Genetic Diversity of Indian Rhinoceros, *Rhinoceros unicornis* (Lin, 1758)

A THESIS
SUBMITTED TO THE GAUHATI UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY IN THE FACULTY OF SCIENCE

Submitted by
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2014

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CERTIFICATE

This is to certify that Mr. Puranjit Das (Enrolment No. Zoo- 52 /09) has carried out the research work under my guidance and supervision and has now submitted the thesis entitled **"Genetic Diversity of Indian Rhinoceros, *Rhinoceros unicornis* (Lin, 1758)"** for the Ph.D. degree under Gauhati University. Mr. Puranjit Das has fulfilled all the requirements as laid down by the Gauhati University for the submission of a Ph. D. Thesis.

Neither the thesis nor any part of it has been submitted to any other University/ Institution for the Ph.D. degree.

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DECLARATION

I, Puranjit Das, hereby declare that the thesis entitled **"Genetic Diversity of Indian Rhinoceros, *Rhinoceros unicornis* (Lin, 1758)"** has been composed entirely by myself and is a result of my own investigations. It has neither been accepted nor submitted for any other degree. All sources of information have been duly acknowledged.

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ABSTRACT

The present studies were carried out in the preparation of the thesis entitled "Genetic Diversity of Indian Rhinoceros, *Rhinoceros unicornis* (Lin, 1758)". The content of the thesis comprises an introduction, review of literature, material and broad methodology with description of study areas and in the subsequent chapters genetic diversity was studied through mitochondrial and nuclear DNA. The studies have utilizes non–invasive sources of DNA. The objectives were to investigate genetic diversity of *Rhinoceros unicornis* found in three wild habitats of Assam. In the first part, genetic divergence of *R. unicornis* was studied through partial sequencing of mitochondrial D-loop region. In the second part, the genetic divergence of Rhinoceros of three habitats was studied through microsatellite genotyping. This study represents the first extensive investigation on the genetic status of the wild Indian rhinoceros taking dung/faecal samples from three wild habitats, using both mitochondrial DNA and nuclear DNA markers.

The one horned Indian Rhinoceros, *Rhinoceros unicornis* is the largest of all Perissodactyl animals characterized by a single large horn, a semi-prehensile upper lip and folded skin. They are now confined in small isolated protected areas in India and Nepal. In Assam, with 2,542 rhinos in Kaziranga National Park, 90 in Pobitora Wildlife Sanctuary and 98 in Rajiv Gandhi Orang National Park, the total rhino population now stands nearly 2,730. However, in historic times (c. 1400 AD), the Indian rhinoceros was found across the Gangetic plain to the Indus River Valley in northern Pakistan and Burma (Myanmar) with more than 450,000 individuals. During 19th century, the rhino

population has reduced significantly due to loss of suitable habitat and poaching. According to official report when hunting of rhino was banned by government in 1908 A.D., the rhino population in Kaziranga National park was estimated to contain less than 20 individuals. But the population has increased in the last century and reach to a suitable position. In the conservation initiatives it is necessary to have detail genetic study and estimate the divergence of the species for future conservation program and propagation of the species. The three rhino habitats of Assam namely Kaziranga, Pobitora and Orang are located distantly and there is a natural barrier "River Bhrahmaputra" in between Kaziranga and Orang national park, thereby they become fragmented.

In the first chapter, genetic divergence study was done through mitochondrial D-loop region. The D-loop region was selected because it evolved with exceptional rapidity, relatively high mutation rate and useful for high resolution analyses of genetic differentiation and population structure. The partial sequencing of D-loop region of mt DNA results 24 haplotypes from 241 sequences (samples) with 21 variable sites obtained in the three wild habitats. In the rhino population of Kaziranga National Park 22 different haplotypes were obtained, 9 haplotypes were detected in Pobitora WLS and 7 haplotypes were found in Orang National Park. All three habitats shared some common haplotypes except two different haplotypes obtained in Orang National Park. Kaziranga National Park represents almost all haplotypes except H19 and H20. Among the 24 haplotypes, a large number individuals are found under the haplotype 9 (hap 09) which is the most common haplotype (17%) distributed in the three rhino habitats. This is followed by H1 (10%) and H12 (8%). The mean haplotype diversity of the three groups of rhino is 0.97571±0.011. The haplotype diversity of Kaziranga group is

0.99567 showing high genetic diversity of the rhino in the habitat. The average nucleotide compositions of all haplotype sequences were 34.18%A, 29.34%T, 12.76% G and 23.72% C and the average nucleotide content of A + T (63.52%) was obviously higher than that of G + C (36.48%). *R. unicornis* group of Kaziranga have higher nucleotide diversity (Pi = 0.01095) than those from the group of rhino found in Orang (Pi = 0.01049) and Pobitora (Pi = 0.00982). The different phylogenetic trees such as Neighbour-joining tree, Maximum Parsimony tree and Maximum Likelihood tree revealed low differentiation between different haplotypes of *R. unicornis* obtained from three different habitats. Most of the haplotypes showed bootstrap values lower than 70, except in the few haplotypes such as H21, H22 and H23. The Medium-joining network showed the relationship among haplotypes. The Standardized variance in allele frequencies (Fst) among three groups, the Kaziranga rhinoceros group found to have little genetic differentiation from Orang rhino group. The result of the AMOVA revealed that 91.62% of the total genetic diversity existed among the individuals and only 8.38 % of the total genetic diversity accounted for differences among groups. Low genetic differentiation in the three groups of rhino was observed, as indicated by the G_{ST} and Snn values of -0.01831 and 0.19518, respectively ($p < 0.001$ for both parameters). The negative Fu's *F*S value of Kaziranga group indicates the population expansion. The Tajima's D values are 0.40849, 1.29517 and 0.98110 for Kaziranga, Orang and Pobitora rhino group respectively. All the 3 values are insignificant though the value of Orang group is larger than the rest indicating randomly evolving mutations. In mismatch distribution analysis the unimodel graph obtained from all three groups of rhino indicating recent demographic expansion. The mt DNA analysis showed that the *R.*

unicornis found in three habitats are genetically diverged but there exist a very little differentiation among the groups.

Microsatellites are usually called Simple Tandem Repeats (STRs). They are highly polymorphic class of genetic markers; consist of short sequence repeat motifs of tandemly repeated di, tri, tetra or penta nucleotide sequences that occurs at large number of loci throughout the eukaryotic genome. The polymorphic microsatellite loci have unprecedented power to detect and describe small genetic differences between populations or groups. For microsatellite analysis in *R. unicornis* population, D-loop haplotype samples were further analysed for any differentiation in nuclear DNA. The isolated genomic DNA samples of all D-loop haplotypes (3 replicas from each haplotype) from three habitats were then amplified using 6 microsatellite loci: Rh1, Rh3, Rh5, Rh7, Rh9 and IR10. Among the 6 microsatellite loci, 5 loci are polymorphic in the rhino groups of all three habitats namely Kaziranga, Pobitora and Orang. In the three rhino groups, the number of alleles per polymorphic loci varied from 2 to 14 and the values of observed heterozygosity and expected heterozygosity ranged from 0.37037 to 0.92593 and from 0.39474 to 0.91579, respectively. Mean P value for Hardy–Weinberg equilibrium (HWE) estimates showed that all three population of rhino conformed to Hardy–Weinberg Equilibrium in the population ($P>0.05$). But few loci showed insignificant value ($P<0.05$) which are exception to the Hardy–Weinberg equilibrium. The deviation from Hardy–Weinberg equilibrium in the three groups of *R. unicornis* in Assam or overall rhino population in Assam was not very significant. The F_{ST} *p*-values showed that differentiation between rhino groups of Kaziranga and Pobitora is not significant. But the group of rhino from Orang NP is found different from Kaziranga and Pobitora. The AMOVA revealed that 87.75 % of the variation was

observed within individuals and 5.50 % among populations. While examining the gene diversity of all 6 loci of all three rhino groups, it has been observed that the Kaziranga rhino group is more diverged than other two groups. The Factorial Correspondence Analysis showed certain degree of differentiations in the individuals of three rhino groups. The analyses of population structure (assuming K= 2 to 5) of the *R. unicornis* of the three habitats of Assam found to have shared common alleles and none the group is distinctly different from one another. The bottleneck analysis showed that the rhino population of Assam has experienced a bottleneck effect in recent past which was not very much severed.

The analysis of mitochondrial and nuclear DNA of *R. unicornis* it has been found that the three rhino groups in Assam are genetically diverged. Both mitochondrial and nuclear DNA analysis showed variability in *R. unicornis* groups from three habitats. The results of this study have important conservation implications and will help in better future management of this species including translocation and captive breeding.

ACKNOWLEDGEMENT

I acknowledge my sincere gratitude to Dr. Umesh C. Goswami, Professor, Department of Zoology, Gauhati University for his patience, motivation, enthusiasm, and constant guidance and supervision in the entire work. I am immensely obliged to him for his valuable suggestions and encouragement with inspiration and helping me at different stages of the study which enabled me to successfully complete this research work. This thesis would not have been possible without the guidance and the help of him.

I would like to extend my sincere gratitude to Dr. Karabi Dutta, Professor and Head of the department of Zoology for giving me the opportunity to avail the departmental facilities and valuable suggestions and help extended during the course of my study. I am grateful to Dr. D. K. Sarma, Dr. A. Dutta, Dr. A. Borkotoki, Dr. M. M. Goswami, Dr. R. K. Bhola, Dr. J. Kalita, Dr. J. C. Kalita, Dr. P. K. Saikia and Dr. D. D. Sarma and other faculty members of the Department of Zoology, Gauhati University for their valuable suggestions and encouragement throughout the period of my study.

I would like to thank Dr. U. Ramakrishnan and the staff of Lab 3 of NCBS-TIFR, Bangalore for providing assistance and laboratory facilities for part of my work.

A special word of thanks is due to Mr. Pidugu Vijay, Mr. Anujit Sarkar and Ms Deepa of Hyderabad for their good advice and support.

I am indebted to Dr. Varsa Srivastava of CDFD, Hyderabad for providing me an opportunity to work in her lab. Her immense knowledge and expertise have been of great value to me.

My sincere thank to Department of Environment and Forests, Government of Assam for giving necessary permission to conduct the study. I am highly indebted to the staff of Kaziranga National Park, Rajiv Gandhi Orang National Park and Pobitora wildlife sanctuary for their encouragement, support and help during sample collection period.

I would like to thank Dr. Dhanjit Kumar Das of National Institute for Research in Reproductive Health, Mumbai, for providing lab facilities and help me during my stay at Mumbai.

I am extremely grateful for the hard work of the following people in obtaining faecal samples from the protected areas: Dr. Mrigen Barman, Mr. Kameshwar Das, Mr. Mrinal Talukdar, Mr. Diganta Choudhury, Mr. Jadav Ch. Das, Mr. Ajanta Choudhury and Mr. Uzzwal Das. I am also very grateful to Mr. Shyam Kumar, Mrs. Bhanita Barman, Ms. Tarali Choudhury, Mr. Bidyut Das and Mr. Falgun Chetia for their help.

My sincere thanks also go to the Head and other members of Dept of Zoology, Bajali College, Pathsala, Assam.

My special thanks to the Administrator of Jawahar Guest House for providing me accommodation during my stay at Bangalore. I would also thank late Dr. Nagaraju for giving opportunity to stay at Research Scholar hostel of CDFD at Hyderabad.

Finally, my warmest thanks are to my wife and other family members for their love, constant support, encouragement and understanding.

Date: ----------

(Puranjit Das)

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LIST OF ABBREVIATIONS

AD	Anno domini
AFLP	Amplified Fragment Length Polymorphism
Arlequin	An Integrated Software Package for Population Genetics Data Analysis
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BSA	Bovine Serum Albumin
bp	Base Pair
Cyt b	Cytochrome b
^{0}C	Degree Celsius
DA	Modified Cavalli-Sforza genetic distance
d.f.	Degrees of freedom
D-loop	Displacement loop
DS	Nei's standard genetic distance
DSW	Stepwise-weighted genetic distance
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
DMSO	Dimethyl sulfoxide
DnaSP	DNA sequence polymorphism
NaCl	Sodium chloride

Exo-SAP	Shrimp Alkaline Phosphatase
F_{ST}	Standardised variance in allele frequencies among populations
ESU	Evolutionary significant units
e.g	For example
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
GenAlEx	Genetic Analysis in Excel
G_{ST}	Coefficient of gene differentiation
gm	Gramme
g	Gravitational force
Ho	Observed heterozygosity
He	Expected heterozygosity
HWE	Hardy-Weinberg equilibrium
IAM	Infinite alleles model of neutral mutation
KNP	Kaziranga National Park
LD	Linkage disequilibrium
Mbp	Mega base pair
µ	Micro
µL	Microliter
M	Molar
ML	Maximum likelihood
MP	Maximum Parsimony
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium Chloride

mL or ml	Milliliter
mM	Millimolar
min	Minute
mg	Milligramme
mg/ml	Milligramme per millilitre
mg/L	Milligramme per litre
mtDNA	Mitochondrial DNA
ng	Nanogram
nm	Nanometer
N	Number of samples
Ne	Effective population size
NJ	Neighbor Joining
NP	National Park
ONP	Orang National Park
PWLS	Pobitora Wildlife Sanctuary
PCR	Polymerase Chain Reaction
pmol	Picomoles
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
SMM	Stepwise mutation model
STR	Simple tandem repeat
Tris	Trizma® base
rpm	Rotations per Minute

Taq	Thermus aquaticus
TBE	Tris Borate EDTA
QTL	Quantitative trait loci
rpm	Revolutions per minute
SD	Standard deviation
SE	Standard error
SMOGD	Software for the Measurement of Genetic Diversity
sq. km	Square kilometre
U/µL	unit per micro litre
UV	Ultra Violet
V	Volt
WLS	Wildlife Sanctuary
P-value	Probability level
π	Nucleotide diversity

CHAPTER- 1

GENERAL INTRODUCTION

Rhinoceros unicornis Linnaeus, 1758 is the largest of all Perissodactyl animals. It is one of world's most endangered mammals. The animal is characterized by a single large horn and a semi-prehensile upper lip which it uses to grasp food. Its skin is folded into shields and studded with wart like tubercles. Horns present in both sexes, grow throughout life, and if lost reproduced (Banerjee *et al*., 1973; Prater, 1980). The legs are shorter in comparison to body size but they are stout and contain odd number of 3 toes in each, hence they are placed under the order Perissodactyla. Several centuries ago, the one-horned Rhinoceros had a wide distribution throughout the north-western, northern, eastern India all along the Indus, Ganges and Brahmaputra River basins. The western limit of its range was the foothills of the Hindu Kush west of Peshawar of Pakistan (Goswami, 1993). The eastern limit of its historic range is uncertain. Some authors believe that it occurred in Cambodia, Laos, Thailand and Vietnam (Mukherjee, 1982). By the first decade of the 20th century there were only a few scattered survivors, comprised of few individuals along the Brahmaputra Valley in Assam (India), and isolated patches in the Nepal terrain (Laurie, 1978; Dinerstein and Price, 1991). Fortunately, the total population increased considerably during the second half of the 20th century. Now the Indian rhino population in Assam is found in Kaziranga National Park, Orang National Park and Pobitora Wildlife Sanctuary. Even after hundred years of success in conservation history for rhino in Assam, the species was wiped out from some other rhino habitats of the state viz. Laokhowa WLS, Burachapari WLS, Kurua

zone and Manas Tiger National Park Reserve. In Manas National Park, all rhinos were eliminated in the political unrest period during 1989-2001. But now a reintroduced population of Indian rhino is growing in the Park.

1.1 Evolution, Taxonomic position and Historic range of Indian Rhinoceros

Rhinoceroses are considered to be prehistoric animals which were available in the earth for millions of years and represent one of the most ancient extant mammalian genera. They were grouped together with horses and tapirs under perissodactyl. Several species of rhinos once roamed the earth, but only five exist on the earth today. These are the white rhinoceros *(Ceratotherium simum*; Lydekker, 1908*)* and the black rhinoceros *(Diceros bicornis*; Drummond, 1826*)* of Africa and the Indian rhinoceros *(Rhinoceros unicornis*; Linnaeus, 1758*)*, the Javan rhinoceros *(Rhinoceros sondaicus*; Desmarest, 1822*)* and the Sumatran rhinoceros (*Dicerorhinus sumatrensis*; Fischer, 1814) of Asia.

Kingdom	: Animalia
Phylum	: Chordata
Class	: Mammalia
Subclass	: Theria
Infraclass	: Eutheria
Order	: Perissodactyla
Suborder	: Ceratomorpha
Family	: Rhinocerotidae
Genus	: Rhinoceros
Species	: *R. unicornis* Linnaeus, 1758

Figure 1.1 Taxonomic hierarchies of *Rhinoceros unicornis*

The evolution of the rhinoceros began during the early Palaeocene, or possibly even earlier in the late Cretaceous (Hooijer, 1968). The Indian rhinoceros is closely related to the Javan rhino and originates from a lineage of Asian rhinos which first emerged 2 – 4 million years ago, whereas the first ancestors of the African species appeared in the mid Miocene 12 – 14 million years ago (Prothero and Schoch, 1989). Molecular estimates, however, suggest the species may have diverged much earlier, around 11.7 million years ago (Tougard *et al.,* 2001). Different studies have hypothesized that they may be closely related to the extinct *Gaindetherium* or *Punjabitherium*. A detailed cladistic analysis of the Rhinocerotidae placed *Rhinoceros* and the extinct *Punjabitherium* in a clade with *Dicerorhinus*, the Sumatran rhino. Other studies have suggested that the Sumatran rhinoceros is more closely related to the two African species and appeared about 15 million years ago (Cerdeno, 1995). According to Shoshani (2006), the two African species did not diverge until the early Pliocene (3.5 – 5 million years ago) and are still closely related enough to hybridise. Moreover both African species and the Sumatran rhinoceros have two horns while the Javan and the Indian rhinoceros have one only (Prothero *et al.,* 1986). The Sumatran Rhino may have diverged from the other Asian rhinos as far back as 15 million years ago (Dinerstein, 2003) and thought to be the oldest and the most archaic form (Lacombat, 2005). Fossils of *Rhinoceros unicornis* appear in the Middle Pleistocene. In the Pleistocene (1,808,000 to 11,550 years BP), the *Rhinoceros* genus ranged throughout Southeast Asia and South Asia, with specimens located in Sri Lanka. Into the Holocene, some rhinoceros lived as far west as Gujarat and Pakistan until as recently as 3,200 years ago (Laurie *et al*., 1983).

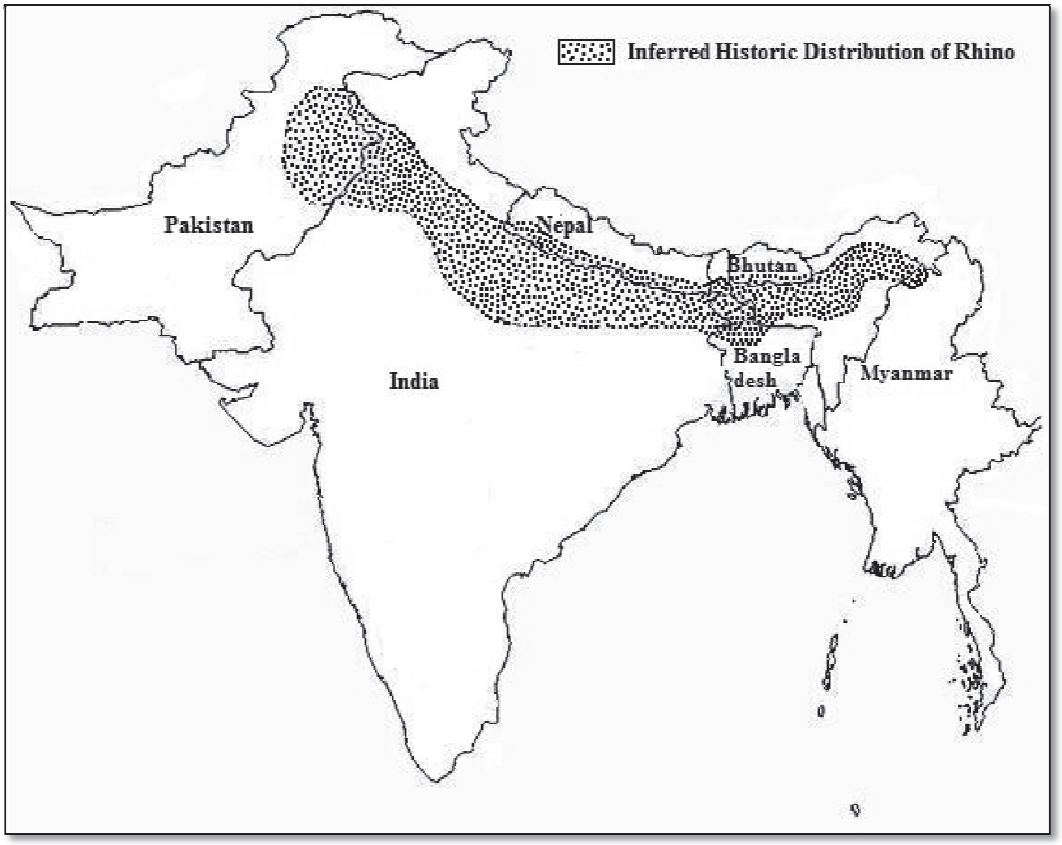


Figure 1.2 Map showing inferred historic range of distribution of *Rhinoceros unicornis*. Historically, the Greater one-horned rhino was abundant in the alluvial grasslands of major river systems (Brahmaputra, Ganges, and Indus) in the northern part of the South Asia subcontinent.

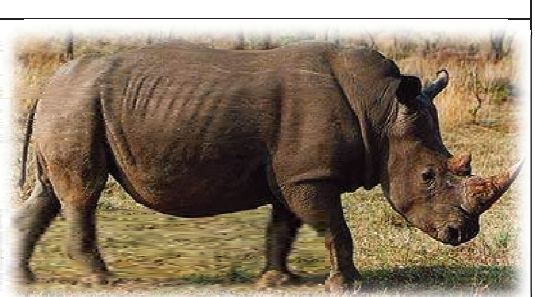
1.2 Status and distribution of different species of Rhino

The Javan Rhino, *Rhinoceros sondaicus* is rarest of all rhinos and has been listed as 'Critically Endangered' by the IUCN (International Union for Conservation of Nature). It is a dusky grey colour and has a single horn. This species, similar in appearance to the closely related greater Asian one-horned rhino, is slightly smaller, with a much smaller head and less developed folds of skin on the neck. In Java, a large portion of the females are hornless, though horned females are known from other parts of the range. Except for mating pairs and mothers with young, the species is solitary.

Javan rhinos favour dense rainforest with a good supply of water and plentiful mud wallows. They prefer low-lying areas (Kemf and Strien, 2002). According to the report of IRF, 2010, there are presently not more than 44 individuals of the species exist on earth. Ujung Kulon National Park in Indonesia is the only habitat of this species *Rhinoceros sondaicus sondaicus*. Another habitat of this species was Vietnam's Cat Loc Reserve where the last Javan rhino subspecies *Rhinoceros sondaicus annamiticus* was poached in 2010. Another already extinct sub-species of Javan rhino, *Rhinoceros sondaicus inermis* was also found throughout Southeast Asia and Indochina.

The Sumatran rhino *Dicerorhinus sumatrensis*, the smallest of all Asian rhino with two horns, anterior is nearly 25cm long, while the posterior horn is usually quite small. Once they roamed widely from the foothills of the Himalayas in Bhutan, eastern India, Myanmar, Thailand, and south through the Malay Peninsula, to the islands of Sumatra and Borneo, and are now found in small populations scattered in Peninsular Malaysia, Sumatra (Indonesia), and Sabah in Borneo (Borner, 1979). Groves (1983) divides the species into three subspecies, *Dicerorhinus sumatrensis* (Sumatra and Malaysia) *Dicerorhinus harrissoni* (Borneo), and *Dicerorhinus lasiotis* (Myanmar and India) based on morphological characters. The Endangered Sumatran Rhino, *Dicerorhinus sumatrensis* has declined from an estimated 600 animals in 1994 to less than 200 individuals that are surviving now in fragmented populations on the islands of Sumatra and Borneo. In Sumatra, Indonesia, between 130 and 175 rhinos are scattered among three populations in Bukit Barisan Selatan, Way Kambas, and Gunung Leuser National Parks. In Sabah, Borneo, Malaysia, approximately 20 Sumatran rhinos remain in fragmented populations; no evidence of rhinos has been seen in peninsular Malaysia for several years.

Table 1.1 Status and distribution of five extant species of Rhino

Species	IUCN Status	Home range	Appro. Popl^{n}	Photograph
Rhinoceros sondaicus	Critically Endangered	Ujung Kulon National Park, Indonesia	*35- 45 individuals	
Dicerorhinus sumatrensis	Critically Endangered	Sumatra and Borneo	*120-180 individuals	
Rhinoceros unicornis	Vulnerable	India, Nepal	*3,624 individuals	
Diceros bicornis	Critically Endangered	South Africa, Namibia, Kenya and Zimbabwe	*4,880 individuals	
Ceratotherum simum	Near Threatened	South Africa	*20,165 individuals	

** Data of 2010 *Data of 2012 (Emslie *et al.,* 2012).

The black rhinoceros is a species of rhinoceros, native to eastern and central Africa including Kenya, Tanzania, Cameroon, South Africa, Namibia, Zimbabwe, and Angola. Black rhinos have been one of the most heavily persecuted species on our planet. Just 100 years ago, Africa's savannahs teemed with rhinos which were estimated more than one million rhinos in sub-Saharan Africa. Poaching escalated during the 1970s and 1980s as Asian and Middle Eastern demand grew for rhino horn. As a result, black rhino numbers declined by a staggering 96% between 1970 and 1992. At present, black rhinoceros are now found in four countries: South Africa, Namibia, Kenya and Zimbabwe and only five populations have been characterized (Emslie and Brooks, 1999). The current black rhinoceros population has been estimated to be approximately 4,880 individuals (Emslie *et al*., 2012). The black rhino has two prominent horns made from keratin with the front horn growing as long as 1.4 metres. The four extant subspecies of black rhinoceros are: *Diceros bicornis bicornis* (South-western)*, Diceros bicornis minor* (South-eastern)*, Diceros bicornis michaeli* (Eastern) and *Diceros bicornis longipes* (Western) based on slight morphological distinctions and geographical distribution (Du Toit, 1987). While sub-species *longipes* is on the verge of extinction, and *michaeli* are dramatically reduced, sub-species *bicornis* and *minor* have fared somewhat better. The vast majority of the latter two sub-species exist in South Africa and Namibia. Most of the South African animals are of the sub-species *minor*, while all of the Namibian animals are of the sub-species *bicornis* (Adcock, 2003).

The white rhinoceros is one of the two species of Rhinos native to Africa. Its current range is primarily Southern Africa. The white rhino has an immense body and large head, a short neck and broad chest. This rhino can exceed 3,500 kg (7,700 lb), have a head-and-body length of 3.5–4.6 m (11–15 ft) and a shoulder height of 1.8–2 m

(5.9–6.6 ft). On its snout it has two horns. The front horn is larger than the other horn and averages 90 cm (35 in) in length and can reach 150 cm (59 in). The white rhinoceros also has a prominent muscular hump that supports its relatively large head. The colour of this animal can range from yellowish brown to slate grey. Most of its body hair is found on the ear fringes and tail bristles, with the rest distributed rather sparsely over the rest of the body. White rhinos have the distinctive flat broad mouth that is used for grazing. The white rhinoceros consists of two genetically distinct subspecies; the northern (*Ceratotherium simum cottoni*) and the southern (*Ceratotherium simum simum*) white rhino. The northern subspecies is believed to have become extinct recently (Timesonline, International Rhino Foundation) but one population of *C. s. cottoni* currently exist in Ol Pejeta (a Kenyan reserve) was created in December 2009 following the translocation from the Czech Republic (Emslie, 2011). The southern subspecies has a population of around 20,150 individuals (IRF, 2010). Presently almost all free-ranging southern white rhinoceroses are confined to the Republic of South Africa. The white rhinoceros is classified as "Near-Threatened" on the IUCN's Red List of Threatened Species (IUCN, 2008). Despite the poaching pressure, the southern white rhino is still increasing and is considered 'Near Threatened' by the IUCN. The majority (98.8%) of White Rhino occur in just four countries (South Africa, Namibia, Zimbabwe and Kenya).

1. 3 Status and distribution of *Rhinoceros unicornis* in India

The greater one-horned rhinoceros, *Rhinoceros unicornis* are now confined in small isolated pockets of protected areas in India and Nepal. Historically, the Indian rhinoceros once existed across the entire northern part of the Indian subcontinent, along the Indus, Ganges and Brahmaputra River basins, from Pakistan to the Indian-Burmese

border; including parts of Nepal, Bangladesh and Bhutan. An estimated 500,000 animals once existed from Pakistan to Bangladesh and Burma and may have also existed in Myanmar and China (Foose and van Strien, 1997).The Kaziranga National Park in Assam, India holds the largest population of more than 2500 individuals. It has a single black horn and a grey-brown hide with skin folds, giving it an armoured appearance. The human population and human impact on the natural habitat have significantly increased in the past few decades, thus increasing the extinction rate of animals and plants.

With 2,544 rhinos in Kaziranga National Park**,** Assam's total rhino population now stands at 2,735. The recent census shows that the Pobitora Wildlife Sanctuary has 90-93 rhinos while the Rajiv Gandhi National Park has recorded 98-100 rhinos. There are another 22 translocated one-horned rhinos in Manas National Park in Assam. Strict protection efforts by wildlife authorities have helped in the recovery of the Indian Rhino which has achieved now status vulnerable in IUCN Red list. There is already an increase of 15 per cent of rhino population in Assam, which is very healthy in terms of conservation of the species. The recent census report suggests that there are about 508 pairs of mother and calf in Kaziranga National Park, which reflects satisfactory breeding of rhinos in Kaziranga National Park due to the ideal habitat.

