G+I	39	713.2	518.5	-218.9
K2+G	35	713.5	538.5	-233.1
JC	33	714.2	549.0	-240.5
K2+I	35	714.6	539.5	-233.7
TN93+G+I	40	715.0	515.3	-216.2
JC+I	34	720.3	550.2	-240.1
K2+G+I	36	720.8	540.8	-233.2
JC+G	34	721.1	551.0	-240.4
GTR	41	727.1	522.5	-218.7
JC+G+I	35	728.0	552.9	-240.4
GTR+G	42	729.4	519.9	-216.3
GTR+I	42	730.2	520.7	-216.7
GTR+G+I	43	733.9	519.5	-215.0

***** T92 is the best fitted model based on the lowest BIC scores (Bayesian Information Criterion). GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor; BIC scores, AICc value (Akaike Information Criterion, corrected) and Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented.

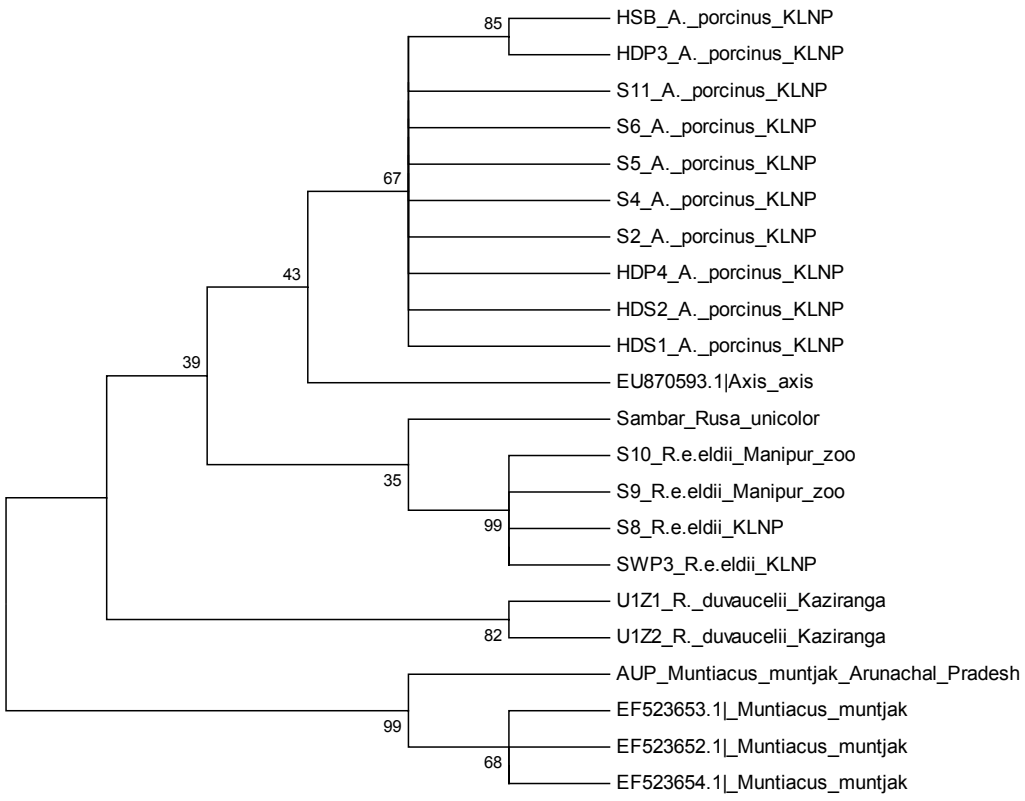


Figure 5.9 Cladogram of sangai with related cervids using Neighbour Joining (NJ) method, based on Tamura 3 parameter (T92) model. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best.

5.5 DISCUSSION

Phylogenetic relationship among Cervidae are of considerable interest because of the value of these species as wild and domestic animals and their suitability for the study of evolutionary patterns and processes (Cronin 1991). Groves & Grubb (1987) noted that the phylogenetic relationships of cervids are not well understood because previous systematic studies often were based on few morphological characters and limited numbers of taxa. Several

molecular investigations have been conducted on the cervidae. They involved mitochondrial and nuclear DNA or amino acid sequence comparisons including fibrinopeptides (Mross & Doolittle 1967), ribonucleases (Beintema et al. 1988), 12S and 16S ribosomal RNAs (Miyamoto et al. 1990, Kraus & Miyamoto 1991), cytochrome *b* (Irwin et al. 1991), k-casein (Cronin et al. 1996) and mitochondrial control-region (Douzery & Randi 1997). However, these studies did not use any sample from wild population of sangai and did not clearly define variability between wild and captive populations.

Mitochondrial DNA has several features rendering it particularly suitable for the analysis of phylogenetic relationships: high copy number, apparent lack of recombination, partially high substitution rate and maternal mode of inheritance (Arnason et al. 2002). To analyse the phylogenetic relationships among sangai deer and between wild and captive and with related cervids two molecular markers mtDNA gene were selected, first the cyt *b* gene because its tempo and mode of evolution is well understood, thought to be relatively constant and similar among large-bodied terrestrial mammals. The cyt *b* gene has been used in numerous studies of phylogenetic relationships among mammals and is the gene for which the most sequence information from different mammalian species is available (Johns & Avise 1998). The sequence variability of cyt *b* makes it most useful for the comparison of species in the same genus or family (Pitra et al. 2004).

The published sequences of cyt *b* gene of three subspecies of Eld's deer were very limited in the GenBank, the genetic relationship within the subspecies of Eld's deer could not be drawn. Therefore, a second marker,

control region (D-loop) gene was selected to construct the genetic relationship of Eld's deer and with related cervids. The control region in many species often evolves faster than the rest of the mitochondrial genome and also to be highly variable. This variability has lead to the expanding usage of control region to examine questions ranging in population structure or among closely related species level for resolving their phylogenetic relationships quite reliably (Ghivizzani et al. 1993, Douzery & Randi 1997).

In the present study the phylogeny of three subspecies of Eld's deer were analysed through mtDNA control region sequences showed monophyletic clades. The Eld's deer from Manipur showed a closest relationship with *R. e thamin* than to *R. e siamensis* from Thailand. The sequences of *R. e siamensis* from Thailand were interspersed with *R. e hainanus* from China forming one clade. This finding was consistent with the study of Balakrishnan et al. (2003). More studies using different classes of genetic markers and incorporation of more sample size from wild and captive population of all Eld's deer may reveal better understanding on its phylogenetics and for planning an effective conservation strategy.

Groves (2006), pointed out that the cervids in Asia have a complex phylogeny that is still in debate. The Eld's deer and barasingha may seem functionally similar, but represent different lines of cervids that have each adapted to seasonally-flooded dry forests (McShea et al. 2005). Based on analysis of mtDNA, Pitra et al. (2004) concluded that the genera within Asia are non-monophyletic, with species forming clades that do not reflect their current classification. For instance, the genetic relationship of sangai with

related cervids suggest that the sangai has close affinity with sambar and hog deer which is closely related to chital, were grouped together as monophyly. On the other hand, swamp deer *R. duvaucelii* which recently revised from *Cervus* to *Rucervus* (Wilson & Reeder 2005) appear closely related to *Muntiacus muntjak* than to Eld's deer. Pitra et al. (2004) and Gilbert et al. (2006) found similar patterns but had less resolution because of the limited number of samples of neotropical deer species in their phylogenetic analysis of old world deer.

The Cervidae first appeared as fossils in the early Miocene (about 25 mya) of Asia, where they expanded into a wide variety of niches and were thus able to dominate over the bovids which arrived later (Cap et al. 2002). During the Miocene, members of this family migrated to North America, after expanding in the Nearctic regions, they crossed to South America during the Pleistocene when the Panama land bridge formed (Cap et al. 2002). Early deer arose in the tropics, and the fossil record demonstrates repeated radiations from tropical climates into more northerly territories during the Pleistocene (Eisenberg 1981). Today, the tropics retained the majority of cervine diversity, although several cold-adapted species have become highly successful (Menon 2003). However, this study suggest that in terms of evolutionary origins, the subspecies *R. e. eldii* from Manipur appears to have diverged earlier than the *R. e. thamin* of Myanmar and *R. e siamensis* / *R. e hainanus* from Thailand and China.

5.6 SUMMARY

The phylogenetic variation in the mtDNA (cyt *b* and control region) genes were analysed for wild and captive populations of sangai and hog deer to understand the genetic relationship and variability among these species. No haplotype variations were detected in the 16 samples of sangai which were examined using mtDNA cyt *b* gene. However, two haplotypes were identified among the 17 individuals of sangai at nt282 position in mtDNA control region of Cerv.tPro / CervCRH gene. Both these variable positions were transition mutation (T→C). Incorporation of more sample size from blood and tissue from wild and captive population of sangai may reveal better understanding on its haplotype diversity and its phylogenetics.

The phylogeny of three subspecies of Eld's deer revealed monophyly. *R. e. eldii* from Manipur showed a closest relationship with *R. e. thamin* than to *R. e. siamensis* from Thailand. The sequences of *R. e. siamensis* from Thailand were interspersed with *R. e. hainanus* from Hainan Island, China. This shows that the status of *hainanus* needs a formal study to examine its accurate taxonomic position and relationship with other subspecies of Eld's deer. Most of the sources of the *siamensis* sequences are not known and only few sequences are available in Genbank and these sequences of *siamensis* showed variation among themselves. The mtDNA control region Cerv.tPro / CervCRH gene showed the populations of sangai were grouped into a single clade consisting of both captive and wild with a high bootstrap support (100%). This indicates that the captive and wild populations of sangai are not genetically divergent as initial experiment has shown. More studies using

different classes of genetic markers and incorporation of more sample size from wild and captive population of all Eld's deer subspecies may reveal better understanding on its phylogenetics and for planning an effective conservation implication.

The divergences times of three subspecies of Eld's deer were calculated with Neighbour joining (NJ) tree using MEGA 5. The results showed that *R. e. eldii* was much diverge earlier than *R. e. thamin* and *siamensis* / *hainanus i.e. R. e. eldii* > *R. e. thamin* > *R. e. hainanus* or *siamensis.* The range of divergence time between *R. e. eldii* and *R. e. thamin* was estimated at approximately 73683 years, while that of *R. e. thamin* and *R. e. siamensis* was approximately 51748 years and that of *R. e. siamensis* and *hainanus* was 18000 years. The genetic relationship of sangai with related cervids using control region gene has close affinity with sambar (*Rusa unicolor*) and hog deer (*A. porcinus*) which is closely related to chital (*A. axis*) were grouped together as monophyly. On the other hand, swamp deer *R. duvaucelii* which recently revised from *Cervus* to *Rucervus* appear closely related to *Muntiacus muntjak* than to Eld's deer. Nevertheless, it becomes difficult to comment on the phylogeny of sangai with related cervids correctly as the numbers of sequences in the databases on control region gene (D-loop) were very low.

Chapter 6

GENETIC DIVERSITY AND POPULATION STRUCTURE

6.1 INTRODUCTION

Genetic variation is both a trait of individuals and a trait of populations (Lacy 1997). Variation within individuals of diploid species is most commonly characterized by the percentage of loci at which an individual is heterozygous. Variation within populations also includes inter-individual variation, and often is quantified by the gene diversity (the heterozygosity expected under Hardy-Weinberg Equilibrium), by the number of distinct alleles per locus, or by the percentage of loci that are polymorphic (Nei 1973). Mean within-individual variation usually is highly correlated with populational (between-individual) variation and all measures of populational variation encompass within-individual variation in the population. Heterozygosity is depleted by inbreeding (mating between relatives), which leads to a greater probability of the two alleles at a locus being identical by descent from an ancestor common to both sides of the pedigree, and by genetic drift (random fluctuations in allele frequencies (Lacy 1997).

Genetic variation is necessary for evolution of populations in response to environmental change (Allendorf & Leary 1986). Consequently, maintenance of genetic variability in natural populations is an essential component of conservation efforts aimed at ensuring long-term persistence of wildlife populations (Frankham 1995). Genetic variation also influences

characteristics that determine the ability of populations to function over shorter time periods (Teska et al. 1990, Rhodes & Smith 1992). The genetic variability is directly assessed through molecular markers, which are heritable characteristics associated with identification and characterization of specific genotype (Avise 2004). The mtDNA has certain limitations as a genetic markers for estimating population genetic diversity (Zhang & Hewitt 2003) for instance, mtDNA represents only a single locus and reflects only the history of female lineages moreover, the effective population size of mtDNA is only a quarter of that of nuclear autosomal sequences. Thus, nuclear DNA polymorphisms are necessary to reveal the genetic diversity and population genetic structure from both sexes within populations (Zhang et al. 2008b).

Microsatellites markers have become the genetic markers of choice in many population genetic studies such as in population differentiation, genetic diversity and stock identification (Queller et al. 1993, Jarne & Lagoda 1996). Microsatellites loci consist of tandem arrays of di, tri or tetra nucleotide sequences and are distributed throughout the nuclear genome. These markers are also useful in assessing population structure and trends, patterns of dispersal, social organization, levels of inbreeding, population relationships, and in planning translocations (Beja-Pereira et al. 2004). Molecular tool development has allowed to obtain knowledge about the genome structure, and to characterize the genetic variation of natural population. Retention of genetic variation in populations of endangered species is a pre-requisite for their future survival. Genetic instability and decline can cause demographic instability and decline, and greater susceptibility to environmental fluctuations and catastrophes. Demographic fluctuations and catastrophe caused

bottlenecks can in turn cause more genetic instability and depletion of genetic variation (Soulé 1986). Moreover, the understanding of genetic population structure is very useful to take on adequate conservation measures.

In this chapter, the genetic diversity and population structure were investigated by employing 25 microsatellite DNA markers to index genetic variability and population structure of Eld's deer, with the objective to estimate the genetic variability of sangai within the species, between wild and captive populations and its sympatric ungulate, the hog deer *A. porcinus*. The chapter in itself was an attempt to answer the following research questions:

As we know, the sangai population had 14 founder individuals in 1975. There is apprehension that the sangai population might be going through a genetic bottleneck and be susceptible to inbreeding depression. What is the degree of genetic variability in the wild and captive population of sangai?

Considering the fact that the hog deer was once widely distributed from the Indus plains of Pakistan, along the Ganges and Brahmaputra plains, in the flood plains of the Irrawaddy and the lower Makong valley of Thailand and Cambodia, up to Vietnam, but recently its range has got fragmented, population has decreased in most places so much so that the species is listed under the endangered category. Is the sympatric hog deer also facing the same genetic loss?

6.2 NUCLEAR DNA (MICROSATELLITES) ANALYSIS

6.2.1 Collection of biological samples (same as Section 5.2.1)

6.2.2 DNA Extraction

The total genomic DNA was isolated from tissue samples of sangai and related cervids (hog deer and swamp deer) using DNeasy blood and tissue Kit (Qiagen) according to the manufacturer's recommendation. Antler and hair samples were extracted with QIA amp® DNA Micro kit (Qiagen) following the manufacturer's recommendation with the exception that 20 µl of 0.1% DTT (Dithiothretol) was added to the first incubation step in order to dissolve the hair and antlers to increase the DNA yield. Qiagen Stool Mini kit was used for extraction of DNA from faecal pellet samples following the manufacturer's protocol, with slight modification. All the extractions were verified with extraction negative controls.

6.2.3 PCR amplification and gel electrophoresis

The PCR amplification was multiplexed with fluorescently labeled 25 microsatellite markers for cervids: RT1, RT6, INRA011, AY302223, BM4208, Ca42, Cervid1, RT27, NVHRT16, NVHRT48, BM6507, OarFCB193, T123, T156, CelJP27, DR/F, T193, T108, T507, BM4107, MAF70, L23481, LSCV085, AF232760 and INRABERN185. The detail of primers source and annealing temperature for each locus is given in Table 6.1. The PCR profile consisted of an initial denaturation at 95 °C for 15 min followed by 45 cycles of denaturing at 94 °C for 30s , annealing at 50-60 °C for 45s, extension at 72 °C for 45s and a final extension 60 °C for 30 min. Negative control reactions were included in each PCR run. The PCR amplified products were separated on

2% agarose gel. Subsequently, the gel was stained with ethidium bromide for visualization of microsatellite loci and allelic pattern. The PCR was performed in three replicates at each locus for each sample.

Table 6.1 List of 25 microsatellite loci for genetic analysis.

Loci	AT*	Size	Dye	Species	Reference
RT1	54	222-240	VIC	Reindeer	Poetsch et al. 2001
RT6	50	93-108	PET	Red deer	Poetsch et al. 2001
BM4208	54	142-178	NED	White- tailed deer	Bishop et al. 1994
INRA011	54	257-277	VIC	White- tailed deer	Vaiman et al. 1992
AY302223	52	128-136	6-FAM	Chital	Gaur et al. 2003
Ca42	60	116-132	PET	Chital	Gaur et al. 2003
Cervid1	54	159-193	6-FAM	White- tailed deer	Dewoody et al. 1995
RT27	56	135-155	6-FAM	Reindeer, Red deer, Roe and Fallow deer	Poetsch et al. 2001
NVHRT16	54	152-192	PET	Reindeer, Red deer, Roe and Fallow deer	Poetsch et al. 2001
NVHRT48	52	105-115	NED	Reindeer, Red deer, Roe and Fallow deer	Poetsch et al. 2001
BM6506	54	184-200	PET	White- tailed deer	Bishop et al. 1994
OarFCB193	54	96-124	VIC	White- tailed deer	Buchanan & Crawford 1993
T123	58	146-178	6-FAM	Wapiti deer	Jones et al. 2002
T156	59	131-227	NED	Wapiti deer	Jones et al. 2002
CelJP27	59	176-196	VIC	Red deer	Marshall et al. 1998
D-F/R	54	150-190	NED	White- tailed deer	Jones et al. 2000
T193	59	172-238	PET	Wapiti deer	Jones et al. 2002
T108	58	131-179	6-FAM	Wapiti deer	Jones et al. 2002
T507	58	143-187	PET	Wapiti deer	Jones et al. 2002
BM4107	50	157-191	NED	Roe deer, Hainan Eld's deer	Bishop et al. 1994
MAF70	50	124-166	6-FAM	Fallow deer, Hainan Eld's deer	Buchanan & Crawford 1992
L23481	50	181-185	VIC	Hainan Eld's deer	Brezinsky et al. 1993
LSCV085	50	166	PET	Hainan Eld's deer	Zhang et al. 2005
AF232760	55	151-175	PET	Red deer	Moore et al. 1992
INRABERN185	52	244-250	VIC	Hainan Eld's deer	Zhang et al. 2005

(*AT- Annealing temperature)

6.3 GENOTYPING AND DATA ANALYSIS

6.3.1 Microsatellites genotyping

The DNA fragments were separated on an automated DNA Sequencer (Applied Biosystem). Different individuals were genotyped as either homozygote or heterozygote on the basis of band pattern, i.e the presence of one or two different alleles respectively, shown for each microsatellite loci. To control for allelic dropout (stochastic non amplification of one allele), three to four PCR replicates were performed according to the concentration of DNA in each sample (Morin et al. 2001). PCR and allele calling were repeated for samples which showed discrepancy in genotypes. The genotypes of each sample were scored as either a heterozygote or a homozygote if it showed consistent allelic pattern at all replicates. Allele frequencies, heterozygosities (observation and experimentation) were calculated and various other parameters of genetic variability were tested and analysed using GeneMapper v.3.7 software (Applied Biosystems).