The Dudhwa National Park, which is located in the foothills of the Himalayas in Uttar Pradesh, has become an ideal home to the famed one horned rhinoceros In 1984, five rhinos were translocated from Kaziranga National Park and after one year 5 female rhinos were bought from Chitwan National Park of Nepal. At present the National Park has 28 rhinos. Dudhwa National Park must be credited for it is the only place where the rhino that had gone extinct was brought back after 100 years and without any aid in the

form of food and water, it survived on its own and reproduced. The Jaldapara and Gorumara sanctuaries of West Bengal are two homes of rhinoceros. According to a census conducted in 2011 there were 155 Rhinoceros in Jaldapara. In 2010 the total number of rhinoceros was 36 in Garumara wild life sanctuary.

1.4 Status of *Rhinoceros unicornis* in Nepal

The Greater One-horned Rhinoceros is the important wildlife resourse of Nepal. The protected areas of Nepal have second largest population of the Indian one-horned rhinos. In Nepal, over 800 rhinos lived in Chitwan valley until 1950s. Rhino numbers dropped to less than 100 as early as mid 1960. But due to intense protection efforts some recovery was brought to the species. According to official sources, at present the population of the greater one-horned rhinos in Nepal has increased to 534. The recent census has revealed an increase of 99 rhinos since the last count in 2008. Chitwan National Park was found to have 503 rhinos, while 24 reside in Bardia National Park, and Shukla Phanta Wildlife Reserve holds seven of the precious pachyderms. The census was conducted by the Department of National Parks and Wildlife Conservation of Nepal, with the technical and financial support from the National Trust for Nature Conservation and WWF-Nepal. Unfortunately the Nepal's rhinoceros population have once again declined due to intense poaching pressure. There has been a dramatic decline in numbers of rhino in Royal Chitwan National Park as a result of the Maoist insurgency in that country. The forest area has been depleted rapidly in Nepal. The unprecedented and increasing loss of forest, rapid human population growth and urbanization has warranted conservation efforts of wild animals in Nepal.

Table 1.2 Population of *Rhinoceros unicornis* in different protected areas of India and Nepal

Name of Protected Areas	Area (Sq. km.)	Geographic Location	Population
Kaziranga National Park, Assam, India	858.98	Latitude 26° 30' N to 26°45' N Longitude93° 05' E to 93°40' E	2,544**
Orang National Park, Assam, India	78.8	Latitude 26° 29' N to 26° 40' N Longitude92°16' E to 92°27' E	100
Pobitora Wildlife Sanctury, Assam, India	38.80	Latitude 26°12'N to 26°15'N Longitude 91°57'E to 92°50'E	93
Manas National Park, Assam, India	519.20	Latitude 26°36 to 26°49'N Longitude 90°48' to 91°15'E	22*
Jaldapara Wildlife Sanctuary, West Bengal, India	216.51	Latitudes 25°58' to 27°45'N Longitude 89°08' to 89°55'E	155
Gorumara National Park West Bengal, India	80	Latitude 26°47' to 26°43' N Longitude 88°52' to 88°47' E	36
Dudhwa National Park, UP, India	490.29	Latitudes 28° 18'to28° 42´N Longitude 80° 28' to 80° 57'E	28
Chitwan National Park , Nepal	932	Latitude 27° 18'to 27° 41'N Longitude 83° 41' to 83° 49' E	503
Bardia National Park, Nepal	968	Latitude 27°23' to27°46'N Longitude 84°53' to 84°97'E	24
Shukla Phanta Wildlife Reserve, Nepal	305	Latitude 28°45' to 28°7' N Longitude 80°06' to 80°21' E	7

*Recently translocated from Pobitora WLS and Kaziranga National Park

** Data of 2013

1.5 Certain behaviours of *Rhinoceros unicornis*

1.5.1 Food and feeding behaviour: There is no detail study on the budget of grass requirement and availability of grasses and food plants in wild rhinoceros. Every year during winter season a grassland management programme is followed by burning the old grasses. However always old grasses are burnt during Jan-march which enhances the growth of new grasses by avoiding formation of any other species of trees. The Indian Rhinoceros is a grazer. Their diet consists almost entirely of grasses, but the rhino is also known to eat leaves, branches of shrubs and trees, fruits and submerged and floating aquatic plants. They may eat soil. Besides they also consume cultivated crops etc. The mostly preferred 10 top ranking food plants of *Rhinoceros unicornis* are *Hemarthria compressa, Hymenachne pseudointerrupta* (Aquatic), *Leersia hexandra* (Aquatic), *Arundo donax* (Aquatic), *Chrysopogon aciculatus*, *Phragmites karka*, *Bracharia ramose*, *Cynodon dactylon*, *Saccharum spontaneum* and *Imperata cylindrica* (Hazarika, 2007). Greater one-horned rhinos eat on average 1.5-2% of their body weight daily. They body weight of rhino in between 4,000-6,000 pounds (1,800-2,700 kg).

1.5.2 Soil eating behaviour: Rhino frequently consume soil from some particular location of the habitat by tip of the tongue. Sometimes, incisor teeth were also used to dig the selected soil. This behavior is known as geophagy and this particular behaviour also seen in other rhino species.

1.5.3 Feeding of aquatic plants: Rhino feed on some aquatic under-water or submerged food plant like *Hydrilla, Vallisneria* etc. This type of feeding is known as dive feeding.

1.5.4 Wallowing: Wallowing is done by Indian Rhino for keeping the body temperature low during hot summer days, and at other times to get rid of ectoparasites which harbor

in their skin folds. Rhino lies in the mud or water holes especially during day hours and spend nearly 60% of the day in water. Wallowing behaviour was also divided into two types (a) Mud wallowing and (b) Water wallowing. Mud wallowing is the process in which the Indian Rhino lies in mud. In water wallowing the Rhino immersed its entire body into the water by keeping only head portion above water surface.

1.5.5 Defecation and urination behaviour: Rhinoceros have a peculiar habit to defecate near other rhinoceros dung forming some piles. A rhino coming up to a dung pile, sniffs at it, may push his horn into it, and then shuffles through it with legs held stiff. Most of the rhinoceroses follow their own dung pile or may also share the same by other rhinos. Dung piles are occurring at the borders of forest and grassland, on riverbanks, near wallows and paths, roads or ditches. Similar observations were also recorded by Laurie (1978) and Dinerstein and Price (1991). Adult males urinate backwards, as far as 3–4 meters behind them.

1.5.6 Home range, Local migration and Stray out behaviour: The home range of an animal is the area where it spends its time; it is the region that encompasses all the resources the animal requires to survive and reproduce. Thus home range is the geographic area to which an organism normally confines its activity. Movement of Rhino from one natural habitat to other is categorized as local migration. The Kaziranga National Park has been providing a suitable condition for rhino and in course of time from Kaziranga National Park the species has started migrating and established itself in other neighbouring wildlife sanctuaries. Occasionally, the animal covered more than 100 km distance during this kind of migration. During their movement, they normally raid the domestic or cultivated crops. During the study period there were several incidences of local migration of rhino were noticed and recorded. In one such incidence

of migration one rhino migrated from Kaziranga National Park to North Lakhimpur district of Assam which is located in more than 250 km away from the national park. Rhino are good swimmer and they even cross big river like Brahmaputra. In another example of stray out one rhino was found to cross river like Brahmaputra and entered in a village of district Jorhat (The Times of India, 23rd Dec., 2013; 26th Dec. 2013, DY365 and 27th Dec. 2013,). In two other incidence of stray out rhino they migrate to Majuli (100 km away from Kaziranga NP) which is a river island of Brahmaputra River. Various studied showed that the rhino could move from Pobitora Wildlife Sanctuary to Orang National Park. (Bhattacharyya, 1991; Talukdar *et al*., 2007; Das and Goswami, 2012b).

1.5.7 Social behaviour: The adult males Indian Rhinoceros are generally solitary, except for mating and fighting. Adult females are largely solitary when they are without calves. Mothers will stay close to their calves. Sub adult males and females mostly form groupings. Indian Rhinos also form short-term groupings, particularly during wallowing. The varieties of vocalizations of Indian Rhinoceros are snorting, honking, bleating, roaring, squeak-panting, moo-grunting, shrieking, groaning etc. The Indian Rhino has pedal scent glands which are used to mark their presence at these rhino latrines. Indian Rhinos are often greeting each other by waving or bobbing their heads, mounting flanks, nuzzling noses, or licking.

1.6 Sexual dimorphism of One-horned Rhino

Sexual dimorphism is the differences in appearance between males and females of the same species, as in colour, shape, size, and structure that are caused by the inheritance of one or the other sexual pattern in the genetic material. In Greater One-horned Rhinos, pronounced sexual dimorphism is apparent in the length of the lower

outer mandibular incisors (tusks), which are longer and wider in males than in females. Female Indian rhino often have slightly narrower longer horns than males. Dimorphism is also apparent in their massive neck and upper shoulder muscles, which are more extensively developed in adult males. These muscles provide the force behind the slashing and gouging with the incisors. The extensive primary and secondary neck and shoulder folds found in dominant males may serve for display in head-on confrontations between rival males, and to deflect the penetration of an opponent's incisors from the neck, chest, and shoulder area. This is the region where most severe attacks first occur before one male inevitably turns and runs from the other. The Greater One-horned Rhinoceros is believed to have poor eyesight. The head-on display, which often precedes combat, occurs when males are within a few meters of one another. A female Indian rhino weighs about 1600 kg; a male weighs about: 2200 kg. The average height of a female is 1.6 m; for a male, it is 1.8 m. Mating takes place throughout the year. A female Indian rhino is fully grown at 4 years in captivity but not until about 6.5 years in the wild. A male Indian rhino is fully grown at 8 years of age in captivity but at about 10 years of age in the wild. The gestation period is between 16 and 18 months and single calf is born during the rainy season (Laurie, 1978). The longevity or life span of a rhino in the wild is 30-35 years and in captivity 45 years or more (Bhattacharyya, 1991). Usually mother rhino keeps her calf away from other rhino and is very aggressive. This period is crucial to new born calf as it is vulnerable to predation. The sexual dimorphism is also seen in breeding biology and foraging ecology. Courtship in Indian rhino is most violent where the male become more aggressive. The 4 years old males born in captivity may become much larger than females but in wild born animal the male smaller than female of same age group (Dinerstein, 1991).

Table 1.3 Home-range sizes reported in the literature and present study (only in *Rhinoceros unicornis*) for the five rhinoceros species

Rhino Species	Sex	Home Range Size (km^2)	Reference
Rhinoceros unicornis	Female	20 km^2	Laurie (1982)
	Sub-adult female	14-18 (in KNP)	This study
	Female (with mature calf)	10-16 (in KNP)	do
	Female (with small calf)	6-12 (in PWLS)	do
		5-10 (in KNP)	
	Male	2–4 km^2 core 3–4 km^2	Laurie (1982)
	Adult male	15-21 (in ONP)	This study
	Sub-adult male	18 (in KNP)	do
	Adult male	20 (in PWLS) 18 (in KNP)	do
Diceros bicornis	Male	2·59–51·8 km^2	Goddard (1967
	Female	2·59–90·6 km^2	
	Female	12·5–47·3 km^2	Kiwia (1989)
	Male	69 km^2	
Rhinoceros sondaicus	Female (no calf)	10 km^2	Schenkel & Schenkel Hullinger (1969b)
	Female (with calf)	2-3 km^2	
	Male	20 km^2	
Dicerorhinus sumatrensis	Female	10–15 km^2	Van Strien (1986)
	Male	50 km^2	
Ceratotherium simum	Female	9–20 km^2	Owen-Smith (1975)
	Male	0·75–2·6 km^2*	
	Male	97 km^2	van Gyseghem (1984)
	Female	30 km^2	

1.7 Exo-skeletal structure: Rhino Horn

Exoskeletal structures of epiblastic origin may be developed on both the inner and outer surfaces of the Malpighian layer of the epidermis. Those developed on the outer surface include hairs, feathers, scales, nails, beaks and tortoise shell; and are specially found in higher vertebrates. Nails, claws, hoofs, and the horns of Oxen are also epidermal, as are such structures as the scales of reptiles, of birds' feet, and of *Manis* among mammals, the rattle of the rattlesnake, the nasal horns of *Rhinoceros*, and the baleen of whales. The rhinoceros horn is made of keratin which is similar to the protein that makes up fingernails and hair. The Javan and Indian Rhinoceros have one horn while the Sumatran Rhinoceros and African species possess two horns. Horns are perhaps the most conspicuous feature of rhinos everyone can recognize. Among the five Rhino species, the Black and White Rhinos have the largest horns. Black Rhinoceroses have two horns; the anterior horn can reach 130 cm in some individuals. The posterior horn is much smaller, ranging from 2–55 cm. Average anterior horn length for the White Rhino is 94–102 cm and posterior horn length is up to 55 cm. Among the Asian rhinos, the Sumatran also has two horns, but they are much smaller than in the other four species. The size of the horns varies, but typically they are larger in males. Often only the nasal horn is conspicuous and the second or frontal horn is much reduced in size. Horn lengths from some museum specimens are large (25–80 cm), but may not be indicative of average size. The Greater One-horned and Javan are intermediate in horn size and only possess one horn. Horn length in Greater One-horned Rhinos averages 25 cm in adult males and 24 cm in adult females; in Javan Rhino males it also averages 25 cm. There are reports of female Javan Rhinos without horns. Horn wear can be extensive in older animals; horns can even be broken off. Broken horns regrow over

time. Indeed rhino horns grow continually from the base (as much as 7 cm per year in White Rhinos), but horn wear may reduce any actual increase in the size of horns (Bhattacharyya, 1991; Laurie, 1978).

1.8 Threats to Rhinoceros

All the five species of rhinoceros have been facing some common threats from time **i**mmemorial**.** Anthropogenic activities are the primary reason for species decline and extinction (Caughley, 1994). Some new threats have also arisen in course of time such as climate change, introduction or invasion of some exotic plant species such as *Mikania micrantha, Ipomoea* and *Mimosa* etc. Poaching is the single greatest threat to the rhino population all over the world, which has led to a massive population decline of all species of rhinoceros. Poaching has put intense pressure on *Rhinoceros unicornis* populations in Assam. Records shows that the intensity of rhinoceros death gradually increase in the last part of nineties century and continues to date. Rhino horn has two main uses: traditional use in Chinese medicine and ornamental use in some countries. Habitat destruction continues to be a threat to the rhino population. The hourly destruction of an estimated 240 acres of natural habitat on earth is directly attributable to the habitat loss of rhino. The ever-present poaching situation is a serious threat to the African black rhino population. During the late 1970s and in the 1980s, the population of black rhino was decreased by 40–90% in some regions. In 1981 only 10,000-15,000 remained, and since 1980 the species has probably disappeared from Angola, Botswana, Chad, Central African Republic, Ethiopia, Malawi, Mozambique, Somalia, Sudan, and Zambia. Kenya's black rhino population now numbers just 620, when a few decades ago it stood at over 20,000. The lowest numbers were seen in the mid-1980s, when just 300 individuals remained.

In spite of the constant pressure of poaching, positive result of conservation efforts have been seen, which has helped the number of greater one-horned grow from 600 to more than 3,169 individuals since 1975. Socio-political unrest is another threat to rhinoceros this was seen in Manas National Park in 1991 during the Bodo movements for which the rhino population was wiped out from the park. In Nepal, there has been a dramatic decline in the numbers (544 to 360) of rhino in Royal Chitwan National Park as a result of the Maoist insurgency in that country. Africa has also experienced decades of political and civil unrest that have had a devastating effect on wildlife including black rhino. Trading of rhino horn and other materials of wild animals in exchange for weapon has been going on among the poverty-stricken people of Africa. Due to scarcity of food and sexual behaviour the rhino tend to come out from wild habitat and meet with accidents. The passing of railways and highways across the protected areas causes several deaths of wild animals in Assam. *Mikania micrantha, Ipomoea* and *Mimosa* are some fast growing alien invasive plants which are not eaten by herbivores including rhino. To undertake a fruitful management of rhinoceros conservation, these disturbances should be overcome by habitat protection as well as by passing stringent legislation and programmes on the translocation of rhinos from high density habitat to the suitable rhino habitat (Das and Goswami, 2012a).

The splitting up of the once continuous habitat is now fragmented due to use by humans for agriculture, development of towns and cities, industries, construction of roads, or other purposes. 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). Loss of habitat and increasingly fragmented

landscapes contribute to species decline by interfering with natural dispersal mechanisms and population dynamics, particularly of highly mobile large mammal species. Habitat fragmentation can interrupt natural dispersal patterns; alter philopatry and mate selection, and effect juvenile survival (Bjornstad *et al*., 1998).

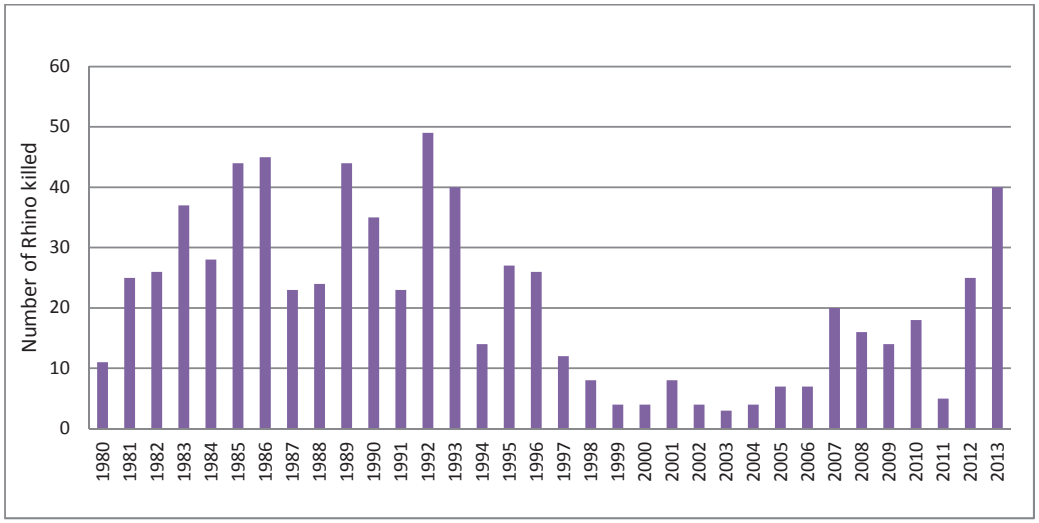


Figure 1.3 Rhinoceros poaching intensity in Kaziranga National Park of Assam since 1980 to 2013.

1.9 Conservation of Rhinoceros

1.9.1 Ex-situ and in-situ conservation approaches

In-situ conservation is the conservation management of species within their natural habitat. Ex-situ conservation is the species conservation initiatives outside the species' natural habitat. Ex-situ conservation involves maintaining individuals in "unnatural" environments under close supervision, i.e. zoos and gardens. The present day conservation efforts of rhinoceros mainly rely on the *in situ* conservation. Besides, formation of new breeding population through translocation to former habitats from

where the rhino population has wiped out due to poaching is also in progress. Captive breeding also help in the expansion of population of rhino.

1.9.2 Rehabilitation Project for Indian Rhino

To promote the survival of Indian rhinos in India some special packages and programmes have been initiated by Government in a collaborative effort with some other agencies like International Rhino Foundation (IRF), WWF and NGOs. The population in Pobitora wild life sanctuary has already exceeded the carrying capacity and the population needs to be reduced both to protect the habitat and to mitigate the ongoing and future rhino-human conflict as animals may move out from the protected areas into agricultural land. Therefore there is a need of long term practical management plan to find a solution. As a consequence, the Assam Forest Department and the various NGOs and institutes interested and involved in conservation of *Rhinoceros unicornis* have agreed that range expansion of the rhino in Assam through translocations from Kaziranga and Pobitora to other appropriate protected areas. Indian Rhino Vision 2020 is a plan which aims to increase the rhino populations in Assam to 3000 by the year 2020. These rhino populations will be built up at the seven protected areas to provide long-term viability to the rhino metapopulation in Assam. The IRV 2020 project will further improve the security of all rhinos in Assam by implementing law enforcement measures, by expanding the distribution of rhinos to reduce risks like disease, inbreeding depression and mass mortality, and by improving the security system for those protected areas where Indian rhinos already live. The project also aims to reduce the rhino population pressures in any single habitat by ensuring a better distribution of the rhino population over suitable ranges. In addition, the project concentrates on integrating the local communities into the conservation effort. IRV 2020 is coordinated

by the International Rhino Foundation, WWF-India and the Assam Forest Department with NGOs. The International Rhino Foundation (IRF) protects and conserves rhinos in areas where they most need attention. Reintroduction and artificial augmentation of populations of endangered species may therefore play an increasingly important role in conservation management and to compensate for compromised gene flow For conservation and safeguarding *R. unicornis* in Nepal, the Government of Nepal has initiated the Greater One-horned Rhinoceros Conservation Action Plan to put effective measures by a more cohesive, participatory and proactive approach towards conservation of the species and its habitat. Awareness and capacity building on rhino conservation has been initiated by some NGOs.

1.9.3 Conservation initiatives for other Rhino species

Several other in-situ Rhino conservation initiatives have been going on in different part of Asia and Africa. These are –

1. North Luangwa Conservation Programme, Zambia for Black rhino
2. Lowveld Rhino Trust, Zimbabwe for Black rhino white rhino
3. Save the Rhino Trust, Namibia for Black rhino
4. Ministry of Environment and Tourism, Namibia for Black rhino white rhino Rhino Protection Unit programme, Indonesia for Sumatran rhinos and Javan rhinos.
5. Sumatran Rhino Sanctuary, Indonesia for Sumatran rhinos

1.10 Molecular Genetics, Genetic diversity and Wildlife Conservation

The wildlife protection is solely depending upon the physical protection of the animals through continuous monitoring of the animals with the help of guards and protection against natural calamities such as flood. Though the physical protection of

wild animals is the sole way to protection but it is not the only way for long term conservation of wild animals. So, genetic study of such wild animal is necessary to find out the best method of conservation and propagation of wild animals. Genetic diversity refers to the variation at the level of heritable characters (polymorphism) and provides a mechanism for populations to adapt to their ever changing environment. The more variation, the higher the chance that at least some of the individuals will have an allelic variant that is suited for the new environment, and will produce offspring with the variant and will in turn reproduce and continue the population into subsequent generations. Genetic diversity comes in many forms. It can be measured in both individuals and populations. Genetic diversity can be haploid (DNA of the mitochondria), diploid, or even polyploid. Genetic traits can be based on the alleles at a single locus or many dozens of loci. Two common terms of genetic diversity are allelic diversity and heterozygosity. Allelic diversity refers to the number of different alleles at any given locus in the population. Heterozygosity is the percentage of loci that are heterozygous in a population or individual (Frankham *et al*., 2002). A heterozygous locus is one in which the two alleles are different (e.g., Aa as opposed to AA or aa). When the alleles are the same, the locus is said to be homozygous. Both allelic diversity and heterozygosity are desirable. Allelic diversity is important for a population's long-term ability to adapt, while heterozygosity is important for more immediate individual health (Allendorf, 1986). When a new allele appears in a population, it has the potential to change the genetic make-up of successive generations. Harmful mutations will likely not persist because the affected individual will either not survive, or will have limited reproductive success. However, some mutations may be passed on to successive generations because an organism with that allele is better equipped to survive in its

environment, that is, it has a selective advantage. Those individuals that produce a greater number of offspring that survive are said to be more fit. Other mutations may have no effect on phenotype, and may persist simply by chance (genetic drift). It is the selective advantage that drives evolution, albeit momentarily, in one direction or another (Russell, 2003). Loss of genetic diversity within populations might be associated with inbreeding depression, which in turn results in reduced fitness and ultimately jeopardizes the population persistence (Bonin *et al*., 2007). Few studies pointed out that intra-specific genetic diversity was also shown to favour species richness and to contribute to ecosystem functioning and recovery (Bonin *et al*., 2007; Dominguez *et al*., 2008).

Genetic variation at the population level consists of the differences in the types of alleles present and their frequencies across all members of a population considered together. Genetic variation within population is caused by change of allele frequencies over time due to selection, random genetic drift and gene flow (immigration from or emigration to other population). Genetic variation can be partitioned into two complementary but disconnected components that have to be assessed separately and differently (Bonin *et al*., 2007). The first is the selected (or functional) diversity arising directly from adaptive evolution due to natural selection and second is the neutral heritage of the population resulting from the effects of neutral evolutionary forces such as genetic drift, mutation, or migration. As a result of this, studies of genetic variability for population management are thus of two kinds. On the one hand quantitative traits that receive particular attention because they are the primary targets of natural selection and the variation at such traits is thus supposed to mirror the adaptive potential of the population or species (Bonin *et al*., 2007). Some authors have underlined the

importance of neutral genetic variation in conservation (Moritz, 1994a and 1994b). Genetic variation within population is important as it is related to heterozygosity, which is known to enhance fitness-related characteristics (Allendorf, 1986). The mean number of alleles (MNA) detected in each population and the expected heterozygosities are good indicators of the genetic polymorphism within the populations. The MNA is the average number of alleles observed in a population, while the expected heterozygosities are the proportion of heterozygote expected in a population. Numbers of alleles per locus per population are obtained by direct counting. Generally, the MNA is dependent on the sample size because of the presence of unique alleles that occur in low frequencies in populations and also because the number of observed alleles tend to increase with increases with population size (Nei, 1987). Therefore the comparison of the MNA between samples of different sizes may not be meaningful unless the sample sizes are more or less the same (Nei, 1987; Hart and Clark, 1989). Variability between populations arises both from random processes (founder effects, demographic bottlenecks, genetic drift and mutations) and from local selection imposed by environment and humans (Hartl and Clark, 1997). The variability between populations is also the result of adaptation of populations to their local conditions. The locally adapted populations may have particular genes or gene combinations critical for viability in their local environment.

1.11 Population genetics and use of molecular tool

A population can be defined as a group of conspecific individuals forming a breeding unit sharing a particular habitat at a certain time (Slatkin, 1993). In a broad sense a population may be defined as an assemblage of living beings that presents a closely interacting system. Thus population is a community of similar individuals living

within a circumscribed area at a given time and capable of interbreeding. Population genetics can be defined as the science of how genetic variation is distributed among species, populations and individuals, and it is concerned with how the evolutionary forces of mutation, selection, random genetic drift and migration affect the distribution of genetic variability (Hansen, 2003). Population genetics seeks to understand the causes of observable genetic variation in populations and to explain the underlying genetic basis for evolutionary change. It includes an empirical aspect, which measures and quantifies the genetic variation in populations, and a theoretical or statistical side, which attempts to explain the variation in terms of mathematical models of the forces that can change gene frequencies. The genetic structure of a population is described by the total of all allele frequencies in the gene pool. In the case of diploid or polyploid sexually interbreeding species, the genetic structure is also characterized by the distribution of alleles into genotypes. The genetic structure of a species can vary both geographically and temporally. The classical and neutral mutation models generate testable hypotheses and are used to explain how much genetic variation should exist within natural populations and what processes could be responsible for the observed variation. According to Russell (2003), mutation, genetic drift, migration, and natural selection are process that can alter the allelic frequencies of population. Population genetics is concerned with the analysis of demographic and evolutionary factors affecting the genetic composition of a population (Jorde *et al.,* 2001). It is the application of Mendel's laws and other genetic principles to entire populations of organisms (Hartl and Clark, 1989). The factors that diminish genetic diversity within populations are genetic bottlenecks, random genetic drift and inbreeding. However, the major threats to genetic diversity that result from human activity are habitat destruction

and degradation, pollution, introduction of exotic species, and over-exploitation (Frankham, 1994). Loss of genetic diversity among-populations occurs when historically divergent and isolated populations experience an artificially high rate of gene flow from other populations. Therefore, biologists suggest some criteria for the urgent need for characterize conservation "units" that are the most worthwhile in preservation of species. Among these criteria, the amount of intra-specific genetic variation is now widely accepted as a key parameter to determine populations to prioritize for protection purposes.

1.12 Importance of genetic diversity in Conservation of wild animals

Genetic variability is thought to be essential for the long-term persistence and adaptability of populations, and thus important in the management of captive and wild populations of endangered 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. When a population is greatly reduced in size, rare alleles in the population are lost if no individuals possessing these alleles survive (Carson, 1983). The declining of rare alleles in the population causes overall declines of fitness in the population (Primack, 1993). Thus genetic effects can have important implications for the persistence of any population. Genetic variability is lost slowly, since loss is dependent on the number of generations the population has spent at its reduced size. When populations become contract and genetic variation is reduced, deleterious alleles may be "purged", leading to only short-term effects. If genetic diversity becomes low at many genes of a species, that species becomes increasingly at risk. It has only one possible choice of information at all or nearly all of its genes—in other words, all the individuals are nearly identical. If

new pressures (such as environmental disasters) occur, a population with high genetic diversity has a greater chance of having at least some individuals with a genetic makeup that allows them to survive. If genetic diversity is very low, none of the individuals in a population may have the characteristics needed to cope with the new environmental conditions; such a population could be suddenly wiped out. The genetic diversity of a species is always open to change. No matter how many variants of a gene are present in a population today, only the variants that survive in the next generation can contribute to species diversity in the future. Loss of genetic diversity may result due to inbreeding and increased genetic drift common to small populations – effects similar to populations exhibiting founder effects – leading to a reduction in heterozygosity (Hedrick *et al.,* 2001; Hawley *et al.,* 2006). To ensure ability to adapt to changing environments and to preserve the possibility of future speciation of a wild animal the management plans should include plans for maintaining existing genetic diversity of the species (Lande, 1988). The loss of genetic variability reduces the capacity of a population to respond to selection. Once gene variants are lost, they cannot be recovered. This is because genetic diversity is the basis for a species evolutionary flexibility and responsiveness to environmental changes. For conservation of animal diversity, it is therefore, important that both within and between populations variation be maintained.

The applications of conservation genetics include analyzing fragmented populations in nature, determining units of conservation in nature, and monitoring captive populations. Some of the most common issues addressed by genetic techniques in conservation are those confronting small or fragmented populations. Genetics can help conservation biologists do viability analyses by testing hypotheses concerning how long genetic variation might persist into the future. This might be done by examining

current levels of genetic variation in a species or population, and integrating these pieces of information with demographic and life history models to examine what happens to genetic variation over time. The use of a conservation genetics approach may be an effective way for assessing the status of populations and species in the wild. Conservation genetics permits scientists to assess the impacts of habitat fragmentation and loss in the wild using both theoretical and empirical methods. Results from these studies allow managers to evaluate the viability of populations and design protected areas for conservation. The conservation initiatives are also concerned with the translocation or reintroduction of animals to areas where they have been extirpated or severely depleted. Such measures require a detailed understanding of the genetics of the populations being reintroduced in order to ensure there is compatibility between populations as well as to maximize genetic variation and minimize the chance of inbreeding among related animals. Determining the extent of genetic variation among captive populations in zoological parks is also essential, because captive populations must have sufficient genetic variation so that they persist into the future without suffering from reduced fitness due to inbreeding and other effects associated with small populations. In some cases, captive populations may be viewed as a source for improving genetically or numerically depleted wild populations (Ballou and Foose, 1994). The primary goal of conservation genetics is to maintain a genetically diverse, healthy and self-sustaining population which is demographically stable.