6.3.2 Discrimination of individuals

In order to ensure that duplicate samples (i.e. sangai/ hog deer that were sampled more than once) were not included in the analysis, only unique multilocus genotypes were used. Unique genotypes were identified using the programs *CERVUS 3.0* (Kalinowski et al. 2007). Samples that had mismatches at up to two loci were re-examined for possible genotyping errors or allelic dropout (Kalinowski et al. 2006). The probability of identity statistic, P(ID), the probability that two different individuals will share the same

multilocus genotype at a given number of loci (Paetkau & Strobeck 1994) and a more conservative variant of P(ID), P(ID-sibs), the probability that a pair of siblings will share the same genotype (Waits et al. 2001) was calculated to ensure that the loci used could reliably discriminate related individuals.

6.3.3 Genetic diversity and Hardy-Weinberg Equilibrium

The observed and effective number of alleles, allele diversity (observed and expected heterozygosity) and percentage of polymorphic loci were computed using *CERVUS 3.0* (Kalinowski et al. 2007). Using allelic frequencies, polymorphic information content (PIC), a measure of marker's informativeness, was calculated with the *CERVUS 3.0* (Kalinowski et al. 2007). Deviations from the Hardy–Weinberg equilibrium (HWE) were conducted using the exact test of GENEPOP 1.2 software (Raymond & Rousset 1995).

6.3.4 Population structure analysis

Wright's F-statistics (Wright 1965), the parameters most widely used to describe population structure (Nagylaki 1998), were initially defined for a three-level hierarchical population structure (individuals, sub-populations and total). F_{ST} is a measure of the relatedness between individuals due to the structure of the population (i.e., non-random distribution of individuals among sub-populations); F_{ST} quantifies the differentiation between sub-populations in the total population (hence S and T). The F_{ST} estimations within the wild samples of sangai and between captive populations were calculated using the GENEPOP 1.2 software (Raymond & Rousset 1995).

6.3.5 Bottleneck detection

Signatures of bottleneck events were investigated by comparing the expected heterozygosity for a sample (H_E) with the heterozygosity that would be expected for a sample taken in a population at mutation-drift equilibrium with the same size and allele number (H_{EQ}) (Wichatitsky et al. 2009). As allele number decreases faster than heterozygosity, a bottleneck is signed by $H_E > H_{EQ}$ in subsequent generations (Cornuet & Luikart 1996). This analysis was performed with the BOTTLENECK 1.2.02 software (Piry et al. 1999) assuming that mutations of microsatellite loci followed either an IAM (Infinite Allele Model), a SMM (Stepwise Mutation Model), or a TPM (Two Phase Model), in the last case of which we assumed that 70% of mutations consist of one step and 30% consist of multistep change with a variance of 30 (default values). Tests were performed using three different methods of Sign test, Standardized differences test and Wilcoxon test (Cornuet & Luikart 1996). Global *p value*s, overall wild samples and overall captive samples of sangai, were obtained with the Fisher procedure (Fisher 1970).

Once all loci available in a sample have been processed, the three statistical tests were performed for each mutation model as explained in (Cornuet & Luikart 1996) and (Luikart et al. 1998) and the allele frequency distribution is established in order to see whether it is approximately L-shaped (as expected under mutation-drift equilibrium) or not (recent bottlenecks provoke a mode shift).

6.4 RESULTS

6.4.1 Screening of microsatellites loci

Of the 25 microsatellites screened, 21 were polymorphic, one was monomorphic and three were non-amplified. In captive sangai, 17 microsatellites were polymorphic, five was monomorphic and three were non-amplified. For the wild population of hog deer, 23 microsatellites were polymorphic, and two loci were non-amplified.

6.4.2 Genetic diversity of sangai in wild population

The number of alleles observed across the 22 loci for the wild population of sangai (n = 23) varied from one to four alleles, whereas the mean number of alleles per locus was 2.64 ±0.15. The average observed heterozygosity, H*o*, was estimated at 0.20 ±0.05 and expected heterozygosity (H*e*) was estimated at 0.38 ±0.04. The polymorphic information content value was 0.32 ±0.03. The result shows that heterozygosity is approximately 40% which indicates a low genetic variability in wild population (Table 6.2). When the Hardy Weinberg equilibrium was checked among 22 microsatellites, eight loci were significantly deviated from HWE which indicates the *p values* were much less than 5%. Remaining 14 loci (Table 6.2) did not significantly deviate from HWE and no significant linkage association was found among all these loci.

Table 6.2. Assessment of genetic diversity at 22 microsatellite loci of sangai in wild population.

Loci	No. of alleles	Size range	Heterozygosity		Cumulative Pid		*PIC*	HWE
			H*o*	**H*e***	**P (ID) ***	**P (ID-sibs) ****		
AY302223	3	194-212	1.000	0.531	0.346	0.576	0.405	NS
BM4208	3	148-164	0.458	0.614	0.226	0.506	0.534	NS
RT6	3	96-120	0.500	0.443	0.385	0.629	0.369	SD
INRA011	2	148-184	0.000	0.156	0.729	0.856	0.141	SD
RT1	3	200-208	0.000	0.358	0.450	0.687	0.322	NS
Ca42	3	176-188	0.083	0.230	0.613	0.790	0.212	SD
Cervid1	2	154-164	0.375	0.510	0.375	0.594	0.375	NS
RT27	3	130-146	0.042	0.520	0.350	0.583	0.399	NS
BM6506	2	200-206	0.042	0.488	0.386	0.607	0.364	NS
OarFCB193	3	94-132	0.042	0.598	0.238	0.516	0.520	NS
T123	3	142-158	0.083	0.160	0.717	0.851	0.150	SD
T156	3	166-182	0.042	0.082	0.846	0.921	0.079	SD
CelJP27	4	186-198	0.125	0.269	0.554	0.757	0.252	SD
D-F/R	4	164-186	0.542	0.756	0.116	0.408	0.692	NS
T193	3	168-228	0.292	0.600	0.244	0.517	0.514	NS
T108	3	128-178	0.000	0.587	0.274	0.531	0.482	NS
T507	2	178-182	0.125	0.361	0.481	0.693	0.291	SD
BM4107	2	152-158	0.087	0.294	0.549	0.744	0.246	SD
MAF70	2	128-132	0.208	0.311	0.529	0.730	0.258	NS
L23481	2	170-182	0.125	0.191	0.679	0.826	0.169	NS
AF232760	2	168-170	0.167	0.223	0.634	0.799	0.195	NS
INRABERN185	1	106	0.000	0.000	1.000	1.000	0.000	NS
	2.64 ±0.15		**0.20 ±0.05**	**0.38 ±0.04**			**0.32 ±0.03**	

H*o* - Observed heterozygosity, H*e* - Expected heterozygosity, *PIC* – Polymorphic information content, HWE – Hardy Weinberg Equilibrium, * Pid - Probability of identity; the probability that two different individuals will share the same multilocus genotype at a given number of loci **P(ID-sibs) - the probability that a pair of siblings will share the same genotype

6.4.3 Genetic diversity of sangai in captive population

When the genetic diversity of the wild population of sangai was compared with the captive population (n = 18 of which six samples from Manipur Zoo, four from Delhi Zoo and eight from Guwahati Zoo), the allele diversity estimates were found to be roughly half of the wild population indicating a much reduced genetic variation (Table 6.3). The number of alleles observed across the 22 loci in 18 individuals ranged from one to four alleles, whereas the mean number of alleles per locus was 2.09 ±0.19. The average observed heterozygosity, H*o*, was estimated at 0.08 ±0.02 and expected heterozygosity (H*e*) was estimated at 0.25 ±0.04. The *PIC* value was 0.20 ±0.03. All the genotypes were tested for deviations from Hardy–Weinberg Equilibrium (HWE), of the 22 microsatellites, most of the loci showed significant deviations from HWE that may be related to both the overlapping of generations and the strong founder event and subsequent inbreeding.

6.4.4 Genetic diversity of sympatric hog deer

Genetic variability in the sympatric hog deer (n = 27) present in the park was also examined. The number of alleles observed across the 23 loci for the hog deer population varied from two to five alleles, whereas the mean number of alleles per locus was 2.70 ±0.18. The average observed heterozygosity (H*o*) was estimated at 0.42 ±0.02 and expected heterozygosity (H*e*) was estimated at 0.51 ±0.03 and the mean *PIC* value was 0.43 ± 0.03 respectively. The heterozygosity is around 50% which showed a moderate genetic variation in wild population of hog deer of KLNP (Table 6.4). When the Hardy Weinberg equilibrium was checked, out of the 23 microsatellites, two loci, T193 and T507, deviated from HWE (both $p<0.001$), no deviation from HWE was observed in the remaining 21 variable loci.

Table 6.3 Assessment of genetic diversity at 22 microsatellite loci of sangai in captive population.

Loci	No. of alleles	Size range	Heterozygosity		Cumulative Pid		*PIC*	HWE
			H*o*	**H*e***	**P (ID)***	**P (ID-sibs) ****		
AY302223	4	194-212	0.500	0.694	0.173	0.456	0.607	NS
BM4208	2	158-164	0.111	0.356	0.487	0.699	0.286	SD
RT6	2	96-120	0.056	0.056	0.896	0.947	0.053	NS
INRA011	4	140-184	0.111	0.162	0.713	0.850	0.154	SD
RT1	1	206	0.000	0.000	1.000	1.000	0.000	NS
Ca42	2	176-188	0.000	0.108	0.806	0.900	0.099	SD
Cervid1	2	154-164	0.333	0.457	0.407	0.629	0.346	NS
RT27	2	130-144	0.000	0.203	0.663	0.817	0.178	SD
BM6506	2	200-206	0.111	0.489	0.388	0.609	0.362	SD
OarFCB193	2	104-132	0.000	0.286	0.560	0.751	0.239	SD
T123	3	148-178	0.111	0.298	0.525	0.736	0.269	SD
T156	2	166-182	0.000	0.108	0.806	0.899	0.099	SD
CelJP27	4	186-198	0.056	0.529	0.321	0.573	0.429	NS
DF/R	1	186	0.000	0.000	1.000	1.000	0.000	NS
T193	2	224-228	0.111	0.108	0.806	0.900	0.099	NS
T108	2	128-178	0.222	0.356	0.487	0.699	0.286	NS
T507	2	178-180	0.000	0.203	0.663	0.817	0.178	SD
BM4107	1	152	0.000	0.000	1.000	1.000	0.000	NS
MAF70	2	128-134	0.000	0.508	0.378	0.597	0.372	NS
L23481	2	164-170	0.000	0.508	0.378	0.597	0.372	NS
AF232760	1	170	0.000	0.000	1.000	1.000	0.000	NS
INRABERN185	1	106	0.000	0.000	1.000	1.000	0.000	NS
	2.09 ±0.19		**0.08 ±0.02**	**0.25 ±0.04**	**0.66**	**0.8**	**0.2 ±0.03**	

H*o* - Observed heterozygosity, H*e* - Expected heterozygosity, *PIC* – Polymorphic information content, HWE – Hardy Weinberg Equilibrium, * Pid - Probability of identity; the probability that two different individuals will share the same multilocus genotype at a given number of loci **P(ID-sibs) - the probability that a pair of siblings will share the same genotype

Table 6.4 Assessment of genetic diversity at 23 microsatellite loci of hog deer population in KLNP.

Loci	No. of alleles	Size range	Heterozygosity		Cumulative Pid		*PIC*	HWE
			H*o*	**H*e***	**P (ID) ***	**P (ID-sibs) ****		
AY302223	5	194-212	0.630	0.732	0.122	0.421	0.675	NS
BM4208	2	162-164	0.370	0.475	0.393	0.615	0.358	NS
RT6	3	98-104	0.407	0.603	0.232	0.512	0.526	NS
INRA011	3	212-220	0.444	0.561	0.304	0.550	0.449	NS
RT1	2	206-208	0.259	0.230	0.625	0.793	0.200	NS
Ca42	2	164-178	0.407	0.440	0.416	0.638	0.338	NS
Cervid1	4	144-168	0.556	0.711	0.146	0.437	0.642	NS
RT27	2	130-144	0.333	0.409	0.439	0.659	0.321	NS
NVHRT48	2	102-106	0.370	0.425	0.426	0.648	0.330	NS
BM6506	2	198-200	0.370	0.453	0.407	0.629	0.346	NS
OarFCB193	2	100-126	0.259	0.331	0.509	0.715	0.272	NS
T123	2	148-152	0.259	0.230	0.625	0.793	0.200	NS
T156	2	128-130	0.346	0.449	0.410	0.632	0.343	NS
CelJP27	4	154-164	0.519	0.718	0.145	0.434	0.647	NS
DF/R	3	154-190	0.500	0.675	0.188	0.466	0.588	NS
T193	3	176-186	0.407	0.532	0.307	0.566	0.443	SD
T108	2	128-154	0.556	0.509	0.375	0.593	0.375	NS
T507	3	174-186	0.444	0.654	0.202	0.479	0.568	SD
BM4107	3	152-190	0.333	0.509	0.300	0.575	0.449	NS
MAF70	3	124-128	0.444	0.643	0.208	0.486	0.559	NS
L23481	2	180-182	0.577	0.491	0.384	0.605	0.366	NS
AF232760	2	164-166	0.407	0.331	0.509	0.715	0.272	NS
INRABERN185	4	102-114	0.407	0.662	0.192	0.473	0.580	NS
	2.70 ±0.18		**0.42 ±0.02**	**0.51 ±0.03**	**0.34**	**0.58**	**0.43 ±0.03**	

H*o* - Observed heterozygosity, H*e* - Expected heterozygosity, *PIC* – Polymorphic information content, HWE – Hardy Weinberg Equilibrium, * Pid - Probability of identity; the probability that two different individuals will share the same multilocus genotype at a given number of loci **P(ID-sibs) - the probability that a pair of siblings will share the same genotype

6.5 F_{ST} BASED ANALYSIS OF POPULATION STRUCTURE

The differentiation between captive and wild sangai was measured and tested using sympatric samples (paired tests). A global result was given by the unweighted mean of the F_{ST} estimates computed among pairs of samples of captive and wild sangai and combining the *p value*s with a Fisher's procedure (Fisher 1970). The study involves four data subsets *viz.* KLNP, Manipur Zoo, Delhi Zoo and Guwahati Zoo populations. Parameters comparison between samples taken from captive and samples taken from the wild (KLNP) was made with the procedure ''Comparison among groups of samples'' of F_{ST}. Though many of the pair wise comparisons of F_{ST} were significant, F_{ST} values showed the same patterns of similarity between localities as the individual-based analyses (Nei 1973). The genetic differentiation among wild population of KLNP and Manipur Zoo (0.265) showed the lowest level genetic isolation probably the captive population of Manipur Zoo was preserving some of the rare alleles from wild. The dataset showed that the individuals from Delhi (0.2845) and Manipur Zoos (0.2829) had similar genetic makeup since both the Zoos obtained their founders from the wild *viz.* one pair of founders (1:1) were introduced from the wild to Delhi Zoo in 1962 (Singh 2004). However, the Delhi and Guwahati Zoos populations (0.5755) showed the highest levels of differentiation followed by Manipur and Guwahati Zoos populations (0.5321).

The F_{ST} values were very high for all the dataset, indicating high level of genetic differentiation in sangai populations, probably reflective of inherent skewness due to kinship patterns, as almost all captive individuals are descendent from few wild founders.

Table 6.5 F_{ST} analysis to estimate the genetic differentiation between datasets of sangai in wild and captive population.

Population (dataset)	KLNP	Manipur Zoo	Delhi Zoo
KLNP (N = 24)			
Manipur Zoo (N = 6)	0.265		
Delhi Zoo (N = 4)	0.2845	0.2829	
Guwahati Zoo (N = 8)	0.389	0.5321	0.5755

6.6 BOTTLENECK TESTS AND SIMULATION OF LOSS OF VARIABILITY

6.6.1 Bottleneck detection in wild sangai

For bottleneck estimation in wild population of sangai, Locus INRABERN 185 was eliminated because it was monomorphic, thus 21 loci were considered for analysis. Under the three mutation models in BOTTLENECK 1.2.02 (Piry et al. 1999), 16 of 21 loci showed significant heterozygosity excess. The overall, both Standardized Differences Test (p = 0.00003 for IAM; p = 0.0015 for TPM; p = 0.021 for (SMM) and Wilcoxon signed-rank tests showed evidence of a bottleneck (p = 0.00001 for IAM; p = 0.0003 for TPM; p = 0.004 for SMM). Significant deviations from mutation-drift equilibrium were detected in wild populations of sangai (p *values*<0.05). The total analysis indicated significant heterozygosity excess (H_E>H_{EQ}) 0.470 ±0.03>0.323 ±0.01 (under IAM), 0.470 ±0.03>0.37 ±0.02 (under TPM), 0.470 ±0.03>0.411 ±0.02 (under SMM) respectively (Table 6.6).

Both the IAM and the TPM models showed consistent results, with significant excess in heterozygosity, this was not unexpected, since the SMM is less sensitive than the IAM and TPM in detecting population bottlenecks

(Cornuet & Luikart 1996). Overall *p value*s were significant for all mutation models except the SMM for Sign test (Table 6.7). The allele frequency distribution were established, once all the 21 loci of the wild population sample of sangai have been processed and the three statistical tests were performed for each mutation model as explained in Cornuet & Luikart 1996, it showed recent bottleneck provoking a shifted mode distribution in wild population of sangai.

6.6.2 Bottleneck detection in captive sangai

For bottleneck analysis in captive population of sangai, of the 22 loci, 17 were polymorphic and remaining five was monomorphic, thus 17 loci were considered for the test. Bottleneck events were detected in the museum sample of sangai in Manipur Zoo (preserved in 1950's) ancestors of captive samples of sangai. The overall, Wilcoxon signed-rank tests showed evidence of a bottleneck p = 0.0007 for IAM; p = 0.013 for TPM; p = 0.087 for SMM) (Table 6.9). The *p values* represent probabilities of deviation from the mutation-drift equilibrium obtained with the Wilcoxon unilateral testing (alternative hypothesis of mutation-drift equilibrium defined as $H_E > H_{EQ}$. The overall *p value*s were obtained by the Fisher procedure to combine independent tests. The global analysis indicated significant heterozygosity excess ($H_E > H_{EQ}$) 0.403 ±0.03>0.291 ±0.02 (under IAM), 0.403 ±0.03>0.325 ±0.03 (under TPM), 0.403 ±0.03>0.35 ±0.03 (under SMM) respectively (Table 6.8). The Standardized Differences Test could not be performed since it requires at least 20 polymorphic loci (Cornuet & Luikart 1996). The observed allelic frequency distribution showed a shifted mode due to existence of bottlenecks population in the founder events.