1.13 Aspects of genetic study

The developments of DNA-based genetic markers have a revolutionary impact on animal genetics. DNA genetic markers have now been used for the molecular genetic characterization and genetic diversity studies in numerous species. Allozymes,

mitochondrial DNA, RFLP, RAPD, AFLP, microsatellite, SNP, and EST markers are the popular genetic markers. Out of these the following two markers have been widely used for study of genetic study of a population.

1.13.1 Mitochondrial DNA Analysis in Relation to Genetic Diversity Analyses

To study the genetic diversity, population structure and population evolution of wild animals, mitochondrial DNA has been widely employed because of its higher mutation rate and information of recent evolutionary events (Brown *et al*., 1979; Avise, 1994; Ballard and Whitlock, 2004; Kim *et al.,* 2002). The vertebrate mitochondrial genome is composed of about 15 to 20 kb in different organisms, coding for 40 genes responsible for 2 ribosomal RNAs, 22 transfer RNAs, and 13 proteins essential in respiration (Ferris and Berg, 1987; Hartl and Clark, 1997). The mitochondrial DNA has a non-coding region responsible for replication, known as the "control region" or "D-loop", that evolves 4 – 5 times faster than the entire mtDNA molecule which itself evolves 5 to 10 times faster than nuclear DNA (Brown *et al.*, 1979). The mitochondria do not have repair enzymes for errors in the replication, for the damages of the DNA (Clayton, 1982). Moreover, the mitochondrial DNA has high replication rate because it lacks histone-like proteins and the production of reactive oxygen species (Rose *et al*., 2007). This may result in heteroplasmy, namely, the presence of a mixture of more than one type of mtDNA in the same cell. The consequences of mtDNA heteroplasmic mutations are dependent on the type and location of the mutations, replication rate of the cell and mtDNA segregation.

The D–loop segment exhibits a comparatively higher level of variation than protein–coding sequences due to reduced functional constraints and relaxed selection pressure. The length of the D–loop is approximately 1 kb and it can easily be amplified

by PCR prior to sequencing to determine the molecular diversity. Sequence analysis of the this region of mt DNA has been used to measure molecular diversity, population structure and gene flow among the populations and to identify conservation units for better management of wild species (Onuma *et al*., 2006; Brown *et al*., 1986; Idaghdour *et al*., 2004; Wu *et al*., 2006; Hu *et al*., 2006). Accumulation of mtDNA mutations tends to increase with age. Thus, the mtDNA has a high level of transitions and transversions, as well as high incidences of small length mutations (Cann and Wilson, 1983).

1.13.2 Microsatellites Analysis in Relation to Genetic Diversity Analyses

Microsatellites are tandem repeats of 1–6 nucleotides found at high frequency in the nuclear genomes of most taxa. Microsatellites also called Simple Tandem Repeats (STRs) which are highly polymorphic class of genetic markers (Weber and May, 1989). A microsatellite locus typically varies in length between 5 and 40 repeats, but longer strings of repeats are possible. Dinucleotide, trinucleotide and tetranucleotide repeats are the most common choices for molecular genetic studies. Dinucleotide repeats account for the majority of microsatellites for many species (Li *et al*., 2002). Trinucleotide and hexanucleotide repeats are the most likely repeat classes to appear in coding regions because they do not cause a frame shift (Toth *et al.,* 2000). Mononucleotide repeats are less reliable because of problems with amplification; longer repeat types are less common, and fewer data exist to examine their evolution (Li *et al*., 2002). The DNA surrounding a microsatellite locus is termed the flanking region. Because the sequences of flanking regions are generally conserved across individuals of the same species and sometimes of different species, a particular microsatellite locus can often be identified by its flanking sequences. The majority of microsatellites are found in non-coding regions of genome. However Morin *et al.* (1994) also reported the

presence of microsatellite in protein coding region exhibiting regulatory role in gene expression and trinucleotide repeats have been used for linkage analysis in association with disease susceptibility genes in human (Richards and Sutherland, 1994). Short stretches of DNA, called oligonucleotides or primers, can be designed to bind to the flanking region and guide the amplification of a microsatellite locus with polymerase chain reaction (PCR). Microsatellite are usually not within the coding regions of genes. Therefore, unless closely linked to the coding DNA regions or to regions under selection, microsatellite based variations are neutral and variation in these loci is not affected by selection. Hence, microsatellite loci provide unbiased information about the level of genetic diversity of a genome (Jobling *et al*., 2004). The mutation rates of microsatellites are estimated around 10^3 to 10^4 per locus per generation. Microsatellite follows Mendelian co-dominant inheritance and scoring is done through polymerase chain reaction. Moreover microsatellite variation is independent of age, sex and environmental changes and hence can be detected at the early stage of development. This makes microsatellites useful for studying evolution over short time spans as it is for domestic animals (hundreds or thousands of years), whereas nuclear base pair substitutions are more useful for studying evolution over long time spans (millions of years).

1.14 Aims and Objectives

The aim of the present study was to assess the genetic diversity in the wild population of Greater One Horned Rhinoceros, *Rhinoceros unicornis* found in the three protected areas of Assam, India. The objectives are------

1. To study genetic divergence of Indian rhinoceros population through control region (D-loop) of mitochondrial DNA.
2. To study genetic diversity of Indian rhinoceros by analysis of polymorphic microsatellite of nuclear DNA.

CHAPTER- 2

REVIEW OF LITERATURE

2.1 History, distribution, ecology and behaviour of *Rhinoceros unicornis*

The first scientific description on the Greater Indian One-Horned Rhinoceros was given by James Parsons in 1743 accompanied by a sketch of a male Indian rhinoceros (Parsons, 1743; Thomas, 1801; Clarke, 1973; Rookmaaker, 1978). The life history of the Greater Indian One-horned Rhinoceros was studied by Gee (1953a, 1953b). William Andrew Laurie made a comprehensive study on Ecology and behaviour of *Rhinoceros unicornis* in Nepal (Laurie, 1978) which covered several aspects such as population dynamics, diurnal time budgeting, food and feeding, reproductive and social behaviour of the Indian rhino in Chitwan National Park of Nepal. The historical distribution of Rhinoceros in India, Pakistan, Nepal, China and Bangladesh was studied by Rookmaaker (1980, 1982, and 2002) and Myanmar by Lwin (1989). A number of studies have been conducted by different researcher on the status and distribution of Indian Rhino (Stracey, 1957; Gee, 1959, 1963; Choudhury, 1985, 1989). Certain aspects of biology of Indian Rhino, taxonomic status, causes of mortality and some anatomical studies were performed by Bhattacharyya (1991). Price (1991) studied the demographic and habitat use pattern of the Indian rhino in terai grassland habitat in Kaziranga National Park of Assam, Mary *et al.* (1998) studied the feeding and territorial bahaviour of Indian rhino and Kushwaha *et al.* (2000 and 2002) evaluate the landmass dynamics and habitat suitability analysis for Indian rhino through remote sensing and

geospatial modelling. Studies on the Eco-Status of the Indian Rhinoceros with special reference to altered habitat due to human interference were done by Ghosh (1991) at Jaldapara Wildlife Sanctuary of West Bengal. Hazarika and Saikia (2007, 2009, 2010 and 2011) conducted a number studies on the Habitat classification and Habitat Utilization, home range and Habitat utilization pattern of Great Indian One-Horned Rhino in the Rajiv Gandhi Orang National Park of Assam. Goswami (1993) studied on analysis of certain strategies of conservation and propagation of *Rhinoceros unicornis*. Banerjee (2001) studied on chemical composition of the some food plants of rhino in Kaziranga National Park. In Nepal, an intensive study on Ecology, behaviour, vegetation modifying factors and Habitat/ animal interactions of Rhino was carried out by Dinerstein (1979a, 1979b, 1991 and 2003). In another study conducted by Dinerstein and Price (1991), the demography and habitat use by greater one horned rhinoceros in Nepal was covered. The population ecology, habitat preference, food ecology and variation of home ranges of male and female rhino in Royal Bardia National Park of Nepal were studied by Jnawali (1995). In this study the author also made a comparison between the food plants of rhino found in the Chitwan National Park and Royal Bardia National Park of Nepal.

Phylogenetic study of Rhinoceros species including Indian rhino was done by different authors (Groves, 1983; Cerdeno, 1995; Prothero *et al.,* 1986; Steiner and Ryder, 2011). The study on population differentiation, infant health and size dimorphism of Indian Rhino was conducted by Zschokke and Baur (2002) and Pluhacek *et al.* (2007).

2.2 Genetic studies of Rhinoceros species

The literature on genetic study of Indian rhino is scanty. So this review comprises

various genetic studies conducted on different species of rhino. Amato *et al.* (1993) conducted study on molecular evolution in living species of rhinoceros and its implications for conservation. They provided strong argument on the reintroduction of genetically healthy rhinoceros population in its former habitats and in situ conservation after thorough study of about the habitat regarding environmental factors.

Ashley *et al.* (1990) examined mtDNA RFLPs of black rhinos from three different geographic populations: Zimbabwe (*D. b. minor*), South Africa (*D. b. minor*) and Kenya (*D. b. michaeli*). They found a small amount of intraspecific variation, with only three mtDNA haplotypes; one unique haploptype in Kenya (*D. b. michaeli*), one unique haplotype in Zimbabwe (*D. b. minor*) and one shared with Zimbabwe and South Africa (*D. b. minor*). The DNA sequences of all three haplotypes were similar which led the authors to conclude that the subspecies had recently shared a common ancestor.

Swart and Ferguson (1997) studied the conservation implications of genetic differentiation in southern African populations of black rhinoceros. They concluded that the four populations were con-specific isolated remnants of a large ancestral population; none of the populations belonged to discrete subspecies but were instead part of a west-to-east 'genetic continuum' where by the Etosha (Namibia) and KZN (South Africa) populations are the extremes, but mere subsets of the Zambezi (Zimbabwe) population. They concluded that short-term genetic management for the species was unnecessary due to large genetic variation and no evidence of inbreeding or excess in homozygosity. They recommended immediate genetic management in order to maintain the level of variability in the Zambezi, Zimbabwe population.

Morales and Melnick (1994) had worked on the molecular systematics of the living rhinoceros species. Merenlender *et al.* (1989) studied on allozyme variation and

differentiation in African and Indian rhinoceroses and observed a significant lack of genetic variability across 25-30 loci in the four rhino taxa (*C. s. simum, C. s. cottoni, D. bicornis* and *R. unicornis*). Allozymes are soluble protein-coding enzymes, usually taken from blood, kidney or liver, mixed with a buffer and separated by their charge or molecular weight on an eletrophoresis gel. They concluded that the low levels of genetic variation were likely caused by recent historic demographic bottlenecks.

The genetic analyses comparing the entire 12S rRNA gene and fragments of the cytochrome *b* (*cyt b*) regions of mitochondrial DNA (mtDNA) of five extant rhinoceros species was done by Tougard *et al.* (2001). The analysis comprises phylogenetic analysis of the complete sequences of the mitochondrial 12S rRNA and cytochrome *b* genes. The analysis identified a basal rhinocerotid divergence between the African and the Asian species, with the Sumatran rhinoceros forming the sister group of the genus *Rhinoceros.* The study also provided insight on the evolution different species of rhinoceros.

A significant work was carried out by Xu *et al.* (1996) and Xu and Arnason (1997) where complete nucleotide sequence of the mitochondrial genomes (16,832 bp) of Indian rhinos was studied. They examined evolution of individual peptide-coding genes by comparison with a distantly related perissodactyl, the horse, and the relationships among the orders Carnivora, Perissodactyla and Artiodactyla.

Dinerstein and McCracken (1990) worked on the genetic diversity of one-horned rhinoceros population in Nepal and found high levels of genetic diversity while analysed from protein electrophoresis.

Brown and Houlden (1999) and Cunningham *et al.* (1999) were the first to specifically isolate microsatellite sequences from black rhinos. Brown and Houlden

(2000) sequenced the non-coding mtDNA control region of captive *D. b. michaeli* and wild captured Zimbabwe *D. b. minor* to examine evolutionary relationships. Five haplotypes were found in the nine *D. b. minor* samples with a haplotype diversity of 0.86.

Orlando *et al.* (2003) conducted research on ancient DNA analysis which reveals evolutionary relationship with Wooly Rhinoceros. They sequenced enitre12S rRNA and partial cytochrome b gene from ancient samples and found relation with the modern rhinoceros *Dicerorhinus sumatrensis.*

Hsieh *et al.* (2003) proposed the utility of cytochrome b gene in species identification of rhinoceros horns. The authors used 402 bp fragment of cytochrome b gene. The results showed that among rhinoceros species, the greatest genetic distance was between black and Indian rhinoceros. The results of this phylogenetic study also showed that there were four major branches among rhinoceros species from a common origin. According to authors the method can be applied in the identification of processed products of rhinoceros horns, such as sculptures, daggers, powders or even mixture powdered prescriptions.

Morales and Melnick (1994) studied fossil evidence along with genetic analyses comparing the entire 12S rRNA gene (840-975bp) and fragments of the cytochrome b (total 688bp) regions of mitochondrial DNA (mtDNA) for all rhino taxa species. Morales *et al.* (1997) studied mitochondrial DNA variability and conservation genetics of the Sumatran rhinoceros.

Ali *et al.* (1999) have done genetic assessment of Microsatellite Associated Sequence Amplification (MASA) in Indian rhino. Southern blot analysis of *R. unicornis* genomic DNA with pSS(R)2 and other synthetic oligo probes revealed a high level of

genetic homogeneity, which was also substantiated by microsatellite associated sequence amplification (MASA). Owing to its uniqueness, the pSS(R)2 probe has a potential application in the area of conservation biology for unequivocal identification of horn or other body tissues of *R. unicornis*. The MASA analysis indicates a high level of genetic homogeneity in the *R. unicornis* genome.

Garnier *et al.* (2001) conducted a genetic analysis using ten microsatellites and used DNA from faecal samples to increase the understanding of the mating system, reproductive skew and effective population size of a *D. b. minor* population in Save Valley, Zimbabwe.

Bollongino *et al.* (2003) worked on DNA Typing from rhinoceros horn. Phylogenetic analyses based on mtDNA data, including the entire sequence of the rhinoceros *12S* gene as well as partial regions of cytochrome b and D-loop (total 805bp) genes was done to support the grouping of two rhinoceros species, the Javan and Indian rhinos into a single genus (Orlando *et al.,* 2003; Fernando *et al.,* 2006).

Harley *et al.* (2005) used nine of the black rhino microsatellite markers to establish baseline information regarding levels of genetic diversity and population differentiation in black rhino subspecies (*D. b. bicornis*; *D. b. minor*; *D. b. michaeli* and the now extinct *D. b. longipes* and *D. b. chobiensis* . They found that *D. b. michaeli* had the highest level of genetic diversity with an expected heterozygosity (*H*E) of 0.675 followed by *D. b. bicornis* (*H*E = 0.505) and *D. b. minor* (*H*E = 0.459).

Fernando *et al.* (2006) studied the genetic diversity, phylogeny and conservation of the Javan rhinoceros through mtDNA 12S rRNA gene and the non-coding D-loop region. They found that genetic diversity of Javan rhino in Ujung Kulon is likely to have suffered extreme genetic drift, from the additive effects of founder events,

bottlenecks, and persistent small population size. Phylogenetic analyses based on mtDNA data, including the entire sequence of the rhinoceros *12S* gene as well as partial regions of *cyt b* and D-loop (total 805bp) genes; support the grouping of the Javan and Indian rhinos into a single genus.

Hutchins and Kreger (2006) studied behaviour of rhinoceros which is important for survival both in range-country protected areas and captivity, and according to authors such knowledge should be used to provide the most appropriate animal care and implications for captive management and conservation.

Scott (2008) studied microsatellite variability in four contemporary rhinoceros species and their implications for conservation. The author used 24 taxon-specific rhinoceros microsatellite loci for estimation of comparative microsatellite genetic diversity within and among the four extant species of African and Asian rhinoceros. According to study the African black *michaeli* rhino subspecies had the highest level of microsatellite genetic variability of all available rhinos, while southern white and Indian rhinos were the least variable rhinos.

Nielsen *et al.* (2008) studied characterization of microsatellite loci in the black rhinoceros (Diceros bicornis) and white rhinoceros (Ceratotherium simum) and their use for cross-species amplification and differentiation between the two species. They designed 21 microsatellites for both black and white rhino, seven of which were polymorphic and were used to distinguish the two species from each other.

Van Coeverden de Groot *et al.* (2011) used nine polymorphic microsatellite loci to examine genetic diversity and structure of *D. b. bicornis* individuals of Etosha National Park, Namibia; a population that experienced a significant population increase due to increased protection. The results were to be utilized as a baseline with which

conservation managers can measure changes in the level of genetic variation in the future.

Karsten *et al.* (2011) used 10 microsatellites to evaluate levels of genetic diversity, differentiation and inbreeding among *D. b. minor* in seven game reserves in KwaZulu-Natal (KZN), South Africa and a single population of *D. b. minor* in Zimbabwe that was founded with black rhino from KZN.

Guerier *et al.* (2012) studied southern white rhinoceros in northern Namibia by use of microsatellite genotypes. The study also assess the variability and parentage including genetic diversity, pedigrees and management within the small managed population of southern white rhinoceros

Muya *et al.* (2011) studied the molecular variation and genetic structure in Kenya's black rhinoceros population. The study comprises using 408 bp of mitochondrial control region sequence and nine microsatellite loci. The results suggest that the Masai Mara is more differentiated, inbred and isolated than other subpopulations. It also suggests that there are neither distinct montane and lowland groups nor other detectable historical barriers to gene flow. According to authors future translocations should consider the genetic profile of individuals and the demographic history of both the donor and recipient subpopulations.

Zschokke *et al.* (2003) were first to developed eleven microsatellite loci of R. unicornis which are useful to analyse the genetic variability within and between the populations of Indian rhino. Zschokke *et al.* (2011) studied genetic differences between the two remaining wild populations of the Indian rhinoceros of India and Nepal by using the genetic markers mitochondrial D-loop region and microsatellite analysis in the few captive animals from different zoos of the world. The results of this study

demonstrated that both Assam and Nepal rhinoceros populations have high genetic diversity and the two populations are genetically distinct and that the origin of each individual can be assigned with a high confidence level. According to the authors both populations are presently completely isolated and cannot naturally interbreed and suggest for separate Management Units; and a strict breeding program should be followed to avoid crossing between individuals stemming from different populations.

CHAPTER-3

MATERIALS AND METHODS

3.1 Study areas

In the present genetic study, dung (faecal matter) samples were used as a source of DNA for study of mitochondrial DNA and microsatellites. Fresh dung samples were collected from the three major rhino bearing areas of Assam namely Kaziranga National Park, Rajiv Gandhi Orang National Park and Pobitora Wildlife Sanctuary.

3.1.1 Kaziranga National Park

Kaziranga National Park (KNP) is situated at the civil jurisdictions of Sonitpur, Nagaon and Golaghat districts of Assam within the geographical limits of 26°30'-26°45'N and 93°00'-93°45'E. The Brahmaputra River is flowing in the north and Karbi Anglong hills on the southern boundary of the park. The Diffolu River originating from Karbi Anglong Hills to the south flows east to west and divides the park in two sections before merging into the Brahmaputra. Deopani and Mora- Diffolu are the other two rivers that drain through the park. The original area of KNP is about 429.93 sq. Km. But additions of six new areas including river Brahmaputra and its islands the area of the Park has now increased to 860 sq. km. However, mapping by remote sensing method the total area of Park is recorded as 985 sq. km. (Kushwaha, 2008). Including Laokhowa and Buhrachapori Wildlife Sanctuaries the total area is 1033 sq. Km.

Kaziranga was declared as a reserve forest in January, 1908 with an initial area of 226.2 km^2. Subsequently, more areas were added and it declared as a game sanctuary.

Kaziranga acquired the status of wildlife sanctuary in 1926. The Assam Rhinoceros Bill was passed in 1954 and steps were initiated to conserve the rhino, the flagship species and its habitat. Finally, Kaziranga was declared as national park in February, 1974. In the year 1985, the Kaziranga National Park was declared as World Heritage Site by UNESCO under criteria N (ix) and N(x) of the natural heritage (Vasu, 2003).

The Kaziranga National Park has rich fauna comprising about 15 mammal, 490 bird and 25 reptile species. The park holds the largest population of Indian one-horned rhinoceros (*Rhinoceros unicornis*). The other important mammal species are: tiger (*Panthera tigris*), elephant (*Elephas maximus*), Asiatic water buffalo (*Bubalus arnee*), hog deer (*Axis porcinus*), sambar (*Cervus unicolor*), Hoolock gibbon (*Hylobates hoolock*) wild boar (*Sus scrofa*), capped langur (*Trachypithecus pileatus*) and rhesus maccaca (*Macaca mulatta*), sloth bear (*Melursus ursinus*), otters (*Lutra lutra and L. perspicillata*), Gangetic dolphin (*Plantanista gangetica*), barking deer (*Muntiacus muntjak*) and Malayan giant squirrel (*Ratufa bicolor*).

The vegetation of Kaziranga National Park consists of alluvial inundated grasslands and reedbeds, alluvial savanna woodland, tropical moist mixed deciduous forests and tropical semi-evergreen forests (Talukdar, 1995). Among the different high grass species, *Saccharum spontaneum*, *S. naranga*, *S. procerum, Imperata cylindrica*, *Erianthus ravennae*, *Arundo donax* and *Phragmites karka* predominate. The short grass species grow around beels. Theae comprises: *Hemarthia compressa*, *Cynodon dactylon*, *Cenchrus ciliaris*, *Crysopogon aciculate* and *Andropogon* sp., which have high forage value in contrast to tall grasses.

Geographically, KNP is mainly comprised of alluvial deposits of the mighty river Brahmaputra and its smaller tributaries. KNP mainly comprises of recent composite

alluvial plains and floodplains. The park is characterized by numerous swamps or beels (wetland) complexes, along with a thick vegetation cover. The Brahamputra river flowing along the northern boundary of the Park forming numerous river island (*char*). The landscape of KNP is dominated by riverine habitat (58.55%) i.e. Brahmaputra river and its sand, followed by Grassland (23.27%), Woodlands (12.27%), Wetlands and beels (5.18%). Besides the river Mora and Jiya Diffolu comprises 0.69% of the park (Kushwaha, 2008). The climate of the park is subtropical monsoon, with mean rainfall of 2220 mm, maximum and minimum temperature range between 38-5˚C (Khuswaha and Unni, 1986). The relative humidity is generally high in most part of the year. It rises as high as above 90 percent during monsoon.

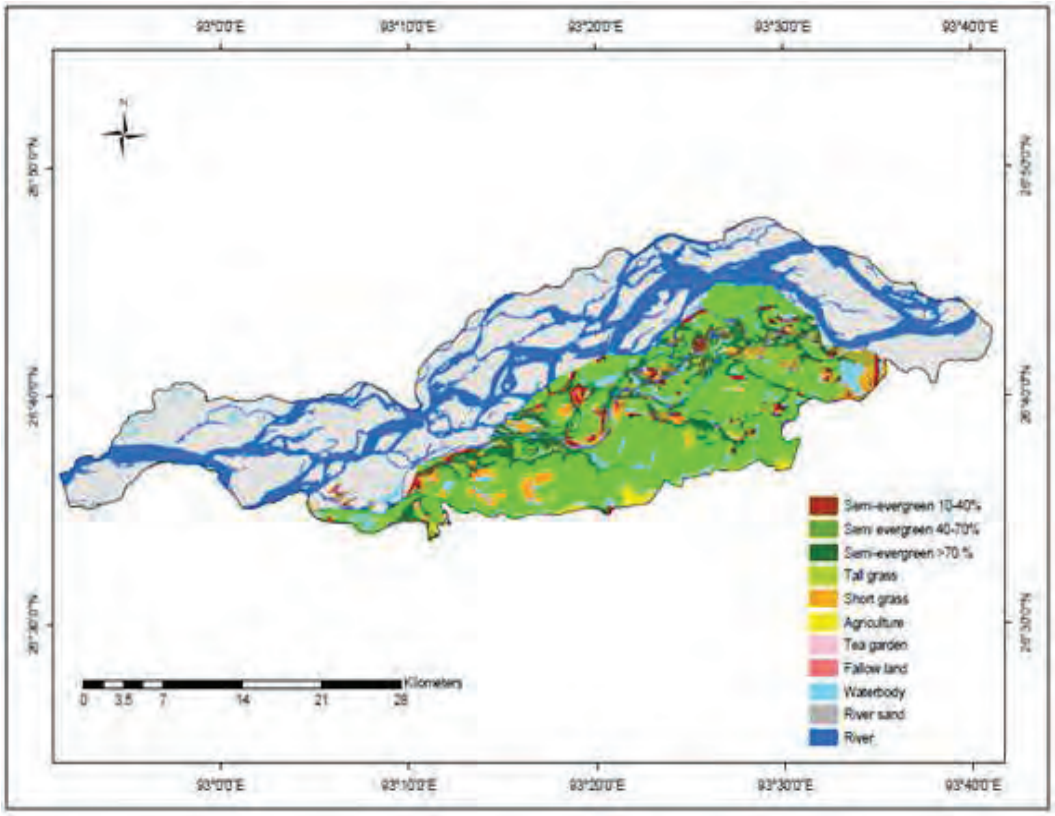


Figure 3.1 Forest and land cover map of Kaziranga National Park (Kushwaha, 2008)

Figure 3.2 Photograph showing grazing by *Rhinoceros unicornis* (a &b) in suitable habitats of Kaziranga (c,d &e) National Park and rhino dung (f)

3.1.2 Pobitora Wildlife Sanctuary

The Pobitora Wildlife Sanctuary is located between the latitude 26º12' to 26º16' N and longitudes 91º58' to 92º05' E in the Mayang Civil Circle of Morigaon District of Assam. The notified area of the Sanctuary is 38.81km². But the actual area covered by the sanctuary is less than the notified area. The river Brahmaputra and Kalong are flowing near the Sanctuary. The Sanctuary was a grazing reserve for cattle before 1971. In 1987 it was declared as Wildlife Sanctuary. The Sanctuary is surrounded by villages and agricultural land. Pobitora. The sanctuary possesses a good network of wetlands and many of these were perennial and as such very favoured areas for Indian rhino. Some of the major wetlands in the sanctuary are Garanga, Haduk, Sitalmari, Pagladova, Duboritoli and Dholi . There are a number of small Hillocks scattered along/ outside the sanctuary are Kasasila Hill, Hatimuria Hill, Kukuri Hill, Boha Hill, Kardia Hill etc (Bhatta, 2011). The soil structure depict that it belongs to *Archaean* Group. It consists of low level alluvium of clay, coarse sand, and gravel and boulder deposits. The clay loam was found in the plain and sandy loam in the hilly forest of the sanctuary. The swampy area is rich in peat deposition. The pH of soil varies from 5.34-5.95 (Bora and Kumar, 2003). The vegetation of the sanctuary is classified into four distinct forest type's i.e. Eastern alluvial grassland, Low alluvial savannah woodland, Barringtoia swamp forest and Northern moist mixed deciduous forest. The entire sanctuary is a basin like structure having woodland (18.44%), grassland (66.91%), wetland (14.91%) & hillock above 15 to 350 msl. Majority of the park area (54.8%) was occupied by grassland of which, 10.23% was found to tall grassland and 17.44% was short grassland. The dominant plant species are- *Bauhinia scandens, Butea paviflora, Byttneria grandifolia, Cayratia pedat, Imperata cylindrica, Cynodon dactylon, Vetiveria zizanioides*,

Sclerostachya fusca, Saccharum spontaneum, Paspalum scorbiulatum, Phragmitis karka, Arundo donax, Echinochloa crusgalli, Panicum auritum Amorphophallus bulbifera, Alpinia nigra, Antidesma acidum, Barringtonia acutangula, Costus specious, Ficus heterophylla, Saccharum spontaneum, Phragmitis karka, Syzygium cumini etc. Pobitora Wildlife Sanctuary also has a very rich collection of faunal species. Besides one-horned Rhinoceros, the sanctuary is a home of home to a large number of amphibian, reptiles, fish and water birds. Mammalian species found in the sanctuary includes Asiatic Water Buffalo, Wild Boar, Leopard, Jungle cat, Jackal etc. The rhinos of the sanctuary were found to be adapted to share the feeding ground with domestic cattle; it was commonly observed that rhinos grazed together with cattle. According to the population census there are 93 rhino in 2011. The sanctuary has high density of rhino in per square kilometre.