Table 6.6 Detection of recent bottlenecking of sangai in wild population assuming different mutation models.

WILD SANGAI							
Loci	**Observed**	**IAM**		**TPM**		**SMM**	
	He	**He*q***	***Prob***	**He*q***	***Prob***	**He*q***	***Prob***
AY302223	0.531	0.371	0.214	0.431	0.321	0.488	0.442
BM4208	0.614	0.372	0.06	0.431	0.104	0.479	0.158
RT6	0.443	0.377	0.434	0.432	0.456	0.488	0.305
INRA011	0.191	0.217	0.486	0.247	0.476	0.259	0.435
RT1	0.465	0.374	0.388	0.434	0.494	0.48	0.37
Ca42	0.55	0.362	0.15	0.427	0.248	0.488	0.373
Cervid1	0.51	0.222	0.026	0.243	0.031	0.262	0.039
RT27	0.52	0.365	0.244	0.429	0.363	0.489	0.503
BM6506	0.488	0.214	0.089	0.254	0.131	0.264	0.139
OarFCB193	0.614	0.374	0.061	0.425	0.097	0.483	0.154
T123	0.585	0.369	0.099	0.427	0.15	0.48	0.241
T156	0.585	0.371	0.099	0.427	0.16	0.489	0.241
CelJP27	0.676	0.483	0.096	0.55	0.158	0.615	0.326
DF/R	0.756	0.49	0	0.54	0.002	0.613	0.012
T193	0.6	0.373	0.079	0.431	0.116	0.479	0.173
T108	0.587	0.363	0.102	0.429	0.158	0.49	0.24
T507	0.361	0.213	0.25	0.231	0.297	0.258	0.35
BM4107	0.162	0.218	0.53	0.262	0.395	0.266	0.389
MAF70	0.191	0.225	0.503	0.241	0.491	0.26	0.435
L23481	0.254	0.215	0.392	0.244	0.468	0.255	0.495
AF232760	0.191	0.219	0.499	0.243	0.55	0.258	0.43
	0.470 ±0.03	**0.323 ±0.01**		**0.37 ±0.02**		**0.411 ±0.02**	

He - Expected heterozygosity, He*q* - Heterozygosity equilibrium, *Prob* – probability, IAM - Infinite Allele Model, TPM - Two Phase Model, SMM - Stepwise Mutation Model.

Table 6.7 Bottleneck detection of sangai in wild population indicating overall *p* values were significant for IAM and TPM mutation models.

Mutation models	Heterozygosity excess	Heterozygosity deficiency	*p values*		
			IAM	TPM	SMM
SIGN TEST	17	4	0.0031	0.0089	0.192
STANDARDIZED DIFFERENCES TEST	17	4	0.00003	0.0015	0.021
WILCOXON TEST	14	7	0.00001	0.0003	0.004

IAM - Infinite Allele Model, TPM - Two Phase Model, SMM - Stepwise Mutation Model.

Table 6.8 Bottleneck detection of sangai in captive population assuming different mutation models.

Loci	CAPTIVE SANGAI						
	Observed	IAM		TPM		SMM	
	He	H*eq*	*Prob*	H*eq*	*Prob*	H*eq*	*Prob*
AY302223	0.694	0.508	0.091	0.573	0.171	0.623	0.26
BM4208	0.356	0.235	0.303	0.255	0.352	0.273	0.396
RT6	0.246	0.232	0.456	0.255	0.514	0.278	0.494
INRA011	0.5	0.506	0.422	0.571	0.236	0.623	0.113
Ca42	0.457	0.241	0.188	0.27	0.24	0.273	0.223
Cervid1	0.457	0.232	0.169	0.265	0.208	0.283	0.245
RT27	0.322	0.229	0.341	0.26	0.41	0.286	0.484
BM6506	0.489	0.235	0.107	0.26	0.137	0.269	0.161
OarFCB193	0.286	0.231	0.391	0.255	0.459	0.276	0.488
T123	0.451	0.395	0.461	0.443	0.427	0.498	0.297
T156	0.246	0.227	0.43	0.253	0.501	0.278	0.563
CelJP27	0.529	0.517	0.463	0.571	0.298	0.622	0.162
T193	0.246	0.233	0.441	0.274	0.562	0.274	0.564
T108	0.356	0.227	0.283	0.257	0.361	0.276	0.387
T507	0.203	0.235	0.51	0.254	0.492	0.276	0.438
MAF70	0.508	0.237	0.069	0.263	0.074	0.274	0.083
L23481	0.508	0.238	0.071	0.254	0.081	0.276	0.085
	0.403 ±0.03	**0.291 ±0.02**		**0.325 ±0.03**		**0.35 ±0.03**	

H*e* - Expected heterozygosity, H*eq* - Heterozygosity equilibrium, *Prob* – probability, IAM - Infinite Allele Model, TPM - Two Phase Model, SMM - Stepwise Mutation Model.

Table 6.9 Bottleneck detection of sangai in captive population indicating *p* values represent probabilities of deviation from the mutation-drift equilibrium obtained with the Wilcoxon unilateral test.

Mutation models	Heterozygosity excess	Heterozygosity deficiency	*p* values		
			IAM	TPM	SMM
SIGN TEST	15	2	0.00056	0.144	0.391
STANDARDIZED DIFFERENCES TEST	11	6	No test	No test	No test
WILCOXON TEST	10	7	0.00007	0.013	0.087

IAM - Infinite Allele Model, TPM - Two Phase Model, SMM - Stepwise Mutation Model.

6.6.3 Non bottleneck detection in wild population of hog deer

Twenty three loci were found to be polymorphic in wild population of hog deer and only seven loci showed significant heterozygosity excess (Table 6.10 and 6.11). The three statistical tests were performed for each mutation model (Cornuet & Luikart (1996) and (Luikart et al. 1998), the overall, Wilcoxon test showed no evidence of a bottleneck in the past (p = 0.894 for IAM; p = 0.993 for TPM; p = 0.999for SMM) (Table 6.11). The global analysis indicated no significant heterozygosity excess ($H_E<H_{EQ}$) 0.252 ±0.025<0.284 ±0.018 (under IAM), 0.252 ±0.025<0.323 ±0.021 (under TPM), 0.252 ±0.025<0.359 ±0.026 (under SMM) respectively (Table 6.9). The observed allelic distribution revealed that the hog deer population does not encounter a genetic bottleneck in the recent past. The allele frequency distribution showed approximately L-shaped (as expected under mutation-drift equilibrium). Although the hog deer population has been gradually declining in the recent years, the population has been found retaining some of its rare alleles and 50% genetic diversity in the wild.

Table 6.10 Non-bottleneck detection of hog deer in KLNP indicating (H_E<H_{EQ}) mutation-drift equilibrium obtained with three mutation models.

Loci	HOG DEER						
	Observed	IAM		TPM		SMM	
	He	Heq	Prob	Heq	Prob	Heq	Prob
AY302223	0.207	0.362	0.27	0.421	0.14	0.484	0.055
BM4208	0.331	0.211	0.298	0.24	0.358	0.265	0.402
RT6	0.268	0.353	0.383	0.427	0.2	0.488	0.088
INRA011	0.234	0.368	0.295	0.406	0.182	0.488	0.063
RT1	0.14	0.22	0.481	0.231	0.427	0.264	0.345
Ca42	0.171	0.215	0.511	0.238	0.461	0.243	0.45
Cervid1	0.326	0.48	0.212	0.542	0.085	0.606	0.024
RT27	0.171	0.214	0.514	0.242	0.466	0.249	0.432
NVHRT48	0.171	0.22	0.531	0.237	0.474	0.26	0.405
BM6506	0.073	0.225	0.33	0.251	0.262	0.251	0.235
OarFCB193	0.257	0.22	0.395	0.239	0.445	0.249	0.48
T123	0.23	0.205	0.397	0.234	0.486	0.249	0.518
T156	0.382	0.221	0.246	0.243	0.286	0.261	0.338
CelJP27	0.205	0.361	0.255	0.419	0.136	0.482	0.046
D-F/R	0.336	0.366	0.447	0.423	0.319	0.485	0.157
T193	0.297	0.357	0.414	0.426	0.235	0.481	0.127
T108	0.107	0.202	0.476	0.241	0.354	0.257	0.309
T507	0.532	0.361	0.207	0.423	0.281	0.476	0.396
BM4107	0.208	0.372	0.275	0.424	0.136	0.485	0.047
MAF70	0.507	0.361	0.284	0.42	0.402	0.478	0.514
L23481	0.292	0.211	0.335	0.235	0.402	0.262	0.468
AF232760	0.307	0.208	0.311	0.239	0.376	0.253	0.422
INRABERN185	0.037	0.212	0.232	0.235	0.175	0.252	0.17
	0.252 ±0.025	**0.284 ±0.018**		**0.323 ±0.021**		**0.359 ±0.026**	

He - Expected heterozygosity, H*eq* - Heterozygosity equilibrium, *Prob* – probability, IAM - Infinite Allele Model, TPM - Two Phase Model, SMM - Stepwise Mutation Model.

Table 6.11 Detection of non bottlenecking in hog deer population at KLNP indicating *p value*s greater than 5% (*p values*>0.05) from the mutation-drift equilibrium obtained with the three test.

Mutation models	Heterozygosity excess	Heterozygosity deficiency	*p* values		
			IAM	TPM	SMM
SIGN TEST	8	15	0.176	0.043	0.020
STANDARDIZED DIFFERENCES TEST	7	16	0.185	0.014	0.00006
WILCOXON TEST	7	16	0.894	0.993	0.999

IAM - Infinite Allele Model, TPM - Two Phase Model, SMM - Stepwise Mutation Model.

6.7 DISCUSSION

The analysis reveals that in KLNP sangai gene pools are affected by habitat fragmentation. Microsatellite DNA analysis revealed a reduced genetic variability in captive sangai as compared to the wild population however the sympatric hog deer maintaining a moderate genetic variability in KLNP. The allelic profile of sangai showed reduction of genetic diversity approximately 40% in wild indicating low genetic variability and a significant loss of genetic diversity in captive population due to inbreeding depression in captivity. Similar studies conducted by Zhang et al. (2005) revealed that the subspecies of *R. e. thamin* and *R. e. siamensis* still harbors a substantial amount of genetic diversity compared to *R. e. hainanus* which is known to have suffered a severe genetic bottleneck and whose current population was founded by about 20 individuals.

On the similar lines, Zhang et al. (2008a) employed 10 microsatellite DNA loci to index genetic variation in one source (Datian) and two introduced populations of Hainan's Eld's deer (Bangxi and Ganshiling), the source population harbored all 40 alleles, while the Bangxi and Ganshiling translocated populations contained 24 and 26 alleles, respectively. The genetic variability was low for each of the three populations. The results suggest that founder effects and genetic drift have affected the two translocated populations and it recommends the three populations be managed as a meta-population for conservation.

In another study Zeng et al. (2007) revealed that genetic diversity was extremely low in Pére David's deer (*E. davidianus*) populations in China and suggests that effective management of a species of low genetic diversity should consider the genetic background of each founder to make sure genetic variations are preserved in both source population and relocated population.

The sangai population has been occupying a distinct place in 14 Zoos *viz.* New Delhi, Guwahati, Punjab, Mysore, Kolkata, Patna, Hyderabad, Ahmedabad, Guwahati, Kanpur, Lucknow, Bhillai and Nandankanan Zoo. The largest number of sangai in captivity is found in the National Zoological Park in New Delhi, which hosts approximately 60 individuals. The captive sangai are from two lineages of a pair each from Kolkata (1:1, 1956) and Delhi (1:1, 1962) transferred from the wild in KLNP (Singh 2004). The population growth of its two lineages at present is very encouraging. Franklin's (1980), experience with captive animals suggests that isolated populations should have at least 50 breeding individuals and preferably 500 individuals to

maintain genetic variability. Lande (1995) concluded that "effective population size" will need to be on the order of 5,000, rather than 500, to ensure long-term viability. In future translocations, the founder population structure should be of particular concern. To establish a viable population, a minimum of 15–30 genetically effective founders is recommended (Frankham et al. 2002) and that the founder population should be greater than the original founder individuals.

The sangai population indicated high F_{ST} values (Wright 1965) showing high level of genetic differentiation in both captive and wild populations probably reflective of inherent skewness due to kinship patterns, as almost all captive individuals are descendent from few wild founders. The captive populations of sangai are from two lineages of a pair (Alipore Zoo, Kolkata 1:1 1956) and (Delhi Zoo 1:1 1962) transferred from the wild. The Delhi and Guwahati Zoo populations showed the highest levels of differentiation. The differentiation between the Guwahati and Kolkata Zoo population requires a formal study, since in the present study there were no samples from Alipore Zoo, Kolkata Zoo, hence level of genetic differentiation between them could not be drawn. The genetic differentiation among wild population of KLNP and Manipur Zoo showed the lowest level of genetic isolation probably the captive population of Manipur were retaining some of the rare alleles from wild. The Delhi and Manipur Zoos individuals showed similar genetic makeup since the Delhi Zoo obtain their founder from the wild *viz.* one pair of founders (1:1) were introduced from the wild in 1962 (Singh 2004). However the Delhi and Guwahati Zoos populations showed the highest levels of differentiation followed by Manipur and Guwahati Zoos populations. The F_{ST} values were

very high for all the dataset, indicating high level of genetic differentiation in sangai populations, probably reflective of inherent skewness due to kinship patterns as almost all captive individuals are descendent from few wild founders.

Bottleneck detection is critical for the interpretation of historical demography of populations and can be a valuable tool for endangered species management. Our data indicate that rapid growth of a population after a severe bottleneck can yield significantly reduced genetic diversity but no signature of a bottleneck within a relatively short time. That effect may be exacerbated by addition of rare alleles to the population through mutation or immigration (Busch et al. 2007). Cornuet & Luikart (1996) and Luikart et al. (1998) proposed three tests for detecting recent population bottlenecks (severe reductions in population size) from microsatellite allele frequencies. These tests are based on the fact that populations that have experienced a recent reduction in effective population size exhibit a more rapid reduction of allelic diversity than heterozygosity (i.e. gene diversity, H*e*) at polymorphic loci. Hence, in a recently bottlenecked population, gene diversity is higher than the equilibrium heterozygosity (H*eq*) estimated from the observed allele numbers under the assumption of mutation drift equilibrium (Luikart & Cornuet 1998).

The present study examines the recent reductions in population size using the generalized stepwise mutation model that predict a shifted mode in the allelic distribution experiencing a recent bottleneck in the past population of wild as well as ancestors of captive population of sangai. The sangai

population has experienced a recent genetic bottlenecking due to founder events and genetic drift since historical records also supported the population that has recovered from only 14 individuals in 1975 (Ranjitsinh 1975) to approximately 80 individuals now (Angom & Hussain unpublished) in the Park. The consequences of bottlenecks may include the loss of rare alleles, followed by the reduction in the mean number of alleles and heterozygosity, the fixation of deleterious alleles, and potentially inbreeding depression. However, although the reduction of genetic diversity has been demonstrated by molecular data for several deer populations that experienced founder effects (Broders et al. 1999, Webley et al. 2004, 2007) other instances such as the white-tailed deer reintroduction in Mississippi (De-Young et al. 2003) were not associated with a significant reduction in genetic diversity.

6.8 SUMMARY

Genetic variation was quantified in 41 samples of sangai representing wild (n=23) and captive (n=18) of which six from Manipur Zoo, four from Delhi Zoo and eight samples from Guwahati Zoo populations. Twenty-seven samples of hog deer from KLNP were also genotyped to assess the allelic composition and variability among the species and with sympatric sangai population. Twenty-five microsatellite loci were screened, 21 were polymorphic, one was monomorphic and three were non-amplified of the collected wild sample of sangai. In captive sangai, 17 microsatellite loci were polymorphic, five was monomorphic and three were non-amplified to the collected sample. However, in hog deer population, 23 microsatellites were polymorphic, and two loci were non-amplified to the collected sample.

The genetic diversity estimates of wild sangai calculated using *CERVUS 3.0* revealed mean numbers of allele at 22 loci were 2.64 ±0.15, observed heterozygosity (H*o*) was 0.20 ±0.05, expected heterozygosity (H*e*) was 0.38 ± .04 and *PIC* was 0.32 ±0.03 respectively. The genetic variability of captive sangai at 22 loci showed mean numbers of allele were 2.09 ±0.19, observed heterozygosity (H*o*) was 0.08 ±0.02, expected heterozygosity (H*e*) was 0.25 ±0.04 and *PIC* was 0.20 ±0.03 respectively. However, the diversity estimates of hog deer revealed mean numbers of allele at 23 loci were 2.70 ±0.18, observed heterozygosity (H*o*) was 0.42 ±0.02, expected heterozygosity (H*e*) was 0.51 ± 0.03 and *PIC* was 0.43 ±0.03 respectively. The allelic diversity of sangai showed reduction of genetic diversity approximately 40% in wild indicative low genetic variability and a significant loss of genetic diversity in captive population due to inbreeding depression in captivity. However the sympatric hog deer showed a moderate genetic variation around 50% at KLNP.

The F_{ST} estimations within the wild samples of sangai and between captive populations were calculated with GENEPOP 1.2 software using pair wise analysis. Parameters comparison between four data subsets *viz.* KLNP, Manipur Zoo, Delhi Zoo and Guwahati Zoo populations were made with the procedure ‘‘Comparison among groups of samples’’ of F_{ST}. The F_{ST} analysis showed genetic differentiation among wild population of KLNP and Manipur Zoo (0.265) were lowest probably the captive population of Manipur were preserving some of the rare and significant alleles from wild population. The Delhi (0.2845) and Manipur Zoos (0.2829) individuals having similar genetic makeup since both the Zoos obtain their founders from the wild. However the

Delhi and Guwahati Zoos populations (0.5755) showed the highest levels of differentiation followed by Manipur and Guwahati Zoos populations (0.5321). The F_{ST} values were very high for all the dataset, indicated high level of genetic differentiation in sangai populations, probably reflective of inherent skewness due to kinship patterns as almost all captive individuals are descendent from few wild founders.

Evidence for a genetic bottleneck was evaluated using software BOTTLENECK 1.2.02 which assumed that a signature of a severe reduction in effective size of a population was an excess of H_E relative to H*eq*. For bottleneck estimation in wild population of sangai, the allele frequency distribution showed a recent bottleneck provoking a shifted mode in wild population of sangai. Bottleneck events were detected in the ancestors of captive samples of sangai. The observed allelic frequency distribution showed a shifted mode characteristics due to existence of bottlenecks population in the founder events. Although the hog deer population has declined in the recent years the population has been retaining some of its rare alleles and 50% genetic diversity in the wild. The observed allelic distribution revealed the hog deer population does not encounter a genetic bottleneck in the recent past. The allele frequency distribution showed approximately L-shaped (as expected under mutation-drift equilibrium).