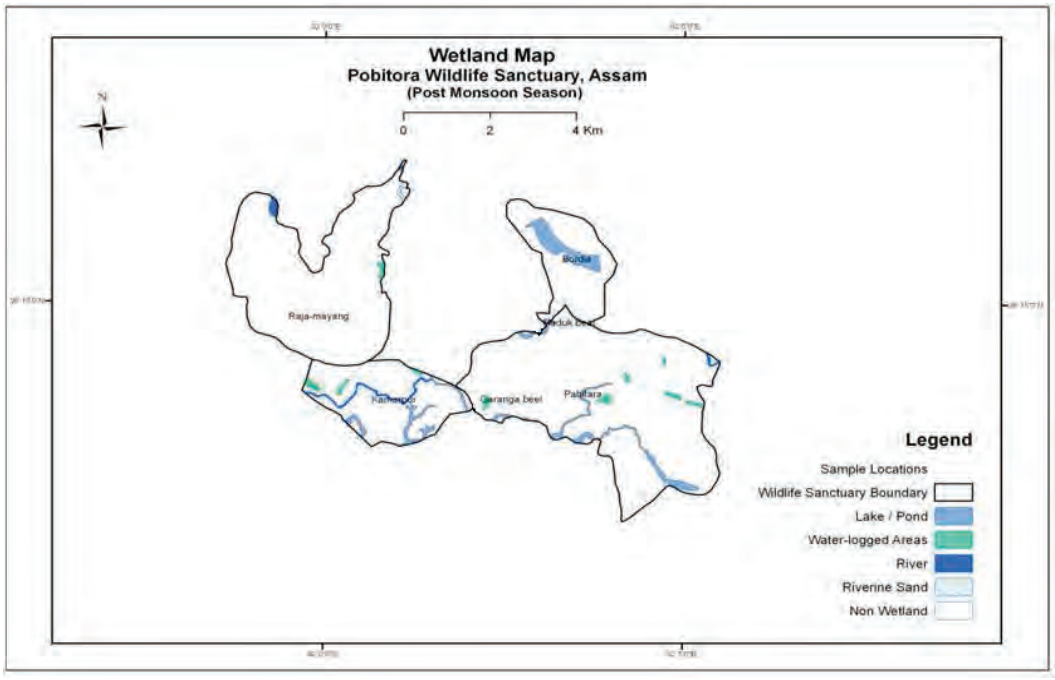


Figure 3.3 Map of Pobitora Wild Life Sanctuary (Source: Wetland map of Assam, ARSAC, 2011)

3.1.3 Orang National Park

The Orang National Park (also called Rajiv Gandhi (RG) Orang National Park) is situated in the north bank of the river Brahmaputra and within the administrative boundary of Darrang and Sonitpur districts of Assam, India and within the geographical limits of 26° 29' N to 26° 40' N latitude to 92° 16' E to 92° 27' E longitude. The National Park is comprises with alluvial soil located in the floodplain of the river Brahmaputra. The park is surrounded by human population except the southern boundary. The northern side is bounded by Nalbari and Rongagora villages of Darrang district. The eastern side is bounded by Borsola villages of Sonitpur district and river Pachnoi. The western side is bounded by river Dhansiri and Bogoribari village of Darrang district and the southern side is bounded by the river Brahmaputra.

According to Champion and Seth (1968) the habitat of Orang National Park is composed of mainly Eastern Himalayan Moist Deciduous forest, Eastern Seasonal Swamp forest, Khair-Sisoo Forest, Eastern Wet Alluvial Grassland and Plantations. The plant species commonly found in Orang National Park are *Legerstroemia parviflora, Terminalia belerica, Sterculia villosa, Salmalia malabarica, Semecarpus anacardium, Schima wallichi, Zizyphus mauritiana* etc. Among the grasses *Phragmites karka, Saccharum procerum, Saccharum spontaneum* and *Imperata cylindrical* are dominant.

The Greater One-Horned Rhino is the flagship species of the Orang NP. The other fauna sharing the habitat are Royal Bengal Tiger (*Penthera tigris),* Asiatic Elephant (*Elephas maximus)*, Hog Deer (*Axis porcinus),* Wild Pig (*Sus scrofa)* etc. The park also witnesses a diverse range of avifaunal diversity. Among the reptiles, *Genus Python, Kachuga tecta, Ophiophagus Hannah, Lissemys punctata* are found in the Park (Sarma,

2010). About 26.06% of the park is covered by wet alluvial grassland and 17.97% is covered by dry savannah grassland. About 8.22% of the park is covered by water body and 6.83% is covered by sandy area. The habitat suitability model analysis for rhino shows that 25.13% of the park is most suitable habitat for rhino (Sarma *et al.,* 2011). The rhino conservation in the Park was started with about 35 rhinos in the year 1972 (Talukdar, 2000) and rhino population in Orang National Park has been increasing from 1972 and according to census of 2013 the rhino population in Orang NP is 100.

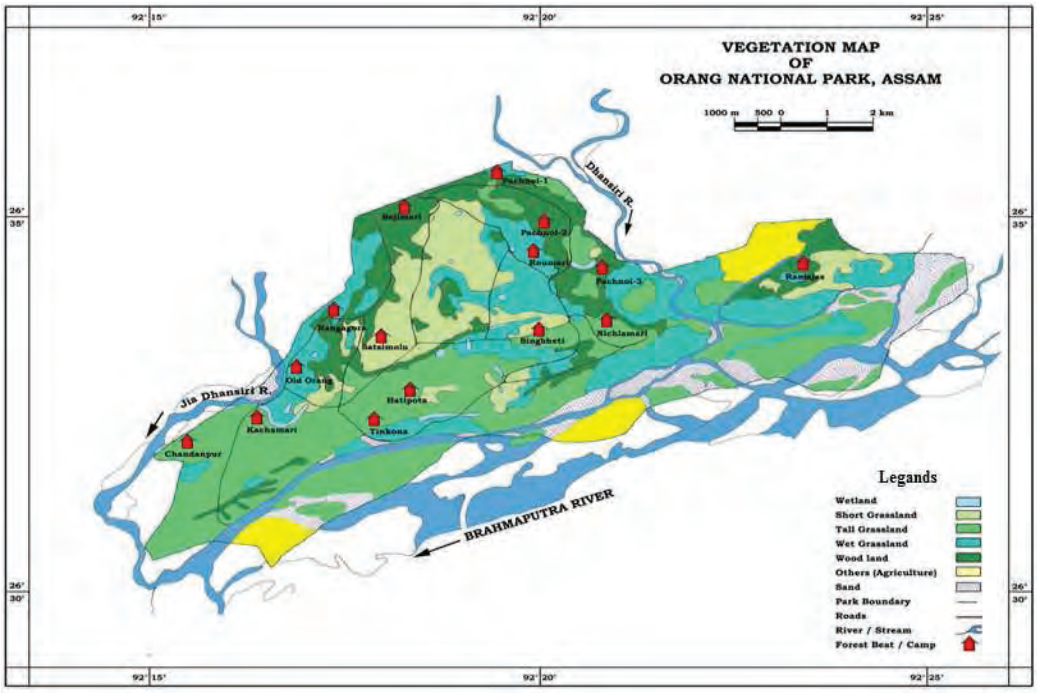


Figure 3.4 Map (vegetation) of Orang National Park (Prepared by Assam Remote Sencing aplication Centre)

3.2 Sample Collection

Presently, Assam holds an estimated 2700- 2750 wild rhino distributed in the above mentioned three protected areas. As there is a constraint in obtaining blood or tissue from endangered large animals such as Rhinoceros; dung sample is used as a source of DNA. Several other researchers (Fernando *et al.,* 2006; Muya *et al.,* 2011) have used dung sample in their researches to successfully amplify both mtDNA and nuclear microsatellites. The Indian rhinoceros of a locality generally defecate in a common place where dung becomes pile up and therefore utmost care was taken while collecting dung. As far as possible, fresh dung samples were collected because freshness enhances the quality and quantity of DNA.

Dung samples were collected in two methods-

1. In the first method dung samples were obtained after monitoring the animal from a safe distance. After defecation, the samples were collected when the animal went back or moving to other place. To identify the dung pile binocular was also used.
2. In the second method fresh dung samples were also collected from field without monitoring the animal. One day old dung samples were also collected.

The samples were collected from December, 2008 to December, 2011 in the three wild habitats of Indian Rhinoceros in Assam namely Kaziranga National Park, RG Orang National Park and Pobitora Wildlife Sanctuary. Monsoon season was avoided while collecting dung samples. The samples were collected from the outermost layer of the dung, as this layer contain the cell of intestinal mucosa. During sample collection high resolution binocular was used for prominent view of animal and Global Positioning System (Garmin etrex GPS) was used to get GPS coordinates. A Sony

digital camera with a zoom lens was used for taking photographs. All information was recorded in prepared datasheet and a notebook.

The geographical coordinates of each sample was recorded. For each sample approximately 10g of dung were placed in 50ml polypropylene tube. The dung samples were labeled properly and stored separately in two chemical preservatives, 95% ethanol and DET buffer. The DET buffer contains: 20% DMSO, 250 mM EDTA, 100 mM Tris, pH 7.5 and NaCl to saturation (Seutin *et al.,* 1991). Samples were then stored at -20°C. A total of four hundred fifty two faecal samples were collected from different locations of three rhino habitats. Additionally, one tissue sample was also obtained from dead animal of Kaziranga National Park which was used as positive reference during PCR.

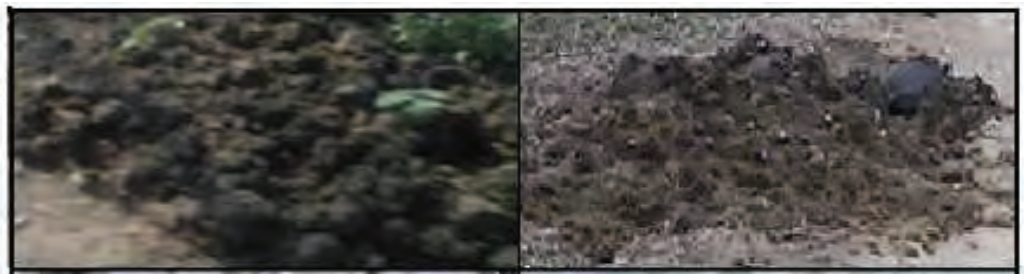


Figure 3.5 Photographs showing dung pile of Rhino. Collection of samples from such dung pile was avoided because it may contain dung from more one animal; which causes mixing of dung.

Figure 3.6 Photograph showing a part of collected dung samples in alcohol that were kept separately in vials and plastic covers to prevent contamination.

Table 3.1 Summary of sampling information of dung samples of *Rhinoceros unicornis* collected from three wild habitats of Assam (Details have been given in Appendix-1).

Name of Protected Areas	Area (Km^2)	Rhino Population	Number of sample collection	No. of Successful amplification
Kaziranga National Park	429.93 860 * 985**	2544	350	241
Orang National Park	78.8	100	50	28
Pobitora Wildlife Sanctuary	38.81	93	52	27
		2737	452	296

*Addition of six new areas including river Brahmaputra and its islands the area of the KNP has now increased to 860 sq. km., **Mapping by remote sensing method the total area of KNP is recorded as 985 sq. km. (Kushwaha, 2008)

In collecting dung samples from wild habitats, the date of sample collection, type of sample i.e., whether the sample is a fresh dropping or not, individual identification mark (in case of direct observation) and the GPS coordinates were also recorded. Each sample bottle was kept in polythene bag separately to avoid contamination from other samples. Disposable sterilized spoon was used for each sample by wearing disposable hand globes while collecting samples.

Figure 3.7 Photograph Indian Rhinoceros of (a) mother and calf, (b) sub adult and (c) adult

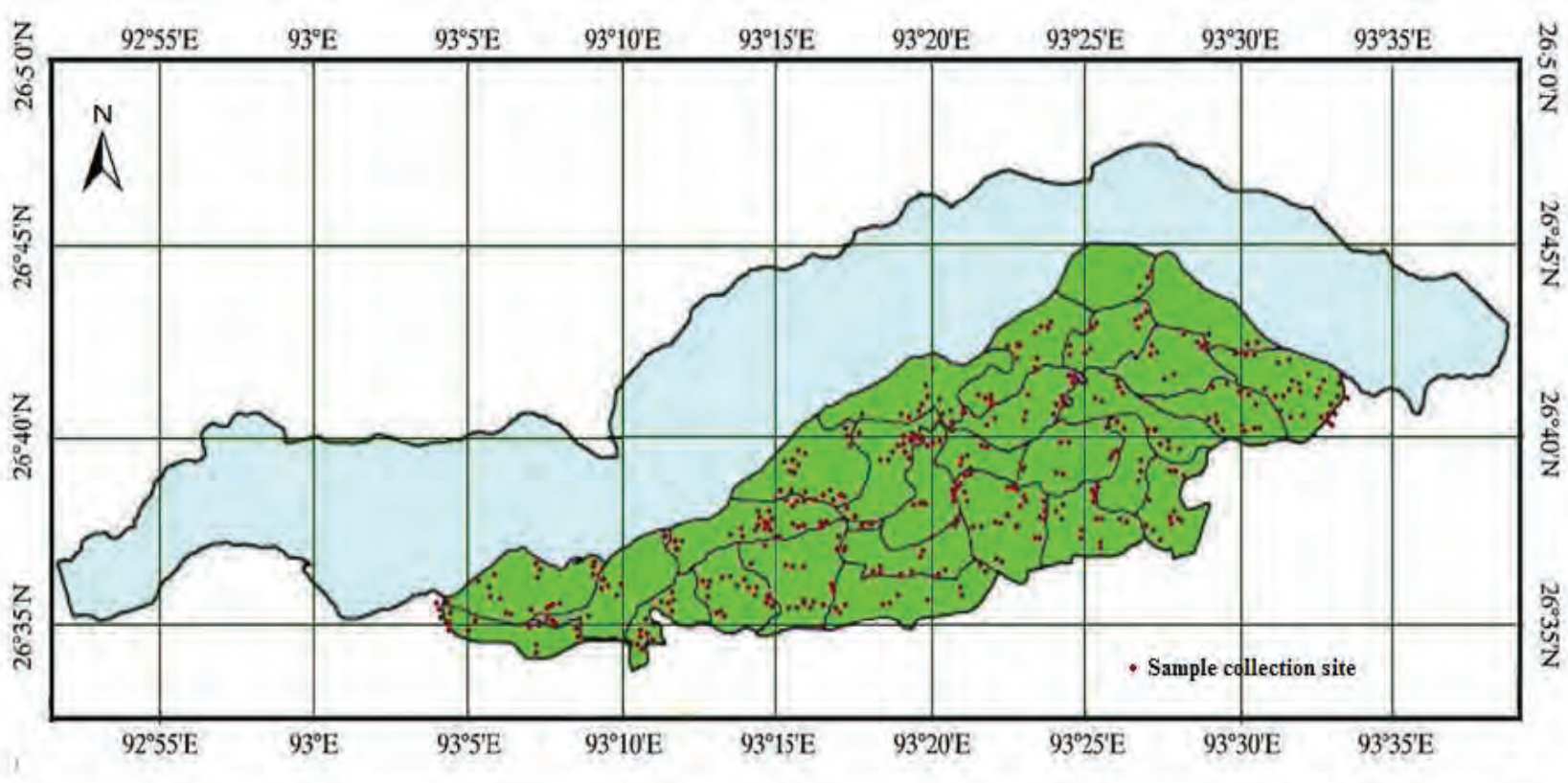


Figure 3.8 GPS coordinates of dung sample collection sites in Kaziranga National Park (modified from Kushwaha, 2008)

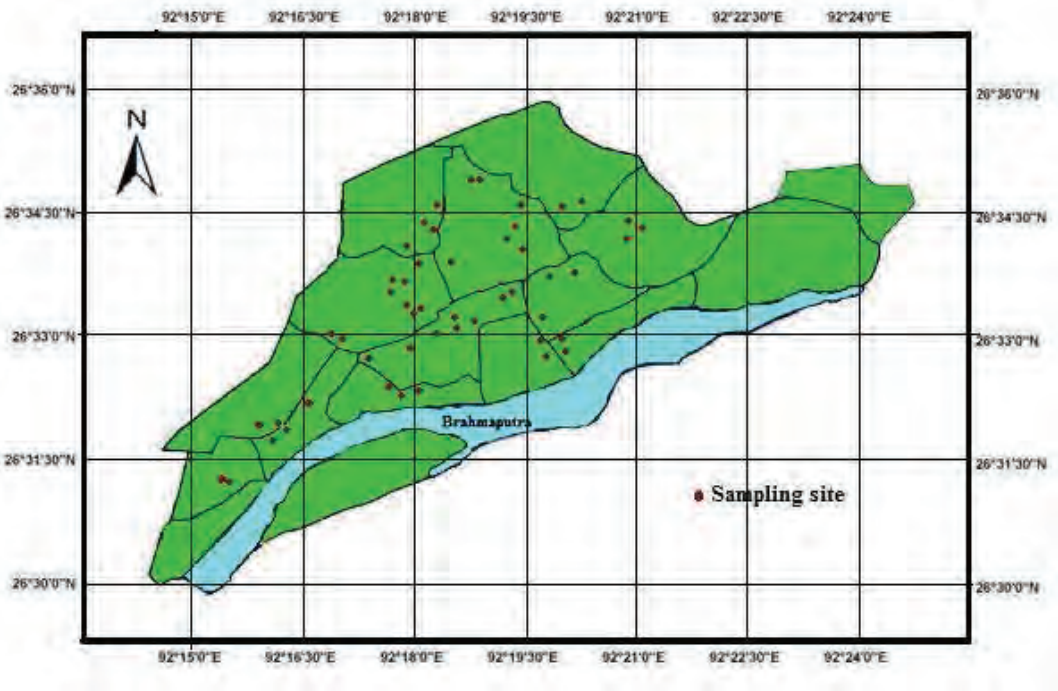


Figure 3.9 GPS coordinates of dung sample collection sites in Orang National Park (Map source: Sarma, 2010)

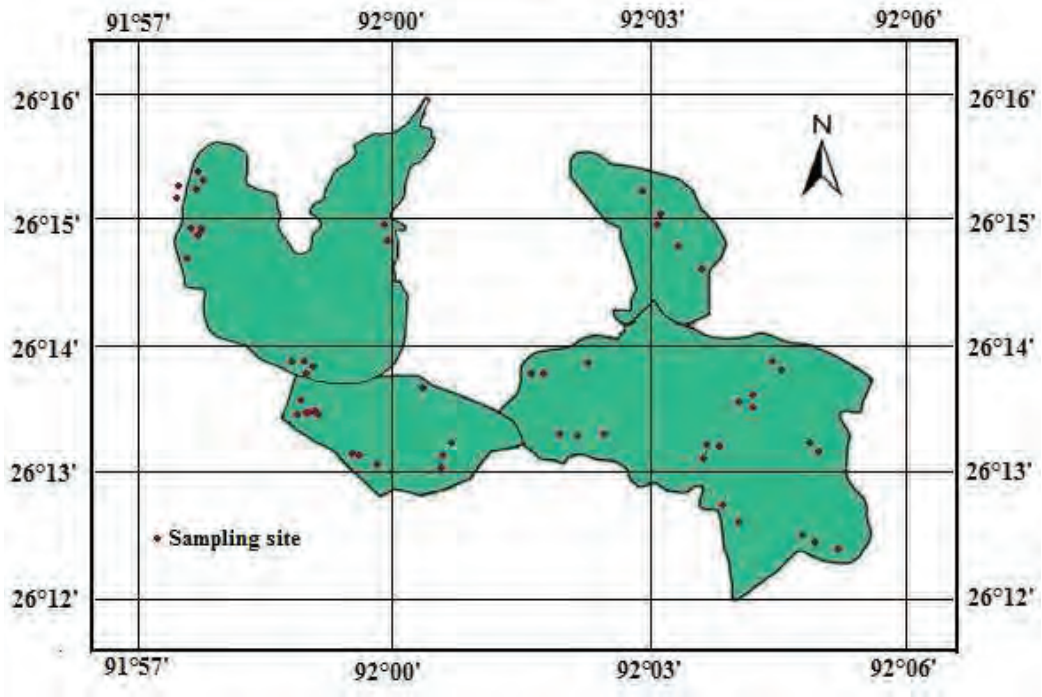


Figure 3.10 GPS coordinates of dung sample collection sites in Pobitora WLS (Map source: Wetland map of Assam, ARSAC, 2011)

3.3 Methodology

The methodology (as chart) of the study is given below

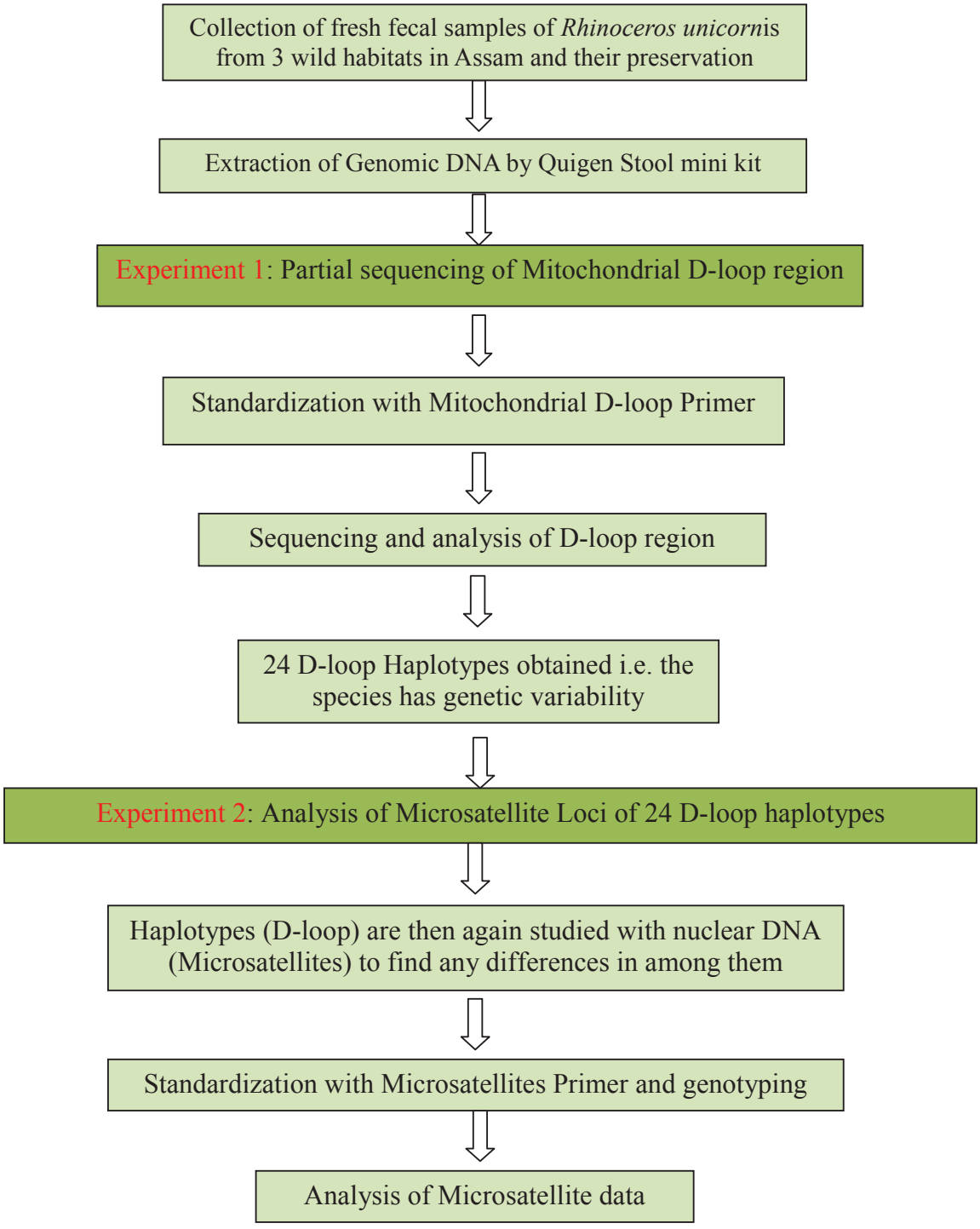


Figure 3.11 Flow chart of the methodology

3.3.1 Extraction of DNA from faecal (dung) samples

DNA extraction from DNA from dung samples by QIAmp DNA stool mini kit with slight alternations in the prescribed protocol. During DNA extraction from dung, in addition to faecal matter approximately 100 µl alcohol was also taken from the bottom of the sample vial. To minimize the possibility of contamination of dung samples, extractions were performed in a lab designated exclusively for extraction of DNA from non- invasive sources. Each group of extraction was accompanied by a negative control. The tissue sample obtained from dead animal of KNP was used as positive reference during PCR whose DNA was extracted by QIAGEN-DNeasy Blood and Tissue kit procedure by following manufacturer protocol.

DNA extraction protocol by QIAmp DNA stool mini kit (QIAGEN)

From faecal samples DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN). The supplied protocols were followed with the following modifications:

1. Three scrapes (approximately 200mg each) were taken from the outside of each faecal in an effort to target epithelial cells rather than DNA from food plants and microbes.
2. 1.5 ml of ASL buffer (provided by the kit) was added to 200 mg of dung sample in 2 ml tube. The sample was homogenized by vortexing and kept overnight at room temperature.
3. The supernatant was pipete out into a new 2 ml tube. 600µl Buffer ASL was added to the pellet and incubated for 1 hour at the same temperature. Vortex vigorously and centrifuged for 2min and pipette the supernatant into the same tube.

4. 1 InhibitEX Tablet was added to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubated the suspension for 10 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.
5. Centrifuged sample at full speed for 7 min to pellet stool particles and inhibitors bound to InhibitEX matrix.
6. Immediately after the centrifuge stops, pipet out all of the supernatant into a new 1.5 ml microcentrifuge tube (not provided) and discarded the pellet. Centrifuged the sample at full speed for 4 min.
7. Pipet out 25 µl proteinase K into a new 2 ml microcentrifuge tube.
8. Pipet out 600 µl supernatant from step 6 to the 2 ml microcentrifuge tube containing proteinase K.
9. Then 600 µl Buffer AL was added and vortex for 15 s. Proteinase K should not add directly to Buffer AL. It is essential that the sample and Buffer AL are thoroughly mixed to form a homogeneous solution.
10. Incubated immediately at 70°C for 30 min. Centrifuge briefly to remove drops from the inside of the tube lid.
11. 600 µl of ethanol (96–100%) was added to the lysate, and mix by vortexing. Centrifuged briefly to remove drops from the inside of the tube lid.
12. Labeled the lid of a new QIAamp spin column provided in a 2 ml collection tube. Carefully apply 600 µl lysate from step 12 to the QIAamp spin column without moistening the rim. The cap was closed and centrifuged at full speed for 1 min. Placed the QIAamp spin column in a new 2 ml collection tube, and discarded the tube containing the filtrate.

13. Carefully open the QIAamp spin column, apply a second aliquot of 600 µl lysate and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate.
14. Repeat step 13 to load the third aliquot of the lysate onto the spin column.
15. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.
16. The QIAamp spin column was open carefully and added 500 µl Buffer AW2. Closed the cap and centrifuge at full speed for 3 min. The collection tube containing the filtrate was discarded.
17. Placed the QIAamp spin column in a new 2 ml collection tube and discarded the old collection tube with the filtrate. Centrifuged at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
18. The QIAamp spin column was transferred into a new, labeled 1.5 ml micro centrifuge tube. Carefully open the QIAamp spin column and pipet out 200 µl Buffer AE directly onto the QIAamp membrane. Closed the cap and incubated for 1 min at room temperature, then centrifuged at full speed for 1 min to elute DNA.

For long-term storage, of eluted DNA was kept at –20°C.

The quality of DNA was checked on a 1% agarose gel. After the gel check, DNA concentrations yielded from the extraction was measured using a Nanodrop Spectrophotometer. Then the extracted DNA samples were stored at 4^0C for further processing.

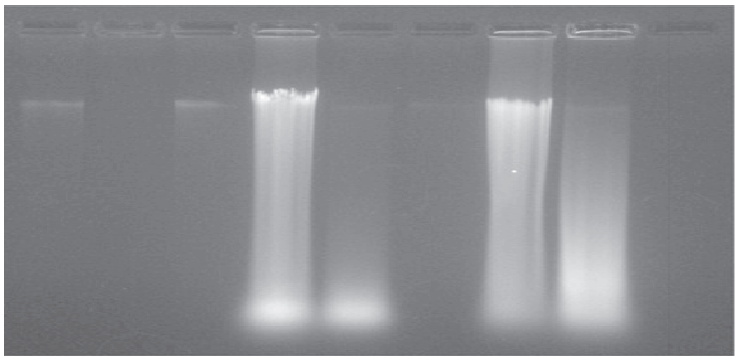


Figure 3.12 Gel photograph showing smear of degraded DNA from fecal samples

3.3.2 Primer Selection and standardization

The name and sequence of Primers selected for amplification of D-loop region and microsatellite genotyping and their standardization have been described in the respective chapters.

3.3.3 DNA sequencing

Amplified cytochrome b regions were sequenced in both the directions to check the validity of the sequence data. The PCR products were sequenced using both the amplifying primers by the ABI Prism Big Dye Terminator Cycle Sequencing kit according to the manufacturer's instructions. The purified products were then analysed with ABI Prism 3100 DNA Analyzer (Applied Biosystems). The DNA was sequenced with a commercial kit. Sequencing was carried out using a DNA polymerase to extend a primer along a single-stranded template in the presence of the four dNTPs (dATP, dCTP, dGTP and dTTP).

Step I

Cycle sequencing reaction

Cycle sequencing involves the linear amplification of double stranded DNAs or single stranded products. The thermocycler performs the denaturation, annealing and extension

steps of a typical PCR, but only a single primer is present in the reaction which results in a linear amplification.

The cycle sequencing reaction comprised-

Template DNA (PCR product)---------2.0µl(40ng)

Primer ----------0.5µl(2.5/mols)

Reaction mixture ----------0.5µl

5X sequencing buffer ----------1.75µl

Water ----------5.25µl

The reaction mixture (RR) contains Taq DNA polymerase, $MgCl_2$ and dNTPs.

Then the samples were kept for cycle sequencing reaction where the thermocycler performs the denaturation, annealing and extension steps. The cycling profiles of the thermocycler were-

I Denaturation 96^0C-----3min.

II Denaturation 96^0C-----10sec.

Annealing 50^0C-----5sec.

Extension 60^0C-----2.30min.

Extension hold 72^0C -----10min.

Hold at 4^0C

Step II

For purification of extension products a precipitation was made with ethanol (absolute) which contained.

3M sodium acetate--------2.0µl

24mM EDTA----------12.0µl

Sample----------10.0µl

Ethanol----------50.0µl

All these were mixed and left at room temperature for 15 minutes. Then solutions were centrifuged at 14000 rpm for 15 minutes and the supernatant was discarded. After this 250µl of 70% alcohol was added to the pallet and spined for 15 min. Again the supernatant was discarded and the pallets were allowed to dry at 90^0C for 1 min. Then to this 15µl of HiDi formamide (ABI) was added and denatured at 95^0C for 2min and finally chilled in ice.

Step III

Capillary electrophoresis

In this step the purified sample was transferred to the 96 well plate sequencer and the run was started. Then the sequence file was created.

The sequences obtained were then open with Chromas software, checked for validity and errors and finally aligned in MEGA and checked in BIOEDIT software.

3.4 Methods of study of Genetic diversity

Information concerned with the genetic diversity of a species comprises variation of genes (hereditary unit) at individual's level within a population or variation between geographical populations. The level of genetic diversity is usually different from one individual to another within a population, and consequently different populations of the same species can differ from one another (Halliburton, 2004). The differences are the result of evolutionary process that reflects adaptation to different conditions of life, locale, and history (Ayala, 1982). Therefore, genetic diversity of a species is an invaluable resource that enables sustainability of the species, and moreover, it is a basic need for successful genetic improvement and management program. Genetic variation within a population is revealed by average number of alleles per locus, average

heterozygosity per individual and proportion of polymorphic loci (Hedrick, 1999). Two approaches were used in this study to analyze the genetic diversity of *Rhinoceros unicornis*: Mitochondrial DNA Analysis and Nuclear DNA (microsatellites) analysis

3.5 Measurements of Genetic diversity from Mitochondrial DNA Analysis

In the present investigation the d-loop region of Mitochondrial DNA was analysed because it is a non-coding control region, is not a gene. This region does not code for a protein product, so there has been no evolutionary need for strict preservation of the base sequence. Therefore, much genetic variation can be expected between individuals of the same species. The haplotype data obtained from sequencing of D-loop region of Mt DNA was analysed by using the following methods-

3.5.1 Genetic Distance

It is a measure of the dissimilarity of genetic material between different species or between individuals of the same species. It calculates the allelic substitutions per locus which have occurred during the separate evolution of two populations or species. The calculation of a genetic distance between two populations gives a relative estimation of the time that has passed since the populations have survived as single cohesive units (Nei, 1983). A number of measures of genetic distance have been suggested over the time by different authors. These measures help to consolidate the data into manageable proportions and aid one in visualizing general relationships among the group of populations (Hedrich, 1999).

3.5.2 Nucleotide diversity (π)

It is defined as the average number of nucleotide differences per site between any two DNA sequences chosen randomly from the sample population, and is denoted by π.

It is a concept in molecular genetics which is used to measure the degree of polymorphism within a population. It was first introduced by Nei and Li (1979).

3.5.3 Construction of Phylogenetic Tree

Statistical tests of phylogenetic trees can be divided into two categories: a test of reliability of a tree obtained and a test of topological differences between two or more different trees obtainable from the same data set. One of the most commonly used tests of the reliability of an inferred tree is Felsenstein's Bootstrap Test (Felsenstein, 1985). In this test, the reliability of an inferred tree is examined by using Efron's bootstrap re-sampling technique (Efron, 1982). A set of nucleotide sites is randomly sampled with replacement from the original set, and this random set is used for constructing a new phylogenetic tree.

3.5.3.1 Neighbor Joining (NJ) Tree

Population relationships are often visualized by constructing a dendogram based on the genetic similarity of the individuals/species. NJ algorithm (Saitou and Nei, 1987) tree combines populations that are closest to each other and also furthest from the rest. It is a fast method even for very large data sets. Furthermore it is useful for bootstrap analysis. Bootstrap analysis is a sampling method which is widely used when sampling distribution is unknown to determine the statistical error.

3.5.3.2 Maximum likelihood (ML) Tree

Maximum Likelihood (ML) evaluates a hypothesis about evolutionary history in terms of the probability that the proposed model and the hypothesized history would give rise to the observed data set. The supposition is that a history with a higher probability of reaching the observed state is preferred to a history with a lower probability. The method searches for the tree with the highest probability or likelihood.

The data are observed gene frequencies or nucleotide sequences; the unknowns are the branching order and branch lengths of the tree (Felsenstein, 1981). Maximum Likelihood is an appealing method of inference as it can incorporate explicit models of evolution and also allows statistical test of evolutionary hypothesis. The advantages of maximum likelihood methods over other methods are: (i) they have often lower variance than other methods (ii) they tend to be robust to many violations of the assumptions in the evolutionary model; (iii) even with very short sequences they tend to outperform alternative methods such as parsimony or distance methods; (iv) the method is statistically well founded; (v) they evaluate different tree topologies, and they use all the sequence information. The disadvantage of this method is that the dependence on a model raises the question of which model to use as the pattern of nucleotide substitution varies from site to site and with evolutionary time (Tateno *et al.*, 1982). Another problem is that it is computationally time-consuming, and it has been shown that more than one maximal likelihood values may exist for a given tree; making it difficult to guarantee that the likelihood value for that tree is actually maximal (Nei, 1996).

3.5.3.3 Maximum Parsimony (MP) Tree

In Maximum Parsimony (MP) Methods, a given set of nucleotide (or amino acid) sequences are considered, and the nucleotides (or amino acids) of ancestral sequences for a hypothetical topology are inferred under the assumption that mutational changes occur in all directions among the four different nucleotides (or 20 amino acids). The smallest number of nucleotide substitutions that explain the entire evolutionary process for the given topology is then computed. The smallest number of substitutions is chosen to be the best tree **(**Fitch, 1971**).**

3.5.4 Haplotype Network

For visualization of positions of different D-loop haplotypes and their relation Haplotype Network and Parsimony network were constructed from all haplotypes obtained in the study. The Medium-joining network was conducted with NETWORK 4.6.1.1(Bandelt *et al.*, 1999) and Parsimony network was constructed with TCS version 1.21 (Clement *et al.,* 2000).

3.5.5 F-statistics

F-statistics (inbreeding coefficients) developed by Wright (1965) and extended by Nei (1977**)** is the most widely used method to measure the genetic differentiation within and between populations (Allendorf and Luikart, 2007). Wright (1965) proposed the quantities to measure the degree of relatedness of various pairs of alleles. Wright's FIT is the overall inbreeding coefficient F which correlates of alleles with individual over populations, Wright' FIS (*f*) is the correlation of alleles within individuals within one population and Wright' F_{ST} is the correlation of alleles of different individuals in the same population. F_{ST} is the correlation between two gametes drawn at random from each subpopulation and measures the degree of genetic differentiation of subpopulations, while FIT and FIS are the correlations between the two uniting gametes or alleles to produce the individuals relative to the total population and relative to the subpopulations, respectively. They are often called fixation index and can be negative, whereas FST is always positive. The formula used in calculating fixation index is as follow as:

$$1\text{-FIT} = (1\text{-FIS})\ (1\text{-FST})$$

F_{ST} is regarded as an inclusive measure of population substructure and is most useful for examining the overall genetic divergence among subpopulations. It is also

called co-ancestry coefficient (θ) (Weir & Cockerham, 1984) or 'fixation index' and is defined as correlation of gametes within subpopulations relative to gametes drawn at random from the entire population. It is calculated using the subpopulation heterozygosity and total population expected heterozygosity. F_{ST} is always positive; it ranges between 0 = panmixis (no subdivision, random mating occurring, no genetic divergence within the population) and 1 = complete isolation (extreme subdivision).

Usually, the genotype frequencies in populations do not follow Hardy-Weinberg equilibrium frequencies in nature and F statistics uses these deviations to measure the inbreeding (which is the tendency for mates to be closely related) within populations. One of these inbreeding coefficients, F_{IS} is a measure of departure from Hardy-Weinberg proportions within local subpopulations and estimated by the formula:

$$F\text{IS} = 1 - \frac{Ho}{Hs}$$

Where *H*o is the mean observed heterozygosity over all sub-populations and *H*S is the mean expected heterozygosity over all sub-populations. *F*IS will be positive meaning there is inbreeding in the examined population which cause heterozygotes deficiency. On the other hand, *F*IS will be negative when there is migration from outside of the population cause an excess of heterozygotes.

F_{ST} is a measure of genetic divergence among sub-populations and can be used as a distance measure. It can be calculated by the formula:

$$F\text{ST} = 1 - \frac{Hs}{HT}$$

Where. *HT* is the expected heterozygosity if the entire base population were panmictic (random mating is observed) and *HS* is the mean expected heterozygosity over all sub-populations.

With using two populations each time, it can be used as a distance matrix to compare pairwise differences among sub-populations. F_{ST} values below 0.05 indicate negligible genetic differentiation whereas >0.25 means very great genetic differentiation within the population analysed. F_{ST} will be between 0, when populations have equal allele frequencies, and 1, when populations are fixed for different alleles. That is why, *F*ST is sometimes also called as fixation index. F_{ST} estimates were calculated using the program FSTAT 2.9.3 (Goudet, 2001), Arlequin 3.0. and GENEPOP version 3.4 (Raymond and Rousset, 1995).

The coefficient of gene differentiation (G_{ST})

The coefficient of gene differentiation (G_{ST}) developed by Nei (1973) is an extension of the (Nei, 1972) genetic distance theory between a pair of populations. G_{ST} can be computed directly from allele frequencies in terms of expected heterozygosities within and between populations. Unlike the F_{ST}, the estimation of heterozygosities in G_{ST} relies only on allele frequencies (Nei, 1987). This method offers several advantages because it is not affected by the number of alleles at the locus and neither is affected by the evolutionary forces such as mutation, selection and migration, which may be taking place in the organism. G_{ST} can be defined as: $G_{ST} = D_{ST}/H_T$

Where *D*ST is average gene diversity between and within populations and H_T is the expected total heterozygosity. The G_{ST} estimates in this study were calculated using the DnaSP 5.0 (Rozas *et al.,* 2003).

3.5.6 Analysis of Molecular Variance (AMOVA)

AMOVA is analysis of variance for molecular markers and one uses a matrix of genetic distances among sampled individuals as a starting point. In *F* statistics gene frequencies are compared among haplotypes or genotypes. However, from molecular

data the amount of mutational differences between different genes can be obtained. Instead of Mendelian gene frequencies, a method that analyses differences between molecular sequences is very useful to estimate the population differentiation. One can achieve this by using Analysis of Molecular Variance (AMOVA) which estimate population differentiation directly from molecular data and testing hypotheses about such differentiation. Several kinds of molecular data, such as microsatellite based data or direct sequence data can be analyzed with this method (Excoffier *et al.,* 2005). The data analyzed with AMOVA uses allele frequency data.

Table 3.2 General AMOVA table for genotypic data, several groups of populations, within-individual level taken from Arlequin package program (Excoffier *et al.*, 2005).

Source of variation	Degrees of freedom	Sum of squares (*SSD*)	Expected mean squares
Among Groups	*G - 1*	SSD(AG)	
Among Populations / Within Groups	*P - G*	SSD(*AP*/*WG*)	
Within Populations	*N - P*	SSD(*WP*)	
Total:	*N* - 1	SSD(*T*)	

SSD(*T*) : Total sum of squared deviations.

SSD (*AG*) : Sum of squared deviations Among Groups of populations.

SSD (*AP*) : Sum of squared deviations Among Populations.

SSD (*AI*) : Sum of squared deviations Among Individuals.

SSD (*WP*) : Sum of squared deviations Within Populations.

SSD (*WI*) : Sum of squared deviations Within Individuals.

SSD (*AP*/*WG*) : Sum of squared deviations Among Populations, Within Groups.

SSD (*AI*/*WP*) : Sum of squared deviations Among Individuals, Within Populations.

G : Number of groups in the structure.

P : Total number of populations.

N : Total number of individuals for genotypic data or total number of gene copies for haplotypic data.

pN: Number of individuals in population *p* for genotypic data or total number of gene copies in population *p* for haplotypic data.

gN: Number of individuals in group *g* for genotypic data or total number of gene copies in group *g* for haplotypic data.

The variance components can be used to calculate a series of statistics called phi-statistics, which summarize the degree of differentiation between population divisions and are analogous to *F*-statistics, such as phi-CT, phi- SC, phi-IS and phi-IT corresponds to the differentiation among groups, among populations-within groups, among individuals-within populations and within individuals, respectively.

3.6 Nuclear DNA (microsatellites) analysis

Microsatellites are short tandemly arrayed di-, tri-, or tetra- nucleotide repeat sequences with repeat size of 1-6 bp repeated several times flanked by regions of non-repetitive unique DNA sequences **(**Tautz, 1989). Microsatellites that have been largely utilized for population studies are single locus ones in which both the alleles in a

heterozygote show co-dominant expression (Balloux and Lugon-Moulin, 2002). Individual alleles at a locus differ in the number of tandem repeats and as such can be accurately differentiated on the basis of electrophoresis or automatic genotyping methods according to their size. Different alleles at a locus are characterized by different number of repeat units. Polymorphism at microsatellite loci was first demonstrated by Tautz (1989) and Weber and May (1989). In genotypic data, parameters made to indicate genetic diversity within a population (or between populations) can be estimated based on the change in allele frequencies as followed as:

3.6.1 Number of alleles per locus

Counting of the number of alleles at each locus in each sample and overall samples is done. For the average number of alleles per locus, the total numbers of alleles were divided by total numbers of locus.

3.6.2 Determination of allele frequencies

The allele frequencies in each sample and overall average can be estimated from banding patterns created by molecular markers. The overall allele frequencies can be presented either by weighted by sample size or non-weighted frequencies.

3.6.3 Allelic richness

The genetic diversity was measured based on allelic richness, which is considered important in the field of conservation genetics, and marker-assisted methods to effectively maximize the number of alleles conserved. Allelic richness is a measure of the number of alleles independent of sample size, hence allowing comparison of this quantity between different sample sizes.

3.6.4 Heterozygosity

It is the average proportion of loci that carry two different alleles at a single locus within an individual. Observed heterozygosity (Ho) can be estimated with co-dominant molecular markers, but estimates are biased by the number of individuals sampled within a population. Expected heterozygosity (He) can be estimated with both dominant and co-dominant markers when assumptions are made about the mode of inheritance, as well as the size and structure of populations.

3.6.5 Hardy-Weinberg equilibrium

In a large random mating population with no selection, mutation or migration, the allelic or gene frequencies and the genotype frequencies remain constant from one generation to the next. Thus a population with constant gene and genotype frequencies is said to be in Hardy-Weinberg equilibrium (HWE) (Falconer and Mackay, 1996). Following factors affect on the HWE

i. Non-random mating: Non-random mating can occur in cases where related individuals have a greater probability of mating with each other than with other members of the population and where individuals that are geographically close are more likely to mate with each other than those that are not geographically close.

ii. Mutation: Mutation brings about genetic variation in a population by producing novel variants of genes. This is the process that produces a gene or chromosome that is different from the wild type.

iii. Migration: The movements of genes caused by individuals moving, including new individuals entering (immigration) or leaving (emigration) a population, introducing or removing genetic material and thereby changing allele frequencies. Migration of genes

into a population results in an increase in a population's genetic variation and the migration of genes out of a population may result in a reduction of the genetic variation.

iv. Selection: Selection is a natural process resulting in the evolution of organisms' best adapted to the environment. Only the individuals that are better adapted to the environment or able to mate successfully could pass their genes on to the next generation. Selection generally results in a reduction of genetic variation in a population.

v. Random genetic drift: Random genetic drift is the change in gene frequencies due to chance or sampling effects. It is very dependent upon population size. Genetic drift is fundamentally the result of a finite population size. Random genetic drift is a stochastic process. The effect of genetic drift is infinitely proportional to the population size. Thus when the population size is small, e.g. due to strong bottleneck effects in the past, there are greater changes in gene frequency under genetic drift at every generation (Cavalli-Sforza *et al.*, 1994). Therefore, the smaller the population size, the greater the chances of sampling errors occurring. In the study of many populations, it is important to determine whether the loci and the populations genotyped were in HWE and whether there were any significant deviations from the HWE.

3.6.6 Linkage disequilibrium

Linkage disequilibrium is often termed 'allelic association' and is a measure of the degree of association between two alleles in a population. Measures of linkage disequilibrium quantify how frequently two alleles are found on the same chromosome in a certain population. A population is said to be at linkage disequilibrium at a set of loci if the alleles are not randomly assorted in the next generation, but are inherited together as a unit. Linkage disequilibrium can be generated by finite population size,

random genetic drift, mutation, selection, non-random mating, and migration. It can have positive or negative values, the tendency for two 'alleles' to be present on the same chromosome (positive LD) or not to segregate together (negative LD). LD is decreased by recombination. Thus, it decreases every generation of random mating unless there are some processes opposing the approach to linkage 'equilibrium'. Linkage disequilibrium explains a situation in which some combinations of alleles occur more or less frequently in a population than would be expected from a random recombination of haplotypes from alleles by their frequencies. Non-random associations between polymorphisms at different loci are measured by the degree of linkage disequilibrium (LD). It is a test of random mating and Mendelian segregation in which independent segregation allele from one genotype to another one (Hedrick, 2005). D is used a measure of the deviation from random association between alleles at two loci (Lewontin and Kojima, 1960). D is known as the coefficient of linkage disequilibrium and is defined in the case of two loci that each have two alleles as:

$$D = (G1G4) – (G2G3)$$

where G1, G2, G3 and G4 be the frequency of the four gametes AB, Ab, aB, and ab respectively.

A population is called as in linkage equilibrium (D=0), if the alleles are associated at random in population. On the other hand, the alleles in two loci are not associated randomly if D is not zero. This is called as in linkage disequilibrium. Linkage disequilibrium estimations were (for total sample size and for each breed separately) done based on 19 loci with FSTAT V.2.9.3 program (Goudet, 2001). The F-statistics and Analysis of Molecular Variance (AMOVA) are also estimated from genotypic data which are discussed earlier.

3.7 Factorial Correspondence Analysis (FCA)

In multidimensional space to see the individuals and to investigate the relationships between the individuals, the Factorial Correspondence Analysis (FCA) (Lebart *et al.,* 1984) is used. The program finds independent axes which are the linear combinations of the alleles such that the maximum genetic diversity observed within the total data could be explained by the first axis. Visualizing on the independent axes for how individuals are related to each other is an informative way to see the amount of inertia, distinctness of the individuals and their relative similarity. GENETIX Software v. 4.05 (Belkhir *et al.,* 1996–2004) was used to make this analysis.

3.8 Population genetic structure analysis

Genetic structure was further investigated through Bayesian clustering of genotypes. The Bayesian clustering method was implemented using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). The number of clusters (K) tested ranged between one and 6; by assuming that at a most structured level, each subpopulation would cluster as its own group. Ten runs for each value of K were performed, in order to verify that the results were consistent across runs. A burn-in period of 100,000 iterations of Markov chain Monte Carlo (MCMC) was used for running the structure program, and this parameter produced consistent results in five runs of each K.

3.9 Analysis of Genetic Bottleneck

A demographic bottleneck occurs when a large population experiences a severe, temporary reduction in size due to environmental or demographic events, like drought, disease outbreak and war. These events may kill a certain percentage of a population and therefore reduce the effective population size. The result is that the genetic variability of all subsequent generations is contained in the few individuals that survive the bottleneck and reproduce. Hence, some genetic diversity is lost in the process. The

magnitude of the loss in diversity depends on the size of the bottleneck and the growth rate of the population afterward (Hunter, 1996). Genetic bottleneck can basically create two problems (Carson, 1983): a loss of certain alleles, especially rare alleles, if no individuals possessing those alleles survive, and a reduction in the amount of variation in genetically determined characteristics due to the presence of fewer alleles and decline in heterozygosity. The overall effect of bottlenecks is the decline in fitness of the individuals in the population. The probable occurrence of bottlenecks of Rhinoceros population in Assam was examined using the software BOTTLENECK version 1.2.02 (Piry *et al.*, 1999).

CHAPTER–4

Genetic divergence study of wild Indian Rhinoceros, *Rhinoceros unicornis* from three Protected Areas of Assam through mitochondrial D-loop region

4.1 Introduction

The Indian one horned rhinoceros (*Rhinoceros unicornis* Linnaeus, 1758) population in Assam is now found in three fragmented wild habitats and has been facing increasing threat from poaching and further habitat loss. In historic times (c. 1400 AD), the Indian rhinoceros occurred along the flood plains from north-western Myanmar across the Gangetic plain to the Indus River Valley in northern Pakistan with a minimal total population of more than 450,000 individuals (Blanford, 1891; Dinerstein and McCracken, 1990; Laurie, 1978). Since the 19th century, due to several reasons their number significantly reduced and fragmented the habitat suitable for the rhinoceros. Due to intense poaching their number further decreased and reduced the population. Today, natural populations of the Indian rhinoceros only occur in the states of Assam Uttar Pradesh and West Bengal in India and the Terai of Nepal (Foose and van Strien, 1997). The rhino population in Assam was estimated to survive few (less than 20) individuals in Kaziranga National Park when hunting was banned in 1908 (Ullrich, 1972; Laurie *et al.*, 1983). Fortunately, the population has increased considerably during the second half of the 20th century in the Kaziranga National Park (KNP) of Assam. Now the rhino population has expanded into neighboring areas of KNP, including the Laokhowa WLS, Pobitora WLS and Orang National Park (Merenlender *et al.*, 1989; van Strien and Talukdar, 2007). But the species has vanished from Laokhowa WLS due to

poaching. Now the Indian rhino population in Assam is restricted to Kaziranga National Park, Orang National Park and Pobitora Wildlife Sanctuary.

Continued habitat loss and fragmentation result in small, isolated wild animals exhibit a greater sensitivity to demographic stochasticity, may have reduced population mean fitness and suffer increased extinction rates because of increased expression of inbreeding depression, decreased levels of genetic diversity and higher probabilities of fixing deleterious mutations relative to pre-fragmentation population structure (Dudash and Fenster, 2000; Frankham, 2010). Genetic diversity in small populations is expected to diminish because of genetic drift and inbreeding. Genetic diversity is necessary to facilitate the development of adequate conservation and management strategies. Genes regulate body size, shape, physiological processes, behavioural traits, reproductive characteristics, tolerance of environmental extremes, dispersal and colonizing ability, the timing of seasonal and annual cycles (phenology), disease resistance, and many other traits (Freeman, 1998). A diverse array of genotypes appears to be especially important in disease resistance (McArdle, 1996). Genetic variation holds the key to the ability of populations and species to persist over evolutionary time through changing environments (Freeman and Herron, 1998). Besides these, to carry out effective conservation programs, understanding the extent of phylogenetic distinction if any between or among the remnant populations is essential (Olney *et al.*, 1994). The study of genetic diversity is also vital for population viability and conservation management programmes (Wilson and Strobeck, 1999).

To study the genetic diversity and population structure of Indian rhinoceros the control or D-loop (displacement) region of mitochondrial DNA was selected. MtDNA has a relatively high mutation rate and shows higher levels of polymorphism compared

to many nuclear genes making it useful when looking for patterns of genetic differentiation (Moritz *et al.*, 1987). The D-loop region of mt DNA evolved with exceptional rapidity and has proved to be useful for high resolution analyses of population structure (Avise, 1994). Animal mtDNA is a closed circular molecule, typically 15-20 kb in length and is composed of about 37 genes. A control region is about 1 kb and initiates replication and transcription. The control region is also called D-loop because at the level of the heavy strand replication origin (OH) contained within the D-loop, the nascent H-strand displaces the parental H-strand, creating a three-stranded structure called a displacement (D) loop. The promoters for heavy (HSP) and light (LSP) DNA strand transcription are also contained in this region, upstream in relation to the OH (Clayton, 1982; 1984). The mt DNA not undergo any form of recombination, making it particularly useful for reconstructing phylogenies. It is more sensitive to changes in population demography because it has a quarter the effective population size compared with nuclear loci. Studies of mtDNA can be used effectively in long-term and short-term management of populations, by measuring genetic variation in the populations and to ascertain evolutionary or phylogenetic conservation value of populations (Moritz, 1994b; Moritz and Cicero, 2004). The control region contains the origin of mtDNA replication, and therefore, it is a triple strand structure (Randi *et al.*, 1998; Larizza *et al.*, 2002). The evolution of the control region of mammalian mtDNA shows some features such as strong rate heterogeneity among sites, the presence of tandem repeated elements, a high frequency of nucleotides insertion/ deletion, and lineage specificity (Pesole *et al.*, 1999; Larizza *et al.*, 2002). Typical mammalian control region shows three domains: extended termination-associated sequence (ETAS, spanning from the tRNAPro gene to the central domain); the central domain (CD); and

the conserved sequence block (CSB, from the CD to the tRNAPhe gene) (Sbisa *et al.*, 1997). In mammals, the substitution rate within the control region is not uniform since two peripheral fragments concentrate as much as the 90% of the variation. These two fragments are always flanking the much more conserved central domain. Therefore, the peripheral regions are useful in the study of population, while the conserved regions are very informative for reconstructing phylogenies among recently diverged taxa (Arnason *et al.*, 1993; Randi *et al.*, 1998; Mate *et al.*, 2004). Moreover, many mammalian control region sequences are currently available, making this region a model for studies of recent mammalian evolution (Sbisa *et al.*, 1997; Matson and Baker, 2001; Larizza *et al.*, 2002).

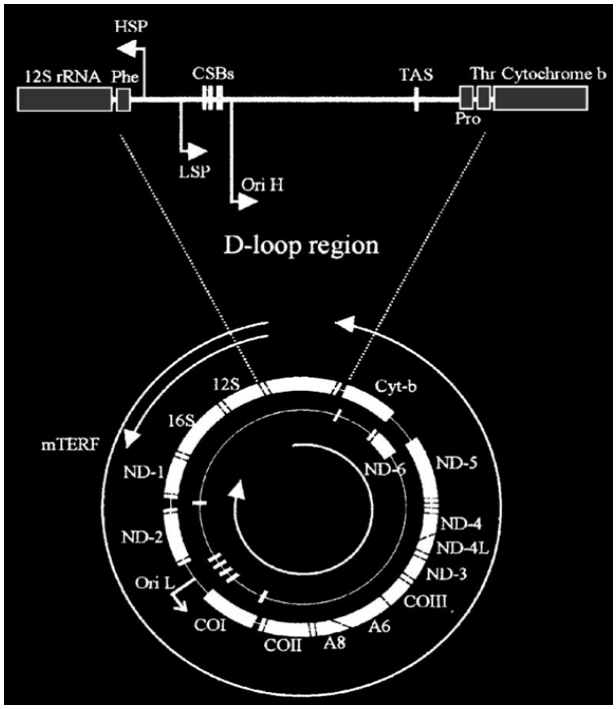


Figure 4.1 Schematic diagram showing the location of the D-loop region of mitochondrial genome (http://lslab.lscore.ucla.edu)

It is hypothesized that the wild rhino population found in the three protected areas is genetically diverged. Therefore, in the present chapter an attempt has been made to

study the genetic diversity of the Indian Rhinoceros population found in Kaziranga National Park, Orang National Park and Pobitora WLS through partial sequencing and analysis of mitochondrial D-loop region taking non-invasive samples from its wild habitats.

4.2 Materials and methods

The present study has utilized non-invasive sampling methods through the collection of fecal samples (Table 3.1, Appendix-1) of the Indian rhinoceros from three wild habitats of Kaziranga National Park, Orang National Park (ONP) and Pobitora Wildlife Sanctuary (PWLS) for genetic analysis using mtDNA. From a total of nearly 2,700 wild rhino population of three protected areas 452 dung samples were collected from where 296 samples gave positive amplification.

The Indian rhinoceros of a locality generally defecate in a common place where dung becomes pile up. Therefore utmost care was taken while collecting dung. Most of the dung samples were collected in fresh condition as far as possible. To obtain genomic DNA, the outermost layer of the dung samples were collected, as this layer contain the cell of intestinal mucosa. For each sample approximately 10g of fresh dung were placed in 50ml polypropylene tube containing 95% ethanol (Merck) and labeled properly. The samples vials were then kept at -20^{o}C until DNA isolation.

For the mitochondrial D-loop analysis, some GenBank sequences have been used as outgroup taxa in the phylogenetic analysis. The complete mitochondrial sequences used in this study are the Indian Rhinoceros (Acc. No. X97336 by Xu *et al.,* 1996) and (Acc. No. NC 001779 by Xu and Arnason (1997) and for African Black rhinoceros (Acc. No. L22010) analyzed by Willerslev *et al.* (2009). The mitochondrial D-loop sequencing was done by Fernando *et al.,* 2006 for Javan rhinoceros (Acc. No.

AY739625-AY739628) and African Black rhinoceros (Acc. No. AY742830-AY742833) were also used in phylogenetic analysis.

4.3 DNA extraction

Genomic DNA extraction was carried out from the alcohol preserved dung samples by QIAmp DNA stool mini kit (QIAGEN Inc.) with slight alternations in the prescribed protocol. During the DNA extraction from dung, in addition to faecal matter approximately 100 µl alcohol was also taken from the bottom of the sample vial. To minimize the possibility of contamination of dung samples, extractions were performed in a lab designated exclusively for extraction of DNA from non- invasive sources. DNA extractions were checked for quality via agarose gel electrophoresis, and quantity was measured using a NanoDrop spectrophotometer prior to use in PCR.

4.4 Primer Selection and standardization

To amplify 420 bp (expected) long D-loop control region located in tRNA-Pro and D-loop of mitochondrial genome, a set of generic primer RH-D-F1 and RH-D-R1 (Fernando *et al.*, 2006) was selected (Table 4.1). The primers were standardized with the quantities of required reagents and for getting appropriate band size and annealing temperature. Two pairs of primers (RDF1 and RDR1; RDF2 and RDR2) were also design based upon complete sequence of mitochondrial genome (X97336) and D-loop sequence of *Rhinoceros unicornis* (AY742825) was used to amplify short fragments of D-loop region of degraded DNA obtained from fecal matter of *Rhinoceros unicornis* (Table 4.2).

Table 4.1 Name and sequence of primer (Fernando *et al.*, 2006)

Name of primer	Sequence (5'→3')	Length	Template size (bp)	Tm
RH-D-F1	CATCAACACCCAAAGCTGAAA	21	420	68^0C
RH-D-R1	ATGGGCCCGGAGCGAGAACGA	21		68^0C

Table 4.2 Name and sequence of newly designed primer

Name of primer	Sequence (5'→3')	Length	Template size (bp)	Tm
RDF1	TCGACCCAAGCGATGTTGAT	20	200	65^0C
RDR1	AAACCCCCACAGTTCATGGG	20		65^0C
RDF2	TCAACCCTCTCACCCAATGC	20	200	65^0C
RDR2	CCAAATGCATGACACCACAGT	21		65^0C

4.5 PCR amplification and Purification of PCR product

From the extracted DNA sample, PCR amplification was performed in 25µl reaction using 2µl genomic DNA extract, 2µl 100µg/ µL BSA, 2.5 µl 1.5X taq buffer A, 0.5 µl 1.5mM $MgCl_2$, 2.5 µl 0.25mM dNTP separate (SIGMA), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.1 µl AmpliTaq Gold DNA polymerase and 14.4 µl water. In every set (7 samples) extraction and PCR experiments, a negative control was included with the samples. Typical problems from faecal samples include contaminants which inhibit PCR, other DNA sources including plant material etc. In some cases the DNA samples did not work well and failed to produce a band. Adequate measures were taken to optimize the PCR.

Table 4.3 PCR reaction reagents and quantities (µL) taken for 25 µl reaction.

Reagents	**Stocks**	**Working solution**	**Quantities** (µL)
Buffer	10X	1.5X	2.5
MgCl2	25 mM	1.5 mM.	0.5
dNTPs (Eppendrof)	2.5 mM	0.25mM	2.5
Primers (F+R) (Sigma-Aldrich)	100 µM	10 µM	1.0
BSA	4 mg /mL	100µg/ µL	2.0
AmpliTaq Gold DNA polymerase (Genei)		5U/µL	0.1
Genomic DNA		10-50ng/ µL	2.0
Water (MiliQ)			14.4
Total:			=25.0

Amplification was performed on Mastercycler employing initial denaturation at 95^0C for 4 min followed by 50 cycles comprising subsequent steps of denaturation at 94^0C for 1 min, annealing at 68^0C (65^0C for newly designed primers) for 1 min and primer extension at 72^0C for 1.5 min. On completion of the cycles, the reaction mixture was incubated further at 72^0C for 5 min and 4^0C and continuously after. Successful sequencing results for the mitochondrial d-loop region were obtained for 296 out of the 452 dung samples. When the first set of primers (Table 4.1) did not work in the PCR, the second two pairs of primers (Table 4.2) were tried for that samples because this primers were so designed that it can amplify small product size (around 200bp) from the

degraded DNA of dung samples; which were thereafter aligned together to get expected size product (400bp). The PCR products (approximately 1.5 µl) were checked for appropriate size with 2% agarose gel (Figure 4.2) stained with ethidium bromide in 1X TBE (Tris-borate EDTA) buffer for 2 hours at a 100v constant voltage. After electrophoresis, the PCR products were cleaned up by adding 3µl of Exo-SAP mixture (Shrimp Alkaline Phosphatase) per 20µl reaction. For purification of a few samples gel extraction method was also followed. Thereafter the products were processed in the PCR machine following the programme 37^0C for 70 min, 80^0C for 25 min and 4^0C forever. Then purified products were again sequenced in both forward and reverse direction in ABI automated sequencer (Applied Biosystems).

Table 4.4 GeneBank Accession numbers of Different species of Rhinoceros used in the study

GenBank Accession Number	Reference	Species
X97336	Xu *et al.* (1996)	*Rhinoceros unicornis*
AY742825	Fernando *et al.* (2006)	*Rhinoceros unicornis*
NC 001779	Xu and Arnason (1997)	*Diceros bicornis*
L22010	Willerslev *et al.* (2009)	*Diceros bicornis*
AY739625--AY739628	Fernando *et al.* (2006)	*Rhinoceros sondaicus*
AY742830-- AY742833	Fernando *et al.* (2006)	*Diceros bicornis*

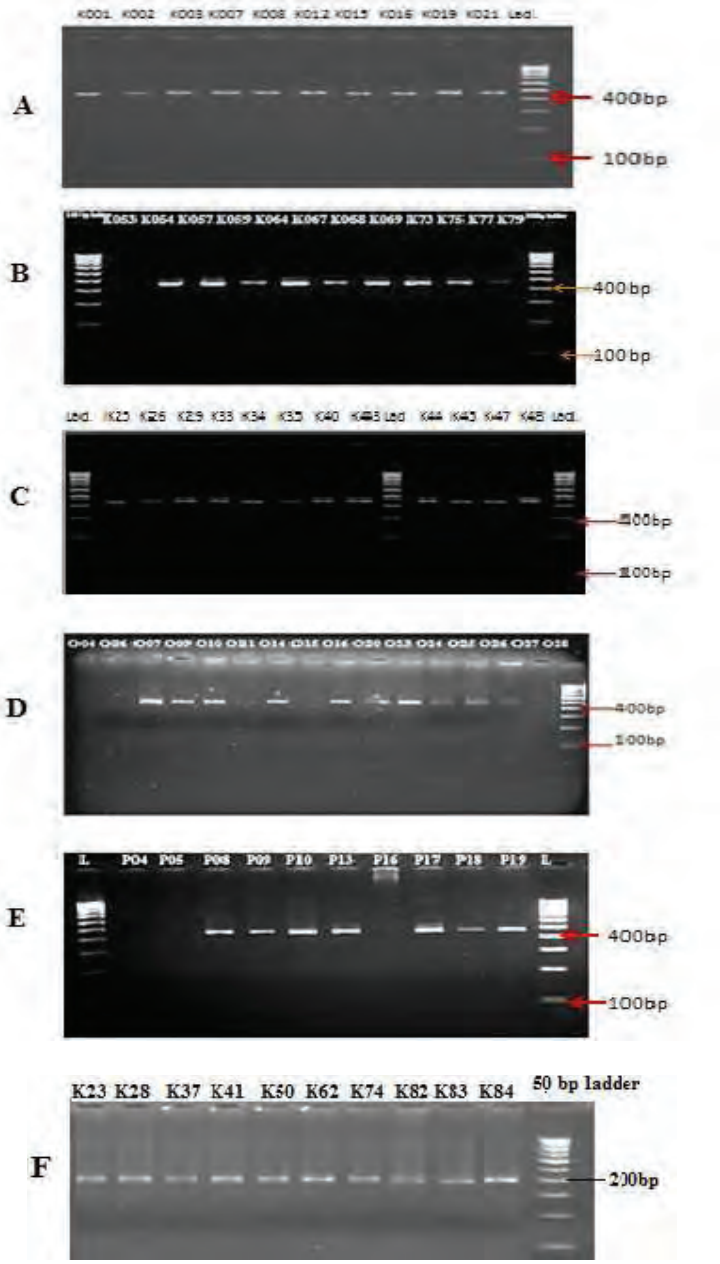


Figure 4.2 Ethidium bromide stained gel photograph of some PCR products of D-loop of 420bp size are shown in the figure A to E with 100 bp ladder was used to find out the amplicon size. Gel photograph A to C showing sequences obtained from Kaziranga, D and E showing sequences obtained from Orang and Pobitora respectively. Gel photograph F showing PCR product of 200bp fragments which is a part of selected region of D-loop sequence, later on which are aligned together with other sequences.

4.6 Analysis of Sequenced Data

The DNA sequences were assembled in BIOEDIT 7.0.9 (Hall 2005) and automatically aligned using CLUSTALW program (Thompson *et al.,* 1994), inbuilt in the genetic analysis package MEGA 5 (Tamura *et al.*, 2011). Sequences were then checked with Finch TV1.4 (Geospiza. com) and then visually refined. From amplified product, 413 bp of the D-loop fragment of *R. unicornis* was selected for analysis. A total of 24 haplotypes were obtained from 296 D-loop sequences. The haplotype sequenced data were then analyzed with the help of BLAST for homology search. In addition to the samples collected in this study, two GenBank data (Acc. No.X97336.1 and NC 001779.1) were used to draw phylogenetic tree. Identical haplotypes, polymorphic sites, haplotype diversity and variance of haplotype diversity within the population were detected in DnaSP 5.0 (Rozas *et al.* 2003). Nucleotide composition of all haplotype sequences, type of substitutions, haplotype distance matrix, Expected heterozygosity diagram generated from haplotypes, number of alleles at different loci, Mismatch distribution, molecular diversity indices, Pairwise Fst Matrix (Weir and Cockerham, 1984) and pairwise differences (Nei's standard genetic distances) andTajima's *D* of three Indian rhino population and for all samples of *R. unicornis* were determined by Arlequin 3.0 (Excoffier *et al.,* 2005). Hierarchical genetic structure was inferred using analysis of molecular variance (AMOVA) for all haplotype data were also calculated in Arlequin 3.0. Fu's Fs test (Fu, 1997) was also calculated in the same program to detect any potential excess of rare alleles, which would indicate a recent population expansion (significance levels were evaluated using 1000 simulations). To explore demographic patterns of rhino populations, distributions of the number of pair wise mutational differences among individuals, mismatch distributions was analyzed.

Different phylogenetic trees were constructed for all haplotypes with the *Diceros bicornis* sequence (Acc. No. L22010) as an outgroup based on the p-distance and Kimura's 2 parameter model using MEGA 5.0. Bootstrap analysis (1000 data sets) was used to assess confidence in the branching order into the dendrogram. Genetic distances among different haplotypes were also calculated by Kimura 2 parameter method using MEGA5.0. A parsimony network linking all haplotypes was developed using NETWORK 4.6.1.1 (Bandelt *et al.*, 1999) and by using the TCS version 1.21 (Clement *et al.*, 2000) for a visualization of the relationship among the haplotypes separately.

4.7 RESULTS

The Nucleotide positions of the sequenced segment of DNA were assigned from 15412th to 15824th position according to the complete rhino mtDNA reference sequence mitochondrial DNA of the GenBank accession no. X97336. The NCBI Blast was done with the haplotypes and result of one haplotype is presented in Figure 4.3. All total 24 haplotypes were obtained from 296 D-loop sequences from three different rhino habitat with 21 variable sites. The same haplotype sequence obtained from different habitat given same ID code placing first letter of the habitats (e.g. H1 from Kaziranga as KH1 etc.). Out of 21 polymorphic sites detected, 8 are singleton variable sites and remaining 13 are parsimony informative sites.

Figure 4.3 Assignment of D-loop segment from 15412th to 15824th position to the complete mitochondrial genome of *Rhinoceros unicornis*

Sequence ID: embX97336.1, Length: Range: 15412 to 15824

Alignment statistics for match Rhino Hap_1			
Score	Identities	Gaps	Strand
747 bits(404)	410/413(99%)	0/413(0%)	Plus/Plus

```
Query  1      CATCAACACCCAAAGCTGAAATTCTACTTAAACTATTCCTTGAACACTCCTCTCTTAAAC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15412  CATCAACACCCAAAGCTGAAATTCTACTTAAACTATTCCTTGAACACTCCTCTCTTAAAC  15471

Query  61     CACAAACCCCCAACTATGTAACATGCCAGTATTAGTGACTCCTATATGTCTCATACATAA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15472  CACAAACCCCCAACTATGTAACATGCCAGTATTAGTGACTCCTATATGTCTCATACATAA  15531

Query  121    TATATTACATCACACTATGGTTATGTACATCGTGCATTAAATTGTTTGCCCCATGCATAT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15532  TATATTACATCACACTATGGTTATGTACATCGTGCATTAAATTGTTTGCCCCATGCATAT  15591

Query  181    AAGCATGTACATTATATTATTGATCTTACATAAGACATTAGGTCATTAATAAGACATAAG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15592  AAGCATGTACATTATATTATTGATCTTACATAAGACATTAGGTCATTAATAAGACATAAG  15651

Query  241    CATTAAGCACAGTGTATGAATATCCTCGACCCAGGCGATGTTGATTAATATCGCATAGTA  300
              |||||||||||||||||||||||||||||| |||||||||||||||| ||||||||
Sbjct  15652  CATTAAGCACAGTGTATGAATATCCTCGACCCAAGCGATGTTGATTAATATTGCATAGTA  15711

Query  301    CATACAGTCATTGATCGTACATACCCCATTCAAGTCAAATCATTTCCAGTCAACATGCAT  360
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  15712  CATACAGTCATTGATCGTACATACCCCATTCAAGTCAAATCATTTCCAGTCAACATGCGT  15771

Query  361    ATCATAACCAATAGTCCGTACCGCTTAATCAGCAAGCCGCGGGAAATCATCAA  413
              |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15772  ATCATAACCAATAGTCCGTACCGCTTAATCAGCAAGCCGCGGGAAATCATCAA  15824
```

4.7.1 Comparison of haplotype sequences with GenBank data through Blast

Basic Local Alignment Search Tool, or BLAST, available to the users through web service BLAST, provided by NCBI is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences (Altschul *et al.,* 1990). Part of BLAST result of one haplotype (hap_1) is presented in the Table 4.5 to know the similarities and dissimilarities of the sequences.

Table 4.5 A part of NCBI Blast result of Rhino hap_1

Description	Max score	Total score	Query cover	Iden-tical	Accession
Rhinoceros unicornis complete mitochondrial genome	747	747	100%	99%	X97336.1
Rhinoceros unicornis tRNA-Pro gene and control region, partial sequence; mitochondrial	708	708	94%	99%	AY742825.1
Rhinoceros unicornis control region, partial sequence; mitochondrial	449	449	58%	100%	JF825418.1
Rhinoceros unicornis voucher Zuchtbuch 191 control region, partial sequence; mitochondrial	449	449	58%	100%	JF825417.1
Rhinoceros unicornis voucher Zuchtbuch 06 control region, partial sequence; mitochondrial	438	438	58%	99%	JF825415.1
Rhinoceros unicornis voucher Zuchtbuch 079 control region, partial sequence; mitochondrial	422	422	58%	98%	JF825395.1

Table 4.5 contd

.

Description	Max score	Total score	Query cover	Iden-tical	Accession
Ceratotherium simum complete mitochondrial DNA sequence	263	263	100%	79%	Y07726.1
Equus asinus complete mitochondrial genome	100	100	18%	90%	X97337.1

4.7.2 Mitochondrial DNA diversity among three groups of *R. unicornis*

In the rhino group of Kaziranga National Park, 22 different haplotypes were obtained, 9 haplotypes were detected in Pobitora WLS and 7 haplotypes were found in Orang National Park. In Table 4.6, the distribution and frequencies of different haplotypes of *R. unicornis* in three protected areas of Assam are presented. Different D-loop haplotypes of *R. unicornis* and their frequencies of occurrences in three protected areas are graphically presented in Figure 4.4. From the figure it has been observed that the Kaziranga National park represent almost all haplotypes except H19 and H20 which are found in Orang National Park. The Percentage of different haplotypes of *R. unicornis* sampled from three habitats viz. Kaziranga, Pobitora and Orang are shown in Figure 4.5.

Table 4.6 Distribution and frequencies of Haplotypes of *R. unicornis* in three protected areas of Assam

Haplotypes	Kaziranga National Park	Pobitora WLS	Orang National Park	Total haplotype sequence obtained
Hap_1	21	3	5	29
Hap_2	16	--	--	16
Hap_3	9	2	--	11
Hap_4	7	--	--	7
Hap_5	13	--	--	13
Hap_6	6	2	--	8
Hap_7	11	--	--	11
Hap_8	6	---	--	6
Hap_9	35	7	8	50
Hap_10	4	--	--	4
Hap_11	12	--	--	12
Hap_12	14	4	5	23
Hap_13	10	--	--	10
Hap_14	7	--	--	7
Hap_15	8	1	--	9
Hap_16	6	--	--	6
Hap_17	7	--	--	7
Hap_18	12	--	--	12
Hap_19	--	--	2	2
Hap_20	--	--	3	3
Hap_21	7	--	2	9
Hap_22	9	3	3	15
Hap_23	12	3	--	15
Hap_24	9	2	--	11
Total D-loop sequence	241	27	28	296

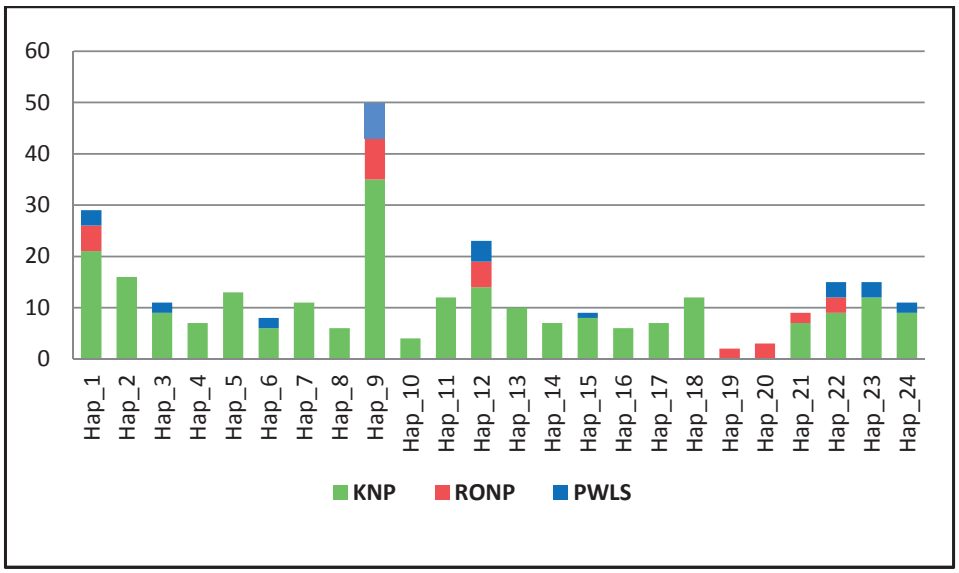


Figure 4.4 Different D-loop haplotypes of *R. unicornis* and their occurrences in three habitats

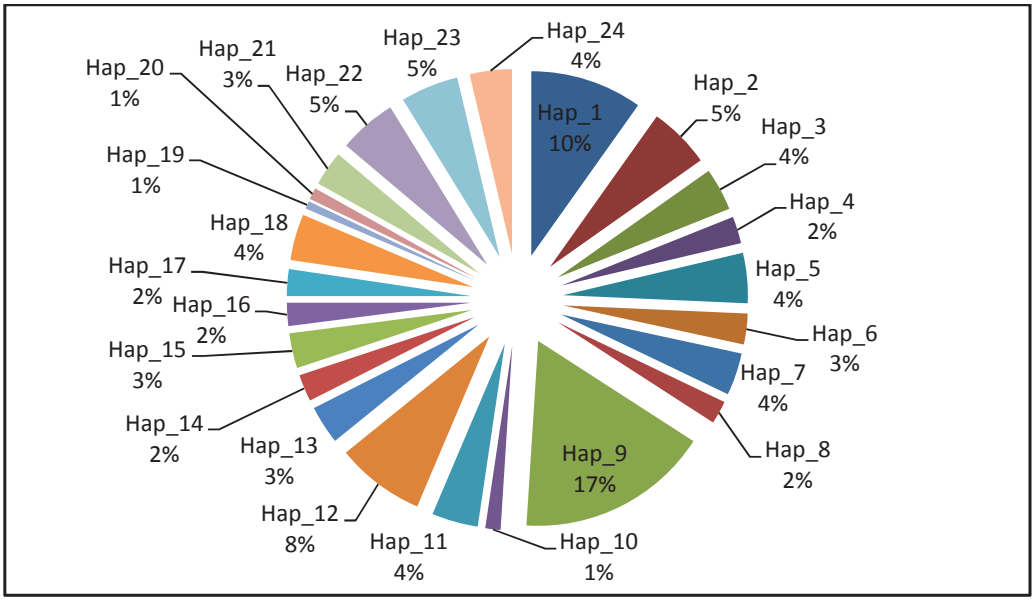


Figure 4.5 Percentage of different D-loop haplotypes sequences of *R. unicornis* sampled from three habitats: Kaziranga, Pobitora and Orang.

The mean haplotype diversity of the three rhino groups is (Hd) 0.97571±0.011. The 21 Polymorphic sites found within 24 haplotypes of *R. unicornis* obtained from three protected areas of Assam and two GenBank sequences is presented in Figure 4.6 with reference to that sequence Acc. no. X97336. In this observation it has been found that the variable positions of nucleotides at certain sites are common in the haplotypes.

Haplotypes	Neucleotide sites
	11111123333334444
	33591234496455560001
	45557280506424783258O
GenBank_R._unicornis_X97336	TACTTAGTTGGGTGTAATGTG
Genbank_R._unicornis_NC001779	.....
Hap_1	..T..G.C.....
Hap_2	..T..GAC.....
Hap_3	..T..G.C..A.....
Hap_4	..TC...C.A.....
Hap_5	..TC...C.....
Hap_6	.....G.....
Hap_7	.G.C...C.....
Hap_8	..TC...C......A.....
Hap_9	.....
Hap_10	CGTC..AC..A.....
Hap_11	.GT...ACC.....
Hap_12	.GT...AC.....
Hap_13	..TC...C...C.....
Hap_14	.....A..G.A.
Hap_15	..T..G.C..A.....A
Hap_16	..T..G.C....C...G....
Hap_17	.....C.G.....
Hap_18	..T..G.C..A.....C..
Hap_19	.GT...AC...CC.....
Hap_20	..T....CC..AC.....
Hap_21	.GT....CC..A.....
Hap_22	.....G.C...A.....
Hap_23	.GT.CG.C..A.....
Hap_24	.....A

Figure 4.6 Polymorphic sites within 24 D-loop haplotypes of *R. unicornis* obtained from three protected areas of Assam, India and two GenBank sequences of complete mitochondrial DNA sequences (Acc. No.X97336.1 and NC 001779.1). Dots (.) denote the nucleotide identical to that of reference sequence (X97336). The top three rows of numbers represent the polymorphic positions and should be read from up to down.

The D-loop haplotypes obtained in the three protected areas have different frequencies. The relative frequencies and shared D-loop haplotypes of three habitats namely Kaziranga (K), Orang (O) and Pobitora (P) were given in the Table 4.7. The Haplotype frequencies of Indian Rhino were calculated in Arlequine with R software from three habitats are individually shown in Figure 4.7. The graphs of observed and expected haplotype frequencies of Rhino groups in the three habitats of Assam were also constructed in Arlequine which are shown in Figure 4.8.

Table 4.7 Relative haplotype frequencies of D-loop haplotypes

Haplotypes	Shared Haplotypes	Rhino Kaziranga (241)	Rhino Pobitora (27)	Rhino Orang (28)
Hap_1	KH1, PH1, OH1	0.0871	0.111	0.179
Hap_2	KH2	0.0664	---	---
Hap_3	KH3, PH3	0.0373	0.0741	---
Hap_4	KH4	0.029	---	---
Hap_5	KH5	0.0539	---	---
Hap_6	KH6,	0.0249	0.0741	---
Hap_7	KH7	0.0456	---	---
Hap_8	KH8	0.0249	---	---
Hap_9	KH9, PH9, OH9	0.145	0.259	0.286
Hap_10	KH10	0.0166	---	---

Table 4.7 contd.

Haplotypes	Shared Haplotypes	Rhino Kaziranga (241)	Rhino Pobitora (27)	Rhino Orang (28)
Hap_11	KH11	0.0498	---	---
Hap_12	KH12, PH12, OH12	0.0581	0.148	0.179
Hap_13	KH13	0.0415	---	---
Hap_14	KH14	0.029	---	---
Hap_15	KH15, PH15	0.0332	0.037	---
Hap_16	KH16	0.0249	---	---
Hap_17	KH17	0.029	---	---
Hap_18	KH18	0.0498	---	---
Hap_19	OH19	---	---	0.0714
Hap_20	OH20	---	---	0.107
Hap_21	KH21, OH21	0.029		0.0714
Hap_22	KH22, PH22, OH22	0.0373	0.111	0.107
Hap_23	KH23, PH23	0.0498	0.111	---
Hap_24	KH24, PH24	0.0373	0.0741	---

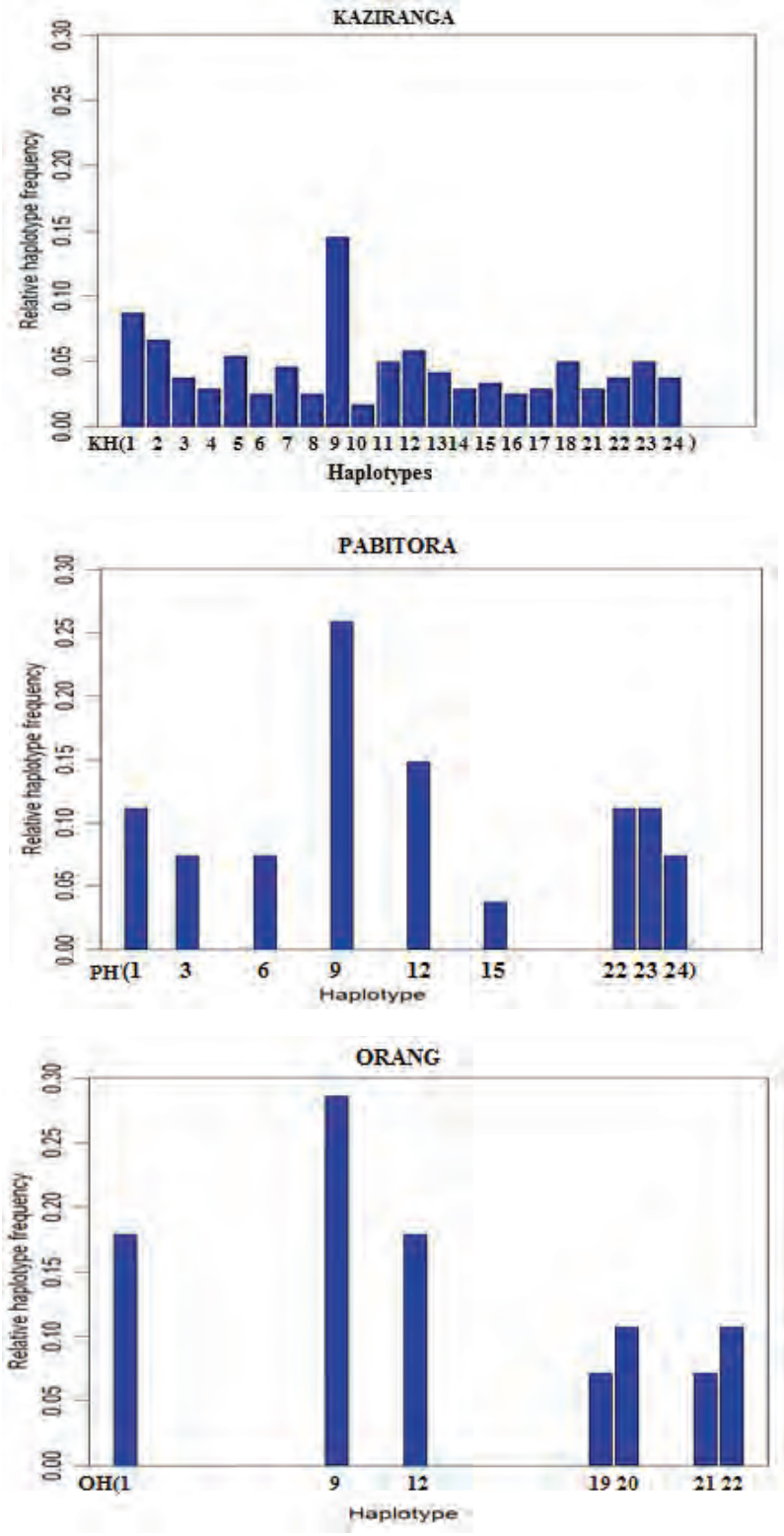


Figure 4.7 Relative haplotype frequencies of Indian Rhino calculated in Arlequine with R software from three habitats

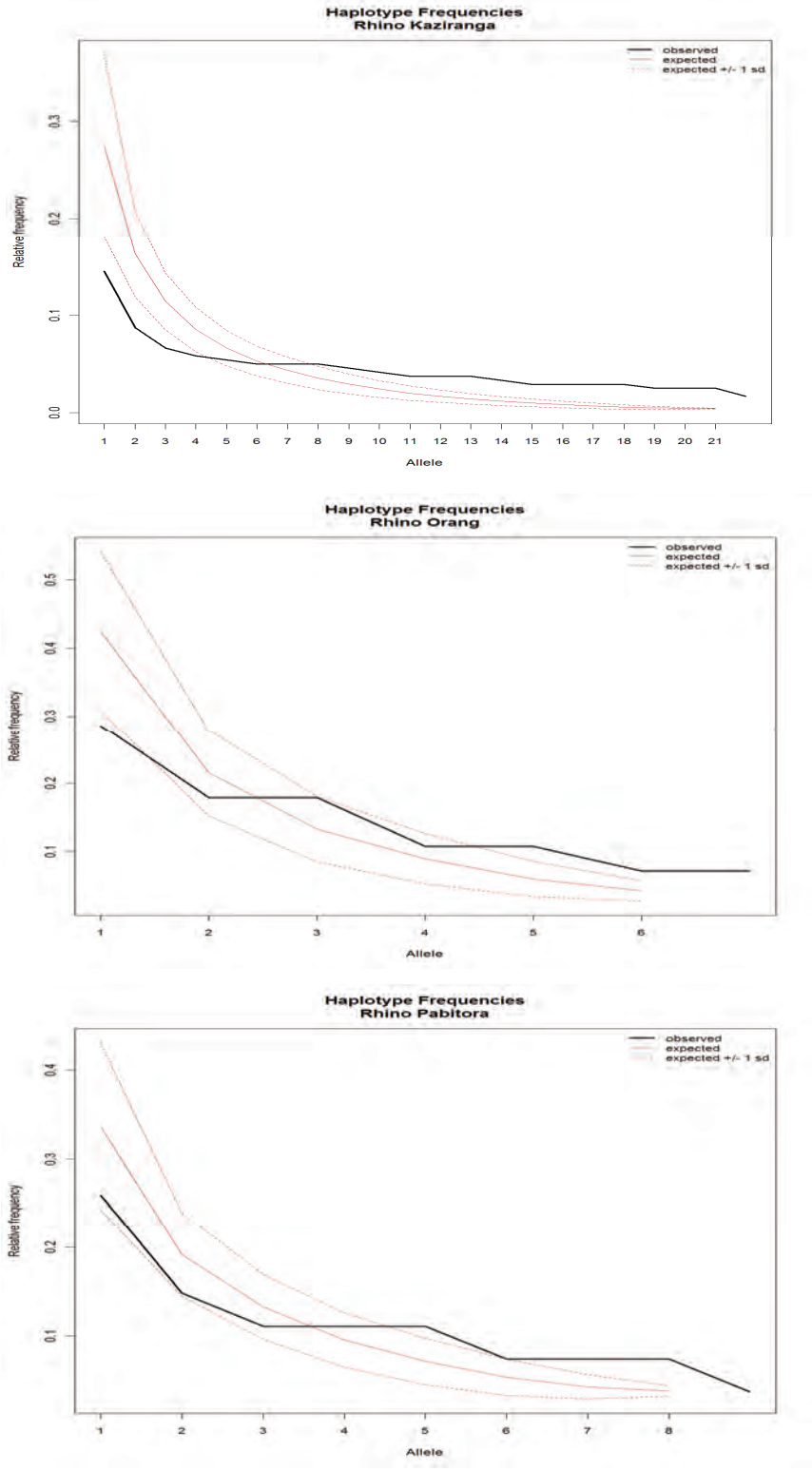


Figure 4.8 Observed and expected haplotype frequencies of Rhino groups in three habitats of Assam

Table 4.8 Estimates of within population variability of Indian Rhinoceros population in three habitats. Standard deviation (SD) values are given in parentheses

Variables	Kaziranga	Orang	Pobitora
Number of Sample	241	28	27
Number of haplotypes	22	7	9
Number of Variable/polymorphic sites	21	10	10
Total number of mutations	22	11	11
Number of transitions	16	9	10
Number of transversions	6	2	1
Singleton variable sites	10	4	4
Parsimony informative sites	11	6	6
Haplotype diversity	0.99567	0.9000	0.9412
Nucleotide diversity (Pi)	0.01095 (0.0053)	0.01049 (0.0054)	0.00982 (0.0046)
Mean number of pairwise differences	3.932613 (1.979008)	3.947090 (2.037406)	3.225071 (1.717077)
Variables	Kaziranga	Orang	Pobitora
Tajima's *D(* test for departure from neutrality)	0.40849	1.29517	0.98110
Standard diversity indices	0.187 (0.1470)	0.359 (0.1355)	0.322 (0.1534)

Table 4.8 contd.

Variables	Kaziranga	Orang	Pobitora
Pi	3.933	3.947	3.225
Theta(S)	3.575 (1.030)	2.826 (1.110)	2.594 (1.230)
Theta(Pi)	3.932 (1.020)	3.947 (1.936)	3.225 (1.151)
Fu's FS	-2.16506	1.50745	-0.20211
Nucleotide composition	C : 23.74% T : 29.33% A : 34.19% G : 12.74%	C : 23.65% T : 29.38% A : 34.17% G : 12.80%	C : 23.76% T : 29.31% A : 34.19% G : 12.74%

The Table 4.8 represent the various estimates of within population variability of Indian Rhinoceros in three habitats. A large number of haplotypes (22) were obtained from the Kaziranga national park where haplotype diversity was estimated 0.99567 showing high genetic diversity of the rhino population. No statistical significance for Fu's Fs or Tajima's D values was observed for whole population or population for each habitat ($P > 0.10$) (Table 4.8). The average nucleotide composition of all haplotype sequences were 34.18%A, 29.34%T, 12.76% G and 23.72% C and the average nucleotide content of A + T (63.52%) was obviously higher than that of G + C (36.48%). The nucleotide compositions of D-loop sequence of all three rhino groups are nearly similar. But rhino group of Kaziranga have a higher nucleotide diversity (Pi = 0.01095) than those from the Orang (Pi = 0.01049) and Pobitora group (Pi = 0.00982).

Different phylogenetic trees were constructed from the 24 haplotype sequences revealed a low differentiation between the different haplotypes of *R. unicornis* obtained from three different habitats. The Neighbour-joining (NJ) tree of all *Rhinoceros unicornis* haplotypes based on the D-loop sequences of this study is shown in Figure 4.9. This model distinguishes between two types of substitutions: transitions, where a purine is replaced by another purine or a pyrimidine is replaced by another pyrimidine, and transversions, where a purine is replaced by a pyrimidine or vice versa. The model assumes that the rate of transitions is different from the rate of transversions. The same haplotypes with some GenBank sequences were used in construction of NJ tree (Figure 4.10) to find out the relation with outgroups (other rhino species). The NJ tree combines groups that are closest to each other and also furthest from the rest. Bootstrap analysis was done to determine the differentiation among the haplotypes. In Figure 4.9 most of the haplotypes showed bootstrap values lower than 70, except in the few haplotypes such as H21, H22 and H23. Figure 4.11 depicts the same type of dendogram with two GenBank sequences of *R. unicornis* (Acc. No.X97336.1 and NC 001779.1) and one other rhinoceros species *Diceros bicornis* (Acc. No. L22010) as an out-group. The dendograms were constructed several times for establishment of relationship among the haplotypes or to find out the relations with other rhino species. The Maximum Likelihood tree and Maximum Parsimony tree based on the Kimura 2-parameter model of *R. unicornis* was also constructed from all D-loop haplotype dataset (Figure 4.12 and 4.13). Maximum Likelihood is an appealing method of inference as it can incorporate explicit models of evolution and also allows statistical test of evolutionary hypothesis. The phylogenetic trees have depicted low differentiation among the haplotypes of *R. unicornis* in Assam.

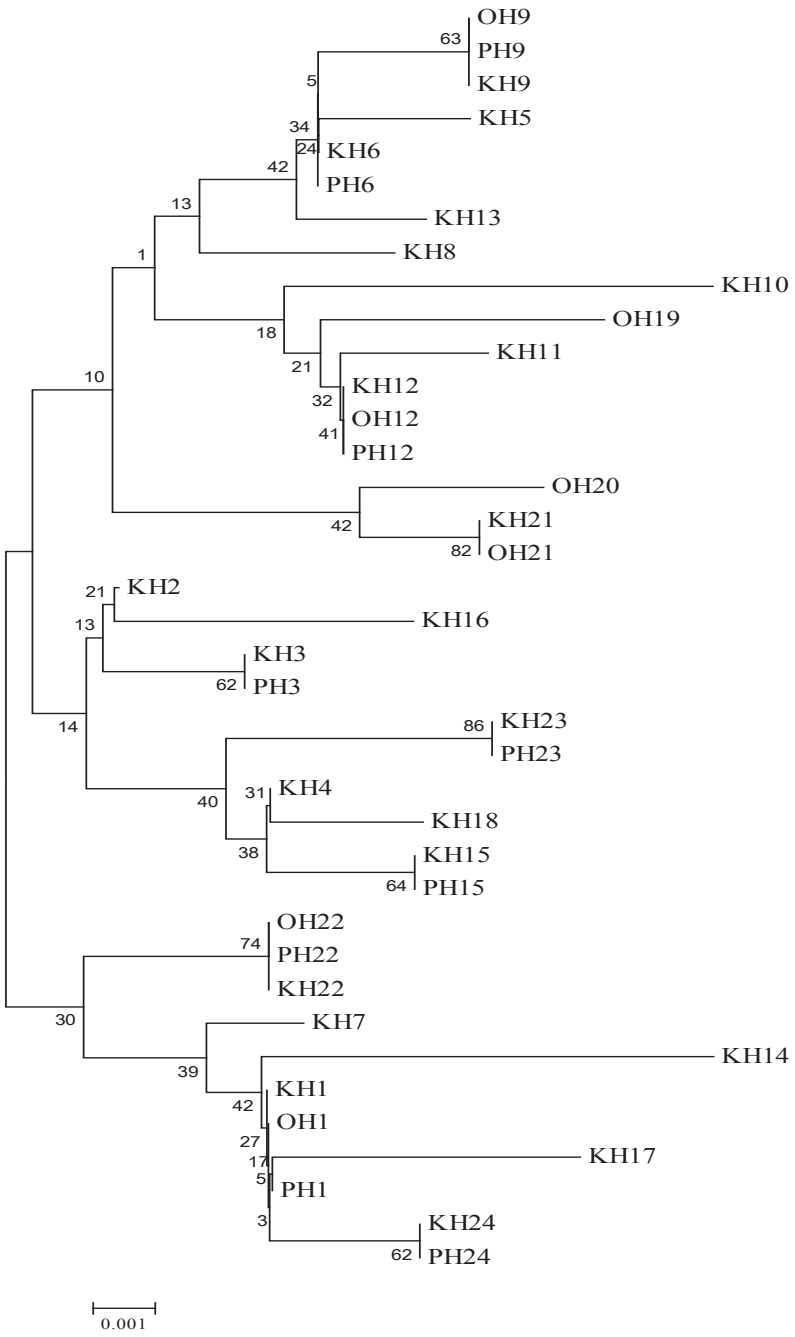


Figure 4.9 The Neighbour-joining tree based on the Kimura 2-parameter model of *Rhinoceros unicornis* haplotypes of D-loop sequences of this study. The values on the branch are bootstrap support based on 1000 replications.

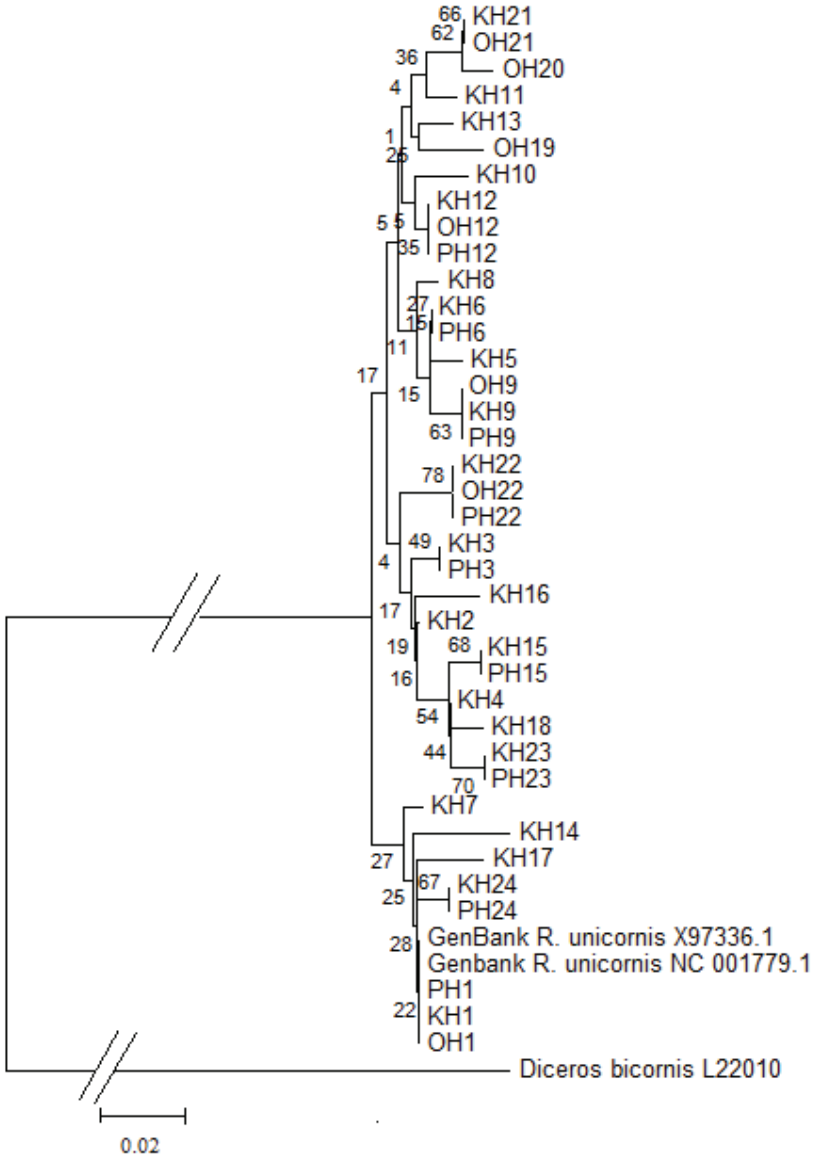


Figure 4.10 The Neighbour-joining tree of *R. unicornis* haplotypes based on the D-loop sequences of this study along with two GenBank sequence (Acc. No.X97336.1 and NC 001779.1) of *R. unicornis* and one out-group, *Diceros bicornis* (Acc. No. L22010). The values on the branch are bootstrap support based on 1000 replications. The tree wide of out-group has been reduced to visualize the differentiation of *R. unicornis* haplotypes.

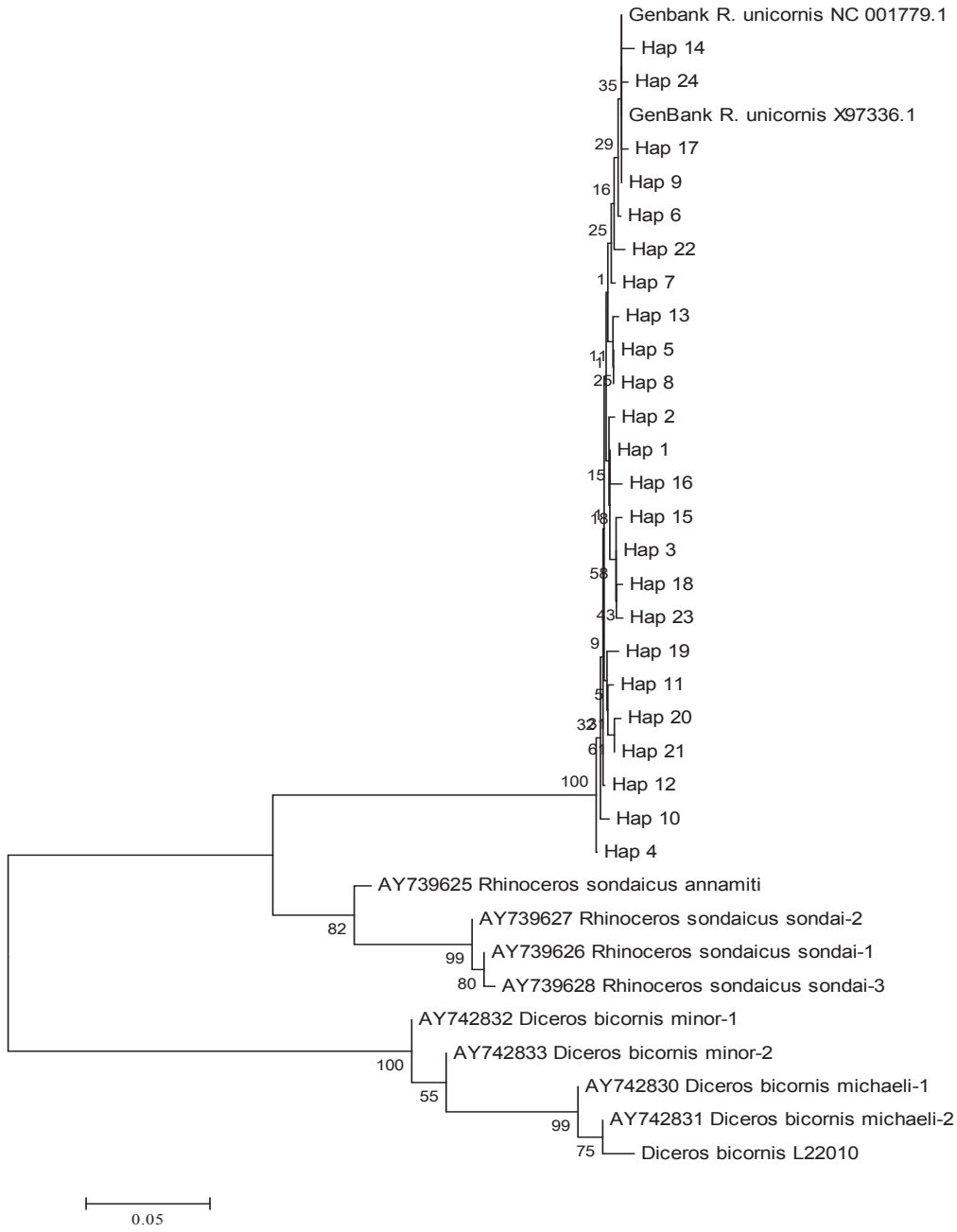


Figure 4.11 The Neighbour-joining tree based on the Kimura 2-parameter model of *R. unicornis* haplotypes of D-loop sequences of this study with some GenBank sequences with sequences of other species of rhino as out-group were also used in construction of the dendogram. The values on the branch are bootstrap support based on 1000 replications.

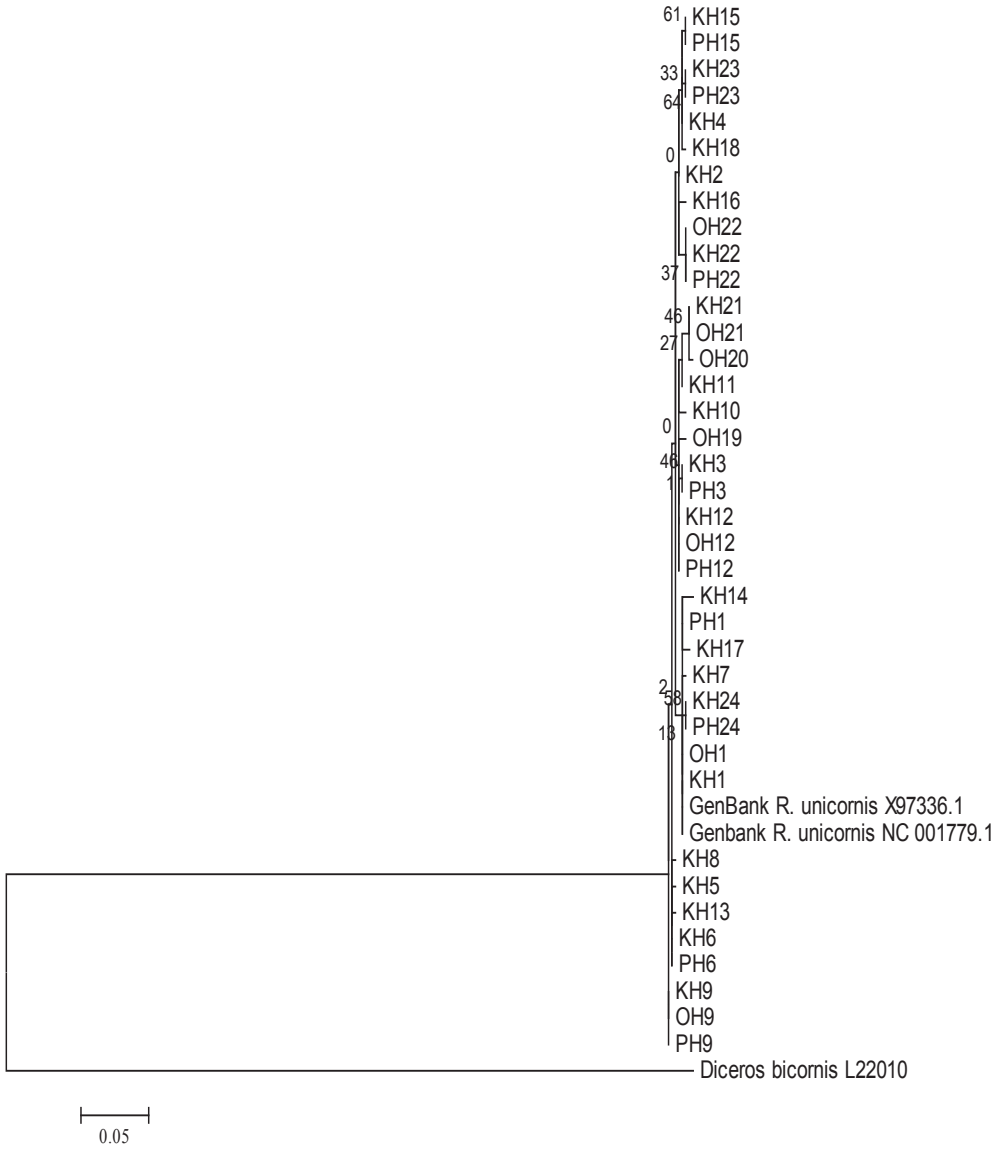


Figure 4.12 The Maximum Likelihood tree based on the Kimura 2-parameter model of *Rhinoceros unicornis* D-loop haplotypes found in this study along with two GenBank sequence (Acc. No.X97336.1 and NC 001779.1) and *Diceros bicornis* (Acc. No. L22010) as outgroup. The values on the branch are bootstrap support based on 1000 replications.

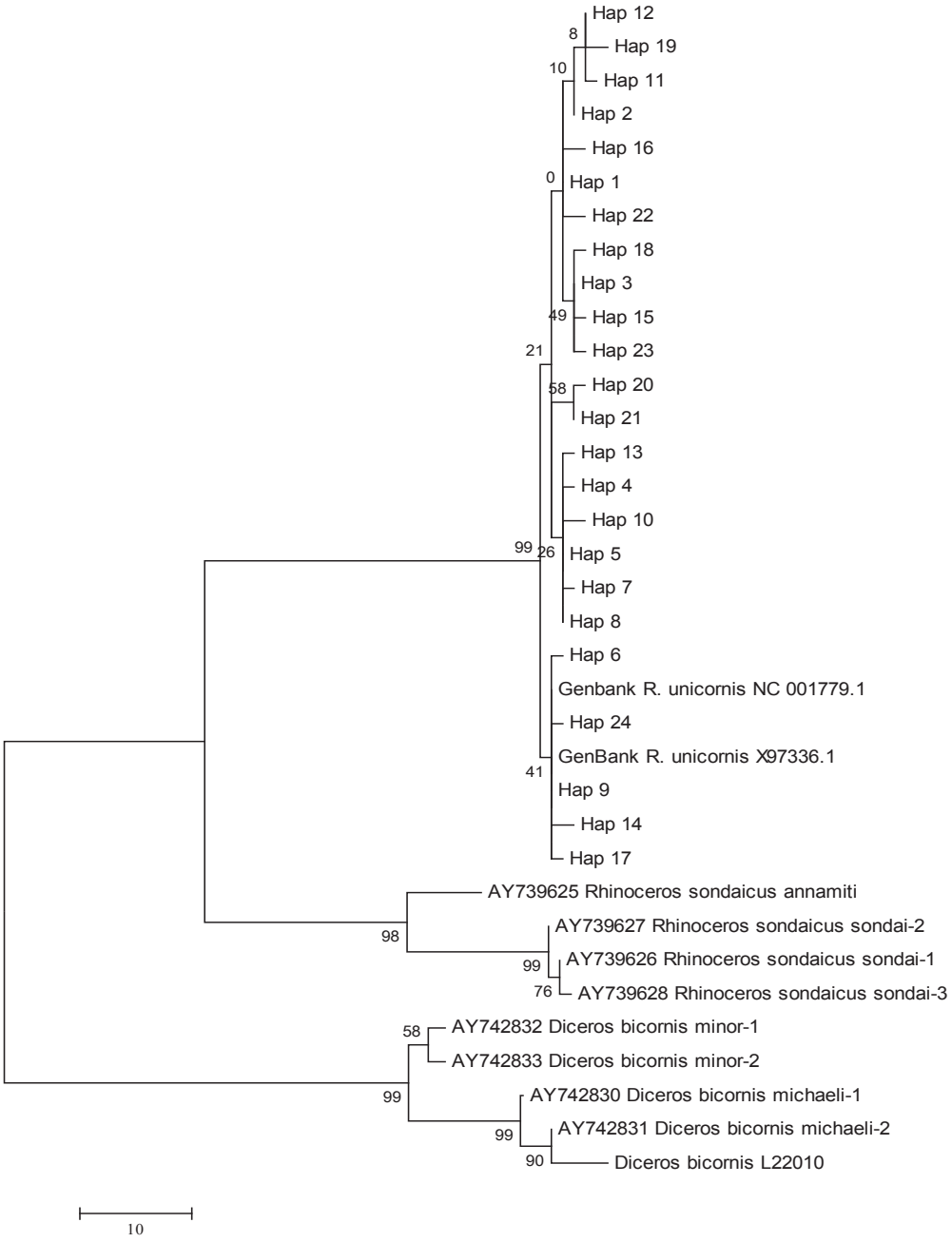


Figure 4.13 Maximum Parsimony tree of *Rhinoceros unicornis* haplotypes based on the D-loop sequences with some GenBank sequences with sequences of other species of rhino as out-group were also used in construction of phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

The Medium-joining network (Haplotype network) of all mtDNA haplotypes based on control region sequences of *R. unicornis* is given in the Figure 4.14. The small black circles is called median vector (mv) seems to be unsampled or hypothetical sequences which have not been found in this study signifies that there could have more D-loop haplotypes in the wild populations. The same haplotypes were also graphically presented in the Figure 4.15 with the help of TCS version 1.21 (Clement *et al.,* 2000).

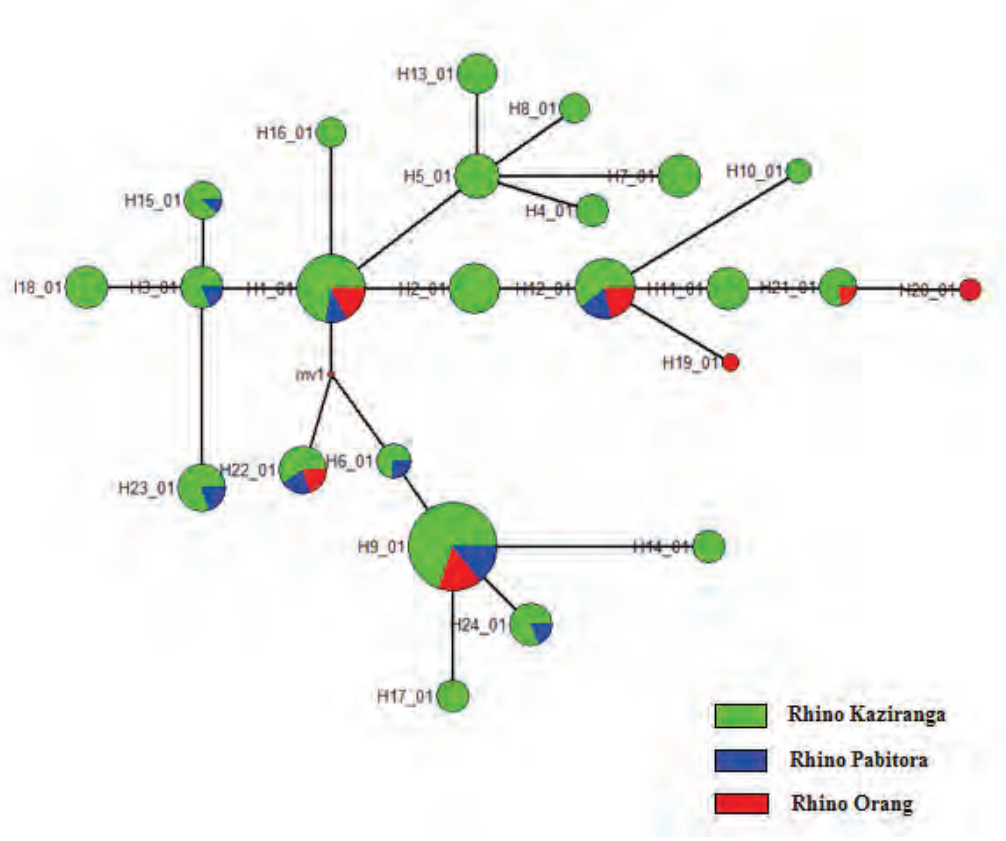


Figure 4.14 Medium-joining networks (Haplotype network) of all mtDNA haplotypes of *R. unicornis* obtained from three protected areas of Assam. Each circle represents a haplotype and its size is proportional to the haplotype frequency. The partially or completely coloured filled circles illustrate the relative frequency of a haplotype in the three habitats. Small black circles is median vector (mv) unsampled, hypothetical sequences which have not been found in this study or extinct ancestral sequences.

In both the network analysis (conducted with NETWORK 4.6.1.1 and TCS) depicts that H9 as the historical haplotype. The other haplotypes have link with H9, on the other hand H1 is another stock in whom a large number of haplotypes have connections.

Parsimony network of 22 haplotypes (only haplotype are shown) of *Rhinoceros unicornis* obtained in Kaziranga National Park and 7 haplotypes from Orang National Park also constructed in the same programme separately (Figure 4.16 and 4.17).

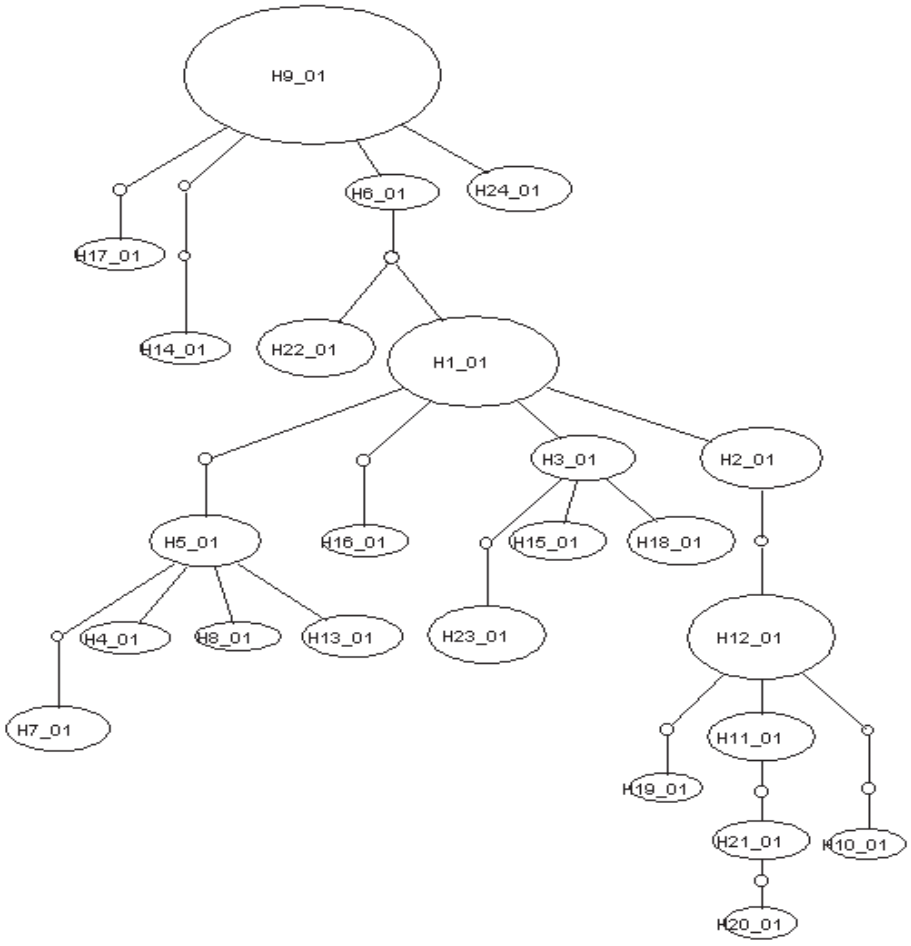


Figure 4.15 Parsimony network conducted with TCS version 1.21 (Clement *et al.* 2000) of all 24 haplotypes of *R. unicornis* obtained in the three habitat. Small circles in the network represent haplotypes not detected in the study.

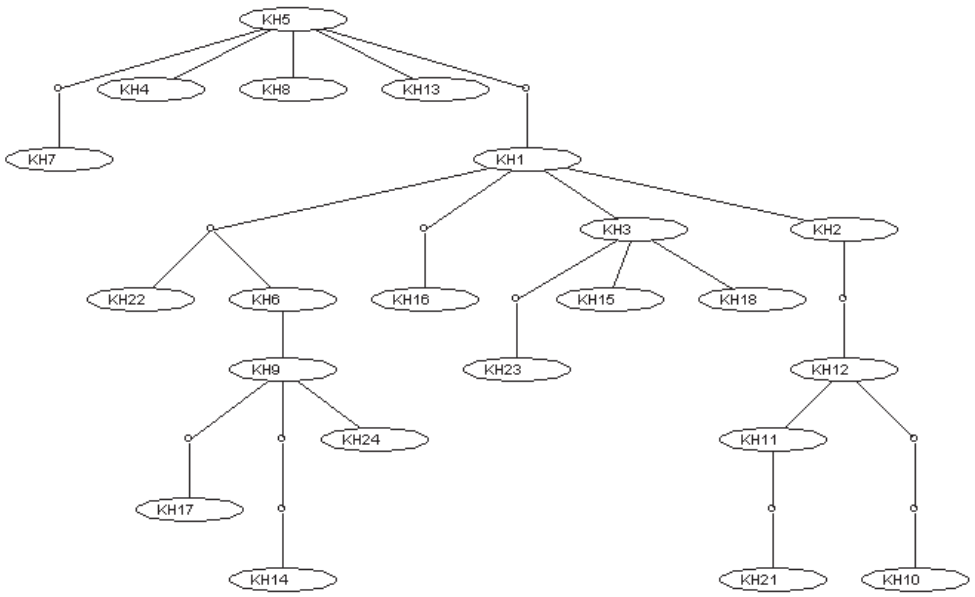


Figure 4.16 Parsimony networks of 22 haplotypes of *R. unicornis* obtained in Kaziranga National Park prepared with TCS version 1.21 (Clement *et al.* 2000). Small circles in the network represent haplotypes not detected in the study.

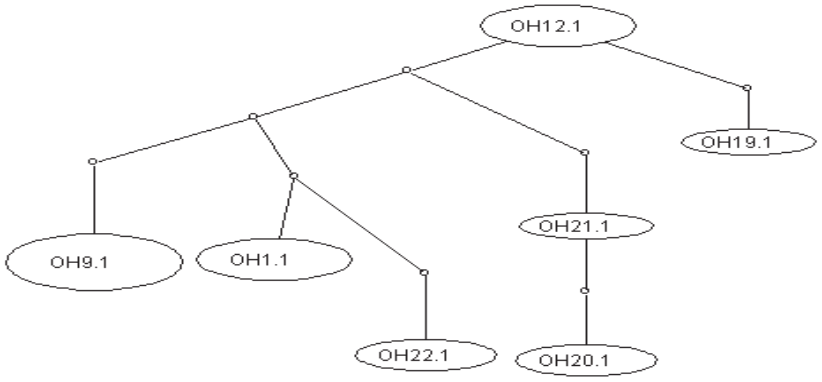


Figure 4.17 Parsimony network of 7 haplotypes of *R. unicornis* constructed with TCS version 1.21 (Clement *et al.* 2000) obtained from Orang National Park. Small circles in the network represent haplotypes not detected in the study.

The Standardized variance in allele frequencies (Fst) among three groups of rhino was calculated in Arlequin based on Kimura 2 parameter from the haplotypic data is presented in the Table 4.9 and Fst value based on F-Statistics is given in Table 4.10. Fst values below 0.05 indicate negligible genetic differentiation whereas > 0.25 means very great genetic differentiation within the population analyzed. When samples from three different locations were considered as three groups it has been found that Kaziranga rhinoceros group have little genetically differentiated from Orang rhino group. However the Fst values of rhino groups from three habitats based on Kimura2 parameter are different from that of F- Statistics. According to Kimura 2 parameter model, there is a difference between Kaziranga and Orang rhino groups (Fst = 0.0393) and Pobitora and Orang (Fst = 0.0220) which signifies little difference (Fst value 0.0 to 0.05; Wright, 1969). While, Fst value between KNP and PWLS was -0.0107, which is not significant and indicates that both the rhino group are genetically indifferent. Therefore it can be inferred that the minor population differentiation may exist between Kaziranga and Orang rhino group. When analysis was done by taking Kaziranga and Pobitora as one group and Orang as other group, the Fst based on F-Statistics was found 0.09259 which is a significant value. In this model the Fst between Kaziranga and Orang is 0.09799 and Kaziranga and Pobitora is 0.08173.

Table 4.9 Population pairwise F_{ST} (based on Kimura 2P) of three Rhino population (below diagonal) and corresponding F_{ST} *p*-values (above diagonal)

	Rhino Kaziranga	Rhino Pobitora	Rhino Orang
Rhino Kaziranga	0	0.9369 (0.0279)	0.1622 (0.0402)
Rhino Pobitora	--0.0165	0	0.0180 (0.0121)
Rhino Orang	0.0393	0.0220	0

Table 4.10 Population pairwise F_{ST} (based on F-Statistics) of three Rhino groups

	Rhino Kaziranga	Rhino Pobitora	Rhino Orang
Rhino Kaziranga	0		
Rhino Pobitora	0.08173	0	
Rhino Orang	0.09799	0.12970	0

Analysis of Molecular Variance (AMOVA) estimates population differentiation directly from molecular data. To explain how the genetic variation of rhino population (within and among the groups), three different AMOVA analyses were performed by Arlequin software that are presented in the Table 4.11, 4.12, 4.13 and 4.14. The result of the AMOVA (Table 4.11) revealed that 91.62% of the total genetic diversity existed among the individuals within populations and only 8.38 % of the total genetic diversity accounted for differences among populations. In order to understand the partitioning of the levels of genetic diversity of the 3 rhino groups (within and among the groups), three different AMOVA analyses were performed by making 2 groups from the 3 rhino groups in Arlequin (Excoffier *et al.,* 2006). The percentage variation is the amount of diversity in the subpopulation or subgroup associated to the partitioned group.

Amova Test 1

Group 1. "All three Rhino group"

Table 4.11 Analysis of molecular variance (AMOVA) among the groups and within populations

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage variation	Fixation Indices
Among groups	1	2.564	0.04273	8.38	0.08381
Within populations	294	137.321	0.46708	91.62	

The first group analysis was carried out to assess the level of partitioning of genetic diversity between three different groups grouped as “Rhino Pobitora+Rhino Kaziranga” as first and second group is Rhino Orang. The results of this analysis (Table 4.12) show that a difference between the groups is 1.98 and difference among populations within groups is 8.09. In the second group analysis, first grouped was “Rhino Kaziranga+Rhino Orang” as first and second group is Rhino Pobitora. The results of this analysis (Table 4.13) showed that a percentage variation between the groups is -1.31 and difference among populations within groups is 9.97. In another analysis the first group was made from Rhino Pobitora” and "Rhino Orang and second group was from Kaziranga Rhino group (Table 4.14). This analysis reveals percentage variations among groups is -3.41 and among populations within groups is 12.47.

Amova Test 2

Group 1. "Rhino Pobitora" and "Rhino Kaziranga"

Group 2. "Rhino Orang"

Table 4.12 Analysis of molecular variance (AMOVA) between the groups “Rhino Pobitora" +"Rhino Kaziranga” and "Rhino Orang"

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage variation	Fixation Indices
Among groups	1	2.912	0.01025	1.98	0.08251**
Among populations within groups	1	2.494	0.04179	8.09	0.10072**
Within populations	293	136.155	0.46469	89.93	0.01984**

**p<0.01

Amova Test 3

Group 1. "Rhino Kaziranga" and "Rhino Orang"

Group 2. "Rhino Pobitora"

Table 4.13 Analysis of molecular variance (AMOVA) between the groups "Rhino Orang" +"Rhino Kaziranga” and "Rhino Pobitora"

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage variation	Fixation Indices
Among groups	1	2.396	-0.00666	-1.31	-0.02142
Among populations within groups	1	3.010	0.05073	9.97	0.02921**
Within populations	293	136.155	0.46469	91.34	0.07958**

**p<0.01

Amova Test 4

Group 1. "Rhino Pobitora" and "Rhino Orang"

Group 2. "Rhino Kaziranga"

Table 4.14 Analysis of molecular variance (AMOVA) between the groups ""Rhino Pobitora" +Rhino Orang" and"Rhino Kaziranga"

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage variation	Fixation Indices
Among groups	1	3.188	-0.01740	-3.41	0.12063
Among populations within groups	1	2.217	0.06375	12.47	0.09069 **
Within populations	293	136.155	0.46469	90.93	-0.03405**

**p<0.01

To explain the population expansion and gene flow among the rhino groups, mismatch statistical analysis was performed. Mismatch distribution is a graphic way of visualizing the signature of a population expansion. If one population expands during two separate periods, there are two collecting phases, which will generate mismatch distributions. It is based on the distribution of the number of pair wise differences between alleles, from which parameters of a demographic (New) or spatial (sudden demographic) population expansion can be estimated.

The Spatial and Demographic expansion model of Rhinoceros group of Kaziranga national Park, Orang national Park and Pobitora WLS are shown in the Figure 4.18, 4.19 and 4.20 respectively. In these graphs, observed mismatch distribution and its

confidence level at 90%, 95% and 99% are plotted. The mismatch distribution is usually multimodel in samples at demographic equilibrium. But it is unimodel in population having passed through recent demographic expansion with high levels of migration between neighbouring groups/populations/ demes. From the graphs plotted, it can be assumed that there is a gene flow among the rhino groups which are distantly located habitats. In Orang rhino group, the mismatch graph showed a multimodel type of curve indicting less expansion of population in recent times. Different variables of demographic expansion model and spatial expansion model of mismatch statistical analysis of three rhino groups are given in Table 4.15.

Dnasp Graph was constructed from pair wise differences of all D-loop haplotypes of Rhinoceros which is shown in Figure 4.21. In this graph the observed pair-wise difference shows higher peaks than the expected one. The differences are more in initial stage and decreases thereafter. The average numbers of nucleotide substitutions per site in the Rhino populations of Assam are given in Figure 4.22 which showed the sites having different substitution rate of nucleotides.

Pairwise mismatch distributions for all three groups of *R. unicornis* were plotted and tested for goodness-of-fit distribution using parametric bootstrapping of 1000 replicates. Low genetic differentiation in the three group structure was observed, as indicated by the G_{ST} and Snn values of -0.01831 and 0.19518, respectively ($p < 0.001$ for both parameters). The Tajima's D value in three rhino groups are 0.40849, 1.29517 and 0.98110 for Kaziranga, Orang and Pobitora groups. All the 3 values are insignificant though the value of Orang group is larger than the rest. An insignificant Tajima's D value -0.59079 was found among the 3 rhino groups, calculated in Dnasp software.

The average number of nucleotide differences between Kaziranga and Orang rhino group is 4.481 and the same differences between Kaziranga and Pobitora rhino group is 4.020. The average number of nucleotide substitution per site between populations (Dxy) Kaziranga and Orang rhino group is 0.01085 while this figure is 0.00973 between Kaziranga and Pobitora rhino group. Randomly evolving DNA sequences perhaps present in Orang rhino group. The value of Theta(S) and Theta (pi) which measures of genetic variation have less difference from each other in the three rhino groups. Values of Theta(S) have little more higher in Kaziranga rhino group which indicates the population is more stable than other two rhino group. On the other hand the Theta (pi) values were almost similar for all the three groups of samples showing that no population seemed distinctly more variable than the other. All groups had comparable values of Theta(S) and Theta (pi), indicating stable populations. The shape of the mismatch distribution has been shown to be influenced by past demographic events such as expansions and bottlenecks which are shown as observed and expected graphs at different confidence intervals.

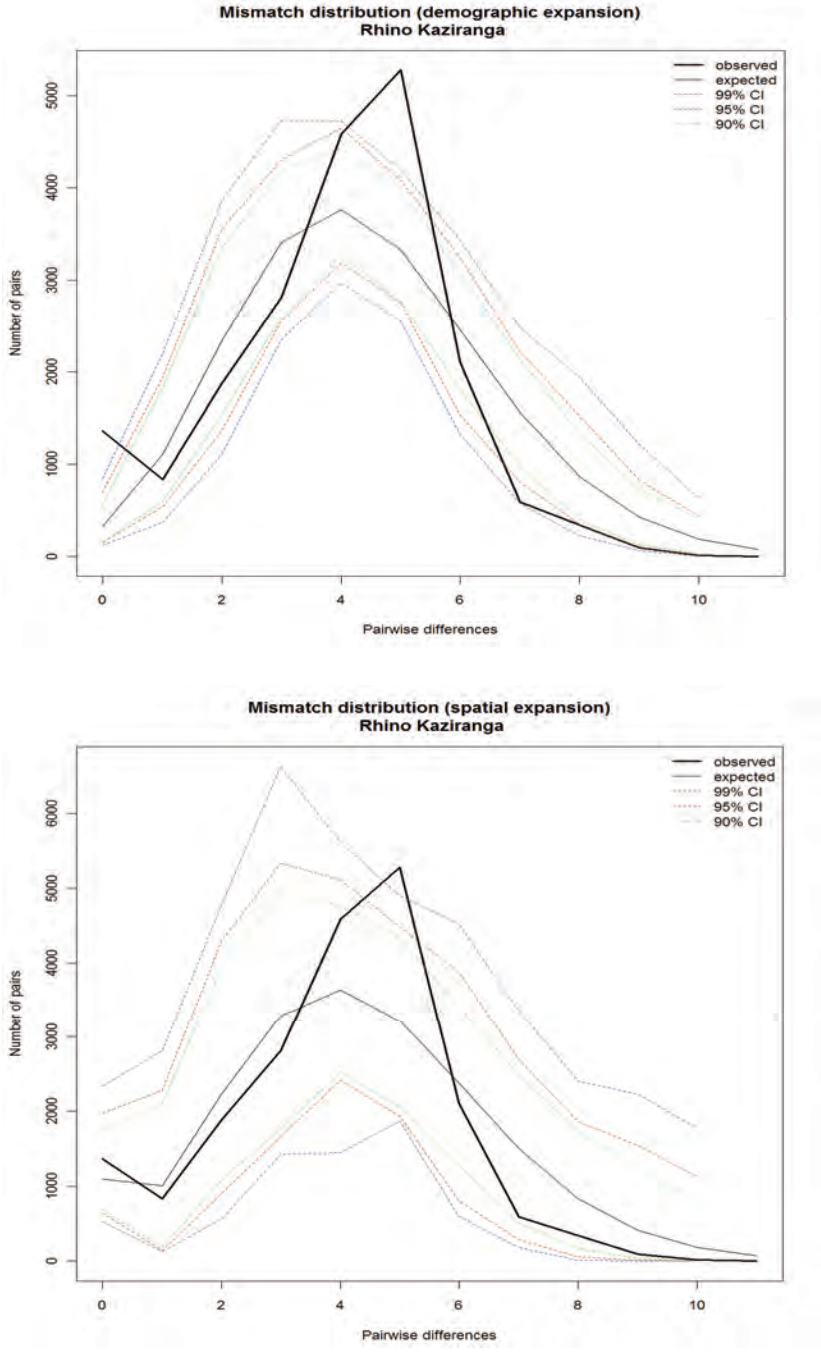


Figure 4.18 Mismatch distribution graphs showing Spatial and Demographic expansion model of Rhino group of Kaziranga national Park. In the x axis the number of differences between pairs of haplotypes and on the y axis their frequencies. Confidence intervals are shown in dotted lines.

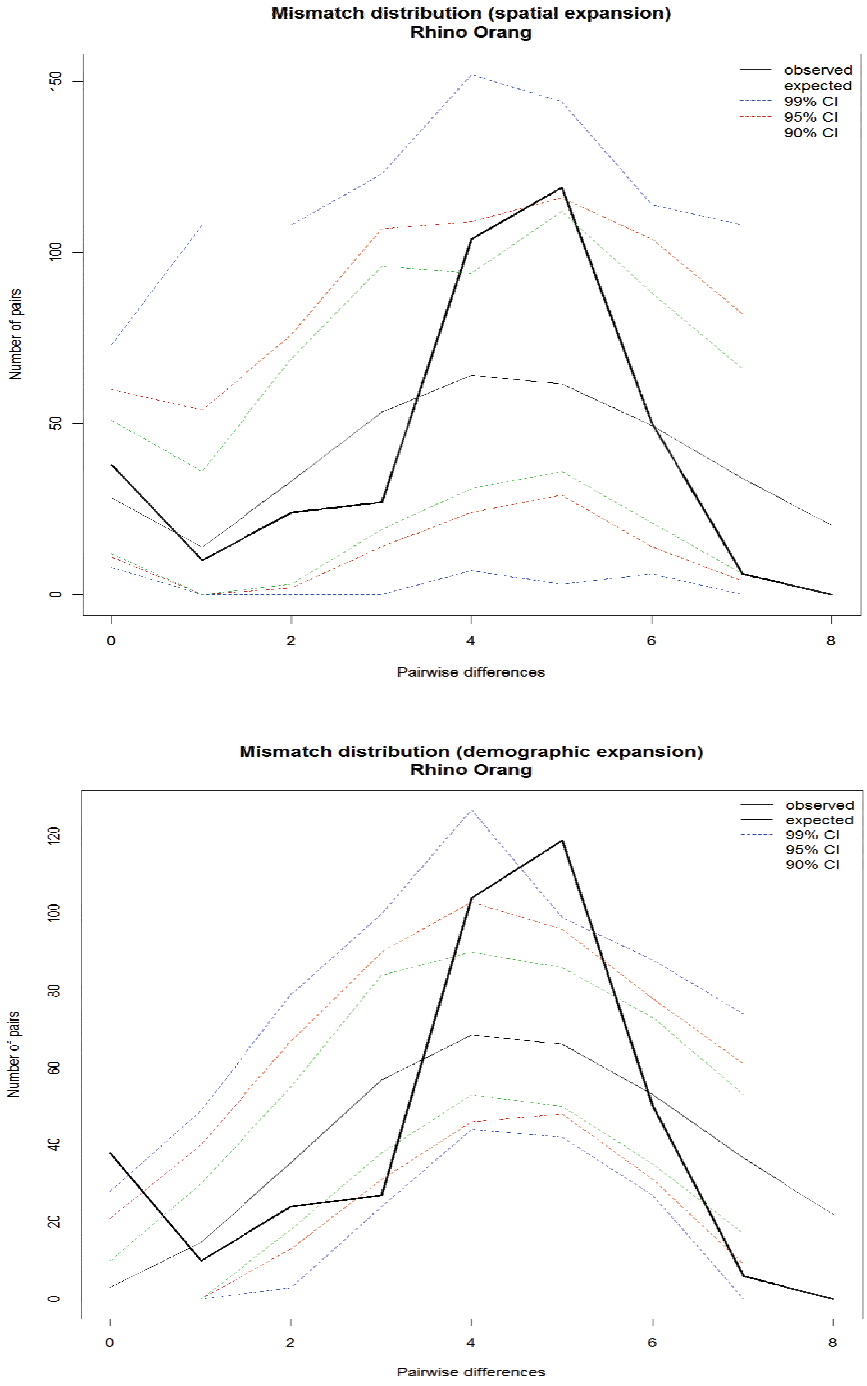


Figure 4.19 Mismatch distribution graphs showing Spatial and Demographic expansion model of Rhino group of Orang national Park. The Y axis stands for the average probability that two DNA sequences differ at a given number of sites represented on the X axis. Confidence intervals are shown in dotted lines.

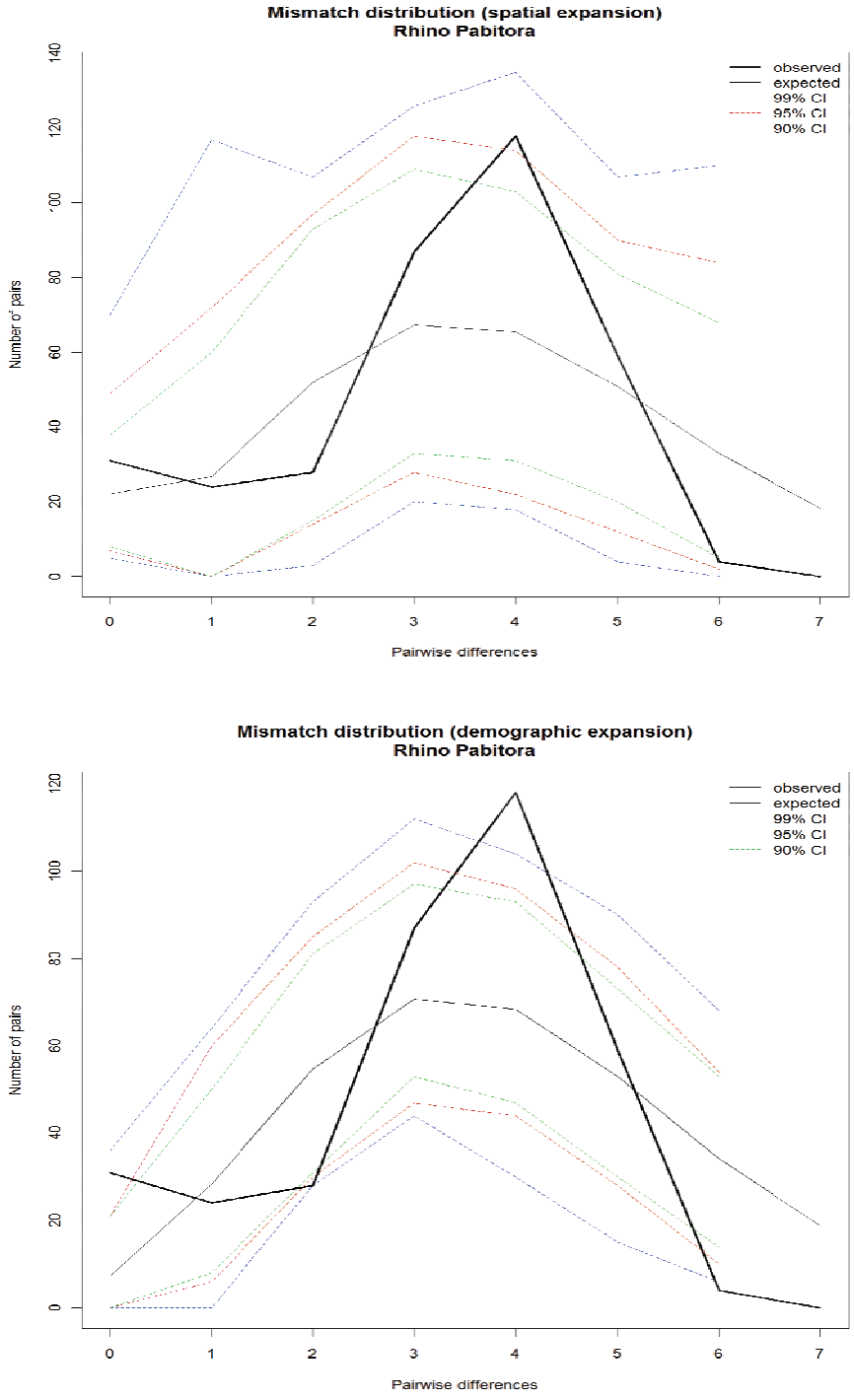


Figure 4.20 Mismatch distribution graphs (expected and observed) showing Spatial and Demographic expansion model of Rhino group of Pobitora WLS. Confidence intervals are shown in dotted lines.

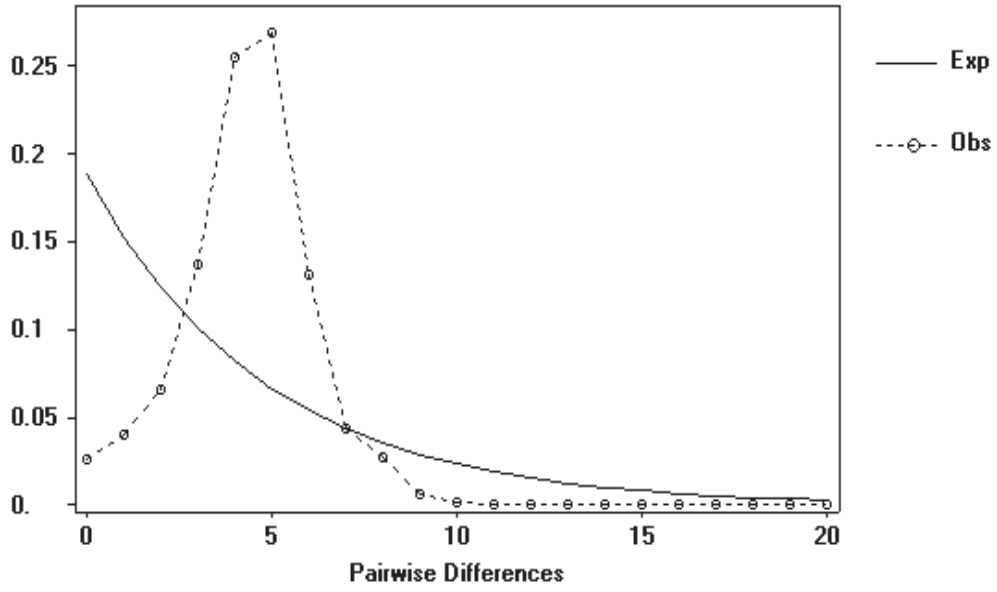


Figure 4.21 Population Size changes Graph showing Pair wise differences of all D-loop haplotypes of Rhinoceros based on constant population size model

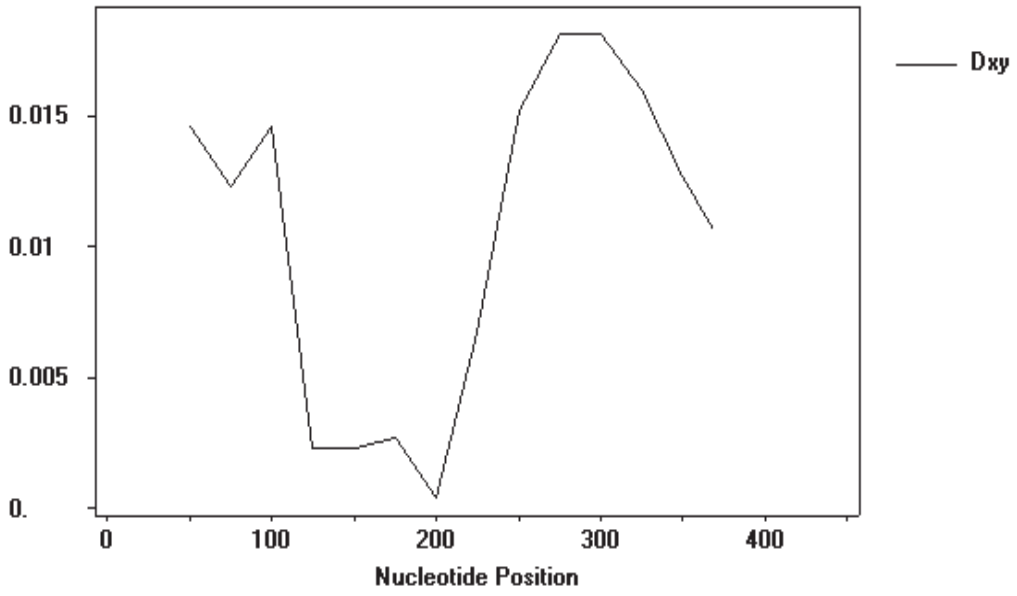


Figure 4.22 Number of nucleotide substitutions per site in the Rhino populations of Assam

Table 4.15 Variables showing Demographic expansion and Spatial expansion model of Mismatch Statistical analysis of three rhino groupns. Theta0 and Theta1 are pre-expansion and post-expansion population's size; Tau is the expansion time.

Statistics	Rhino Kaziranga	Rhino Pobitora	Rhino Orang
Demographic expansion model			
Tau	4.4	3.9	4.8
Tau qt 95%	4.97070	4.63867	5.67773
Theta0	0.030	0.00000	0.00176
Theta0 qt 95%	1.01426	0.42891	0.16875
Theta1 qt 5%	60.2343	22.4765	23.7265
Sum of Squared deviation (SSD)	0.01888	0.05237	0.07985
Model (SSD) p-value	0.01000	0.00000	0.07985
Raggedness index	0.04638	0.08952	0.09711

Table 4.15 contd.

Statistics	Rhino Kaziranga	Rhino Pobitora	Rhino Orang
Spatial expansion model			
Tau	4.42621	3.88511	4.81054
Tau qt 95%	5.24630	5.24450	6.09811
Theta qt 95%	1.96327	1.65990	1.85248
SSD	0.01730	0.03823	0.04585
Model (SSD) p-value	0.02000	0.03000	0.03000
Raggedness index	0.04638	0.08952	0.09711

4.8 DISCUSSION

The present study has revealed mitochondrial DNA diversity in the rhino population of three protected areas of Assam (India) namely Kaziranga National Park (KNP), Orang National Park (ONP) and Pobitora Wildlife Sanctuary (PWLS). A total of 24 different haplotypes were recorded from analysis of 296 D-loop sequences. In earlier studies (Das and Goswami 2012b) only 3 haplotypes (Hap03, Hap04 and Hap05) were found from 14 samples in KNP. The number of D-loop has increased when a large number of sample were collected from the three protected areas. Significant haplotype diversity (0.99567) is found in the present studies, indicating a rich genetic diversity of the wild rhinoceros population in Assam, from which we can infer the availability of greater ancestral lineages. Among the 24 haplotypes, a large number individuals are found under the haplotype 9 (hap 09) and it is thus the most available haplotype distributed in the three rhino habitats.

Different phylogenetic trees were constructed to find out the best evolutionary models for better effect with distant related sequences. To find better effect