Chapter 7

SYNTHESIS AND CONSERVATION IMPLICATION

7.1 INTRODUCTION

Global extinction of species, driven by anthropogenic factors, is occurring at an unprecedented rate (Pimm et al. 2001, Cardillo et al. 2006, Isaac et al. 2007, Morrison et al. 2007). Around 52% of all the threatened mammalian species included in the IUCN Red List of Threatened Species, have shown declining population trends (Hoffmann et al. 2010). These trends indicate that the overall conservation status of mammals is likely to deteriorate further in the near future, unless appropriate conservation actions are devised. The situation is particularly serious for land mammals in South and South East Asia as a result of the combined effects of overharvesting and habitat loss (Schipper et al. 2008). Conservation success will depend on identifying vulnerable species and understanding factors that support their persistence, particularly in human-dominated landscapes such as South Asia (Karanth et al. 2010).

The purpose of the present study was to improve upon the existing ecological and genetic knowledge base of the severely fragmented, small and isolated population of Eld's deer in the Keibul Lamjao National Park, Manipur, India, so as to develop appropriate measures for the conservation of this species and related cervids. Species having small population size are subject to a higher chance of extinction as their small population size makes them

vulnerable to genetic drift, and inbreeding within populations can further decrease individual variability. Since many habitats have been severely reduced and fragmented, some species may have dispersal behaviors that are no longer optimal for the landscapes in which they find themselves, or they may simply be unable to avoid inbreeding. Inbreeding has been observed to cause higher mortality, lower fecundity, reduced mating ability, slower growth, developmental instability, more frequent developmental defects, greater susceptibility to disease, low ability to withstand stress, and reduced intra and inter-specific competitive ability (Allendorf & Leary 1986, Wright 1977, Ralls et al. 1988).

The long term genetic risks to small and isolated populations are more strongly determined by random genetic drift than by natural selection except when selection is strong (Kimura 1983, Lacy 1987). Therefore, deleterious mutations occasionally become fixed in a small population, due to chance drift, replacing more adaptive alleles. As deleterious mutations accumulate, population size may decrease, causing genetic drift to become even more rapid. This feedback has been termed mutational meltdown (Lacy 1997). The time course of mutational meltdown is on the order of hundreds of generations, however, so it would not be a significant contributor to recent and rapid decline of populations (Lacy 1987). More research is needed to determine if, and under what circumstances, populations could be purged of their genetic loads, but data does not yet allow us to presume that any mammalian population will emerge from a bottleneck with constant or recovered fitness and a greater ability to withstand future inbreeding (Hedrick 1994).

Keeping in view the above mentioned literature survey the following synthesis has been made based on the findings of the study for effective conservation of sangai and associated cevids in the Keibul Lamjao National Park and also in captivity.

7.2 DEMOGRAPHIC STATUS OF SANGAI AND HOG DEER

A critical look at the population growth rate of sangai suggested that during 1975-2003 the population growth rate was 10% per annum (Hussain et al. 2006). The population growth during 1984-2003 was 5% per annum. The results of this study (2006 - 2008) indicate that the population of sangai in the Park is more or less stable. This indicates that the population growth rate was higher during 1975 - 2003 and now it has reached an asymptote. There could be several factors that are affecting the population growth rate. Recent studies have suggested that the population dynamics of large herbivores can be strongly affected by a combination of stochastic environmental variations and density dependence (Gaillard et al. 1998). Food resources, habitat quality, weather, disease and parasites, interspecific competition, predation, human activities and population density can account for the demographic variation within a population. The sangai population being small and highly fragmented is subject to a higher chance of extinction because they are more vulnerable to inbreeding depression and genetic drift, resulting in stochastic variation in their gene pool, their demography and their environment. In addition, the long term viability of small populations can impact on population persistence *viz.* lower the fecundity and survival of inbred individuals within a

population, will depress population growth rate, which in turn has contributed to accelerated rates of extinction and reduction in genetic load.

Apparently the following ecological factors are affecting the demographic structure of the sangai in the Park (a) deteriorating habitat condition i.e. thinning of *phumdi* (b) lack of connectivity for recolonization (c) poaching and incidental mortalities (d) increased probability of disease and mortalities. These factors have been discussed below in the light of present study.

7.2.1 Deteriorating habitat condition

The habitat in the Park is deteriorating primarily because of the change in water regime due to construction of the Ithai barrage (Shamungou 1998, Hussain et al. 2006, WII 2009). Due to increased water level, the *phumdi*, which used to settle during lean seasons and get replenished with soil and nourishment, are now continuously floating, resulting in their thinning. Consequently, they are increasingly becoming defunct in supporting the weight of the deer. Besides, the release of water from the Ithai barrage causes drifting of *phumdi* towards north due to water current (Singsit 2003). This reduces the availability of thick *phumdi* to the sangai. However, it is worthwhile to note that the sangai is not entirely dependent upon the *phumdi* for their survival. In fact the ideal home of sangai would be a combination of the *phumdi*, the hard ground pastures and the shallow water bodies (Panwar 1979). However, such habitat in the Park is very limited (WII 2009) owing to geophysical characteristics of the area. During the present assessment it was observed that only 22 ±0.35 km^2 area is available to sangai. Probably this

limits the population growth. Besides, frequent fire in the Park and controlled burning as a management strategy reduces the building materials for *phumdi* leading to its thinning. The huge amount of the pollutants brought in by the rivers, particularly Nambul and Nambol causing further degradation and make the Loktak Lake including the Park more polluted which needs urgent attention (WII 2009).

The Park is surrounded by 36 villages within a 3 km radius from the periphery. There are 7 villages in the north, 10 villages in the west, 9 villages in the south and 10 villages in the east. Despite its protected status, the Park is under enormous pressure from the biomass demands of the people living in these villages. There are no strict regulations from the Park authorities owing to lack of staff and there is no clear cut demarcation of boundary or fencing. Moreover, after the commissioning of the Loktak Hydro-Power Project in 1983, large agricultural areas at the Lake periphery have been submerged which have changed the economic life of the people making them more dependent on the Park. This coupled with the increasing population has caused serious stress to the habitat of sangai, and other wild animals in the Park.

7.2.2 Lack of connectivity for recolonization

One of the primary impacts of many human activities is habitat fragmentation; that is, human use of the landscape creates habitat ‘‘islands’’, and the species within them often have little or no genetic contact with conspecific populations inhabiting other such islands. The Keibul Lamjao

National Park and its primary inhabitants the sangai are classic example. Population genetic theory and experiments predict that most fragmentation events caused by human activities facilitate local extinction (Templeton et al. 2001). As the existing sangai population is growing within a restricted area without any scope for recolonization into the sinks, the population is subjected to density dependant factors and genetic drifts. The Keibul Lamjao National Park is now completely cut off from the surrounding wetlands, particularly to its east such as Khoidum Pat, Loushi Pat and Ikop Pat (Figure 7.1). The entire Park is surrounded by agricultural land and fish ponds which have left no room for the sangai to recolonize in the adjacent areas. Hence it is crucial to identify the connectivity of the Park to the surrounding landscape and to restore such connectivities.

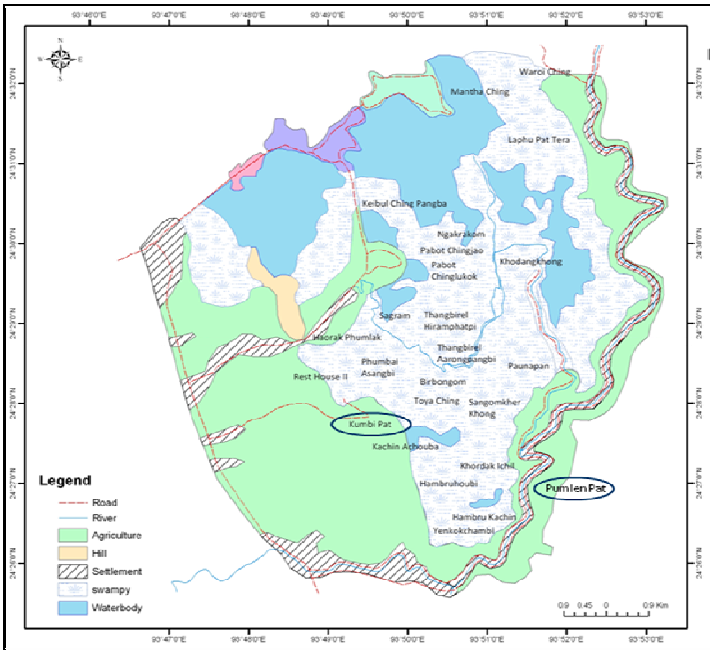


Figure 7.1 Adjacent area of KLNP showing Kumbipat and Pumlenpat for extension where evidences of presence of sangai was found during the study.

The deterioration of habitat in the Park is happening due to thinning of *phumdi*. It is of utmost importance that the liaisons with Loktak Development Authority strengthen so as to find an amicable solution to reduce the water level of the Loktak Lake. Simultaneously, monitoring of the extent of thick phumdi should be carried out. The three hills and the strip of dry land are most important habitat for sangai and these need to kept disturbance free at least during the dry season and early floods. Burning of these strips of lands needs to be stopped.

7.2.3 Poaching and incidental mortalities

The Keibul Lamjao National Park being situated at the border of insurgence prone areas of India and Myanmar, poaching of wildlife is a known issue. The sangai is listed in Schedule I of the Indian Wildlife (Protection) Act, 1972 and considered as endangered by IUCN. The survival of sangai is threatened primarily by poaching. Sangai used to be hunted under a permit by the Manipur State Durbar till early 1900's. It was only in 1934 that the hunting of sangai was stopped. In 1975, Ranjitsinh reported poaching in the Park, the poachers used dogs, and trap. In spite of stringent protection measures poaching in the KLNP is a threat to the existence of the deer. Conservation measures for wildlife were tightened considerably with the introduction of the Wildlife (Protection) Act, 1972 and Manipur Wildlife Protection Rules (1974) under section 64 within state (Shamungou, 1998). In spite of such efforts usually 3 or 4 animals were poached in the Park (as reported during 1983-85). During the present study though cases of poaching was not observed, indirect evidences of poaching such as presence of snares were frequently observed.

7.2.4 Increased probability of disease and mortalities

With increasing number of sangai and hog deer there are frequent reports of mortalities of both the species in the Park (WII 2009). This could be due to the fact that perhaps the density dependent factors are affecting the population. Brucellosis and Tuberculosis are common disease of the livestock in the area that might have contaminated the population (Plate 7). Large scale mortalities such as during flood in 1992 were also observed which led to 20% decline in the population (WII 2009). Due to the absence of a predator in the Park, the wild boar has becomes very ferocious, though not observed directly, the local people and the forest guards of KLNP informed that the wild boar also predates upon newly born fawns and attacks the juvenile of sangai and hog deer. The evidences of poaching and mortality due to disease could be other factors affecting the population. Appropriate protection strategy and disease monitoring plan needs to be developed and implemented to reduce the mortality of sangai and hog deer due to these factors. Very limited control exists at the eastern side of the Park. Improvement in protection measures in the Park and control of grazing in the peripheral villages is required. Social fencing can be created by involving local communities in protection strategy. Increase in front line staff strength and their deployment in the eastern side and appointment of local watchers from the eastern side villages will enhance protection.

(a)

(b)

Plate 7. Mortalities of hog deer in the Park (a) due to snares (b) disease.

During this study it was estimated that less than 100 mature sangai exist in the KLNP. As stated earlier the species is protected under the Schedule I of the Indian Wildlife Protection Act (1972). The IUCN has listed the Eld's deer as 'Endangered'. The resultant categorization as 'Endangered' for the species has compromised the status of some sub species *viz.* the sangai which is geographically isolated, distinct and have drastically low population numbers. Threats posed for this subspecies include habitat degradation, developmental process; human encroachments and low genetic diversity which all have led sangai to a high degree of isolation. In the absence of adequate information and extinction proneness of some subspecies, there is a need to reconstruct the threat status of certain subspecies especially the sangai by upgrading it to 'Critically Endangered' under the IUCN Red list category. During the study, it was observed that the hog deer population in Park is also showing a declining trend. This indicates that the major threats experienced by sangai and hog deer are similar, largely due to habitat degradation and poaching and these needs to be addressed in an integrated manner.

During the study, it was observed that the hog deer population in Park is also declining. This indicates that the major threats experienced by sangai and hog deer are similar, largely due to habitat degradation and poaching. By virtue of its population characteristics there is need to have a second home for sangai which was mooted in 1986 by Rodgers & Panwar (1986). The relocation and reintroduction efforts need to be carried out after careful examination of the sites along with long term scientific research and monitoring. The species needs to be introduced in more or less similar habitat

in wild through conservation breeding programme for rapid multiplication in order to sustain a viable population both in wild and in captivity. It is important to conduct regular monitoring of population that would provide valuable up-to-date information, to help identify the critical population and sites for prioritized conservation actions and to support and guide the protection of the species. It is necessary to study the demographic parameters, population dynamics, requirement of space and forage for sustained reproduction and social structure and behaviour. The other research topics of management interest include plant community structure and productivity of *phumdi*, water quality analysis and extent of pollutant load, disease and its impact on population and impacts of biomass extraction on the Park ecology and attitude of local people towards Park. The WII's research project "Conservation ecology of sangai and its wetland habitat" is presently looking into these aspects.

7.3 PHYLOGENETIC STATUS OF SANGAI AND HOG DEER

The present study intended to examine the genetic variability of sangai within the species, between wild and captive populations and in comparison with related cervids. The information thus obtained will contribute to the understanding of the genetic dynamics and its sustainability of sangai and to designing a conservation strategy for the species in the wild. The studies on the phylogeny of sangai and hog deer are still in their infancy compared with the detailed work and conservation efforts carried out with other cervids elsewhere in the world. There still remain many knowledge gaps in the systematic and genetic status of both these species. For instance, the Eld's deer has a complex phylogeny; many studies (Zhang et al. 2008a, Zhang et

al. 2008b) reported *R. e. hainanus* as a distinct fourth subspecies while other studies does not recognize this (e.g. Balakrishnan et al. 2003). Thus this indicates that the status of *hainanus* needs a formal study to examine its accurate taxonomic position and relationship among the subspecies of Eld's deer. Most of the sources of the *siamensis* sequences are not known and only few sequences are available in GenBank and the sequences of *siamensis* showed variation among themselves. In addition, all the three subspecies are different with respect to their body size, antlers morphology and diet; the difference between *R. e. siamensis* and the other two subspecies *R. e. thamin* and *R. e. eldii* is conspicuous and distinct.

In the present study, the phylogeny of three subspecies of Eld's deer was examined through mtDNA Cyt *b* (150 to 471 bp) and control region (230 to 478 bp) genes. Therefore, probably in future the phylogenetic relationship based on full genomic structure should be investigated between wild and captive stock to understand its haplotype and nulceotide diversity and its effective population size for planning an effective conservation strategy. The phylogenetic trees of Neighbor-Joining (NJ) and Minimum Evolution (ME) with D-loop R / Lo-F gene showed the wild and captive populations of sangai forms a monophyletic clades showing first clade comprising of captive population of sangai from Hyderabad Zoo and Delhi Zoo and the second group forming one clade comprising of sangai from KLNP and Manipur Zoo. However, the result is based exclusively on a short fragment of 230 bp of the primer D-loop R / Lo-F gene. To compare and check the result, a larger fragment of 478 bp of control region Cerv.tPro / CervCRH gene as identified by Balakrishnan et al. (2003) was analysed using Neighbour Joining (NJ) and

Maximum Parsimony (MP) methods. The result showed that the population of sangai was grouped into a single clade consisting of both captive and wild with high bootstrap support (100%). This indicates that the captive and wild population of sangai may not be genetically divergent as evident earlier from the D-loop R / Lo-F gene study.

No haplotype variations were detected among the 16 individuals of sangai using mtDNA cyt *b* gene. However, two haplotypes were identified among the 17 individuals of sangai at nt282 position in mtDNA control region of Cerv.tPro / CervCRH gene. More studies using different classes of genetic markers and incorporation of large sample size from blood and tissue from wild and captive population may reveal better understanding on its phylogenetics and genetic relationship of sangai and related cervids.

The examination of the genetic relationship of sangai with related cervids using control region gene revealed close affinity of sangai with sambar (*Rusa unicolor*) and hog deer (*A. porcinus*), which is closely related to chital (*A. axis*) indicating monophyly. The study by Randi et al. (2001) pointed out that the hog deer (*A. porcinus*) is not closely related with sambar (*R. unicolor*) and also examination of the evolutionary relationships within Cervinae using the mtDNA control region marker revealed paraphyly of the genus *Cervus.* On the other hand, this study revealed that the swamp deer *R. duvaucelii* which was recently revised from the genus *Cervus* to *Rucervus* (Wilson & Reeder 2005) appear closely related to *Muntiacus muntjak* than to Eld's deer. Eld's deer and swamp deer may seem functionally similar, but represent different lines of cervids that have each adapted to seasonally flooded dry forests

(McShea et al. 2001). This needs confirmation by taking more samples as the numbers of sequences in the GenBank on control region gene (D-loop) were very limited.

7.4 GENETIC DIVERSITY AND POPULATION STRUCTURE

One of the most important trait to consider in genetic studies is the retention of genetic variation in populations of endangered species which is a pre-requisite for their continuous survival. The genetic diversity of sangai in the wild showed reduction i.e., approximately 40% is indicative of low genetic variability and significant loss of genetic diversity in captive population probably due to inbreeding depression. However, the sympatric hog deer showed a moderate genetic variation around 50% at KLNP. This indicates that both sangai and hog deer are susceptible to inbreeding depression, decreased fitness through heterozygote benefits and lesser evolutionary potential than species with higher genetic diversity. Although the hog deer population has been gradually declining in the recent years, the population was found to retain some of its rare alleles and 50% genetic diversity in the wild.

The Hainan's Eld's deer whose current population was founded by about 20 individuals in 1970s, is also known to have suffered a severe genetic bottleneck. During this study it was observed that the sangai population in the Park has experienced founder events and genetic drift in the past. To establish a viable population, the founder population during future reintroduction should be greater than the believed to be original 14 founders in the KLNP. To establish a viable population, it is recommended that a

minimum of 15–30 genetically effective founders be used (Frankham et al. 2002). The founder population should compose of a cohort of over 20 same-age individuals with 1:1 sex ratio. Genetic monitoring of both the source and the reintroduced populations should be done prior to reintroduction in order to assess the effectiveness of the conservation program. Based on the present study, the founder population should have both a balanced genetic composition and a large effective population size.

In the future, more research is required to determine the genetic population structure on the basis of genetic markers, variable enough to detect differences between all the three subspecies of Eld's deer (an extensive study of microsatellite loci). An extensive study is required to assess the relatedness and kinships among all the captive population of sangai to determine the genetic population structure of each population/sub population so that changes in genetic variability due to inbreeding depression and founder events in the populations could be identified and appropriate conservation measures are taken.

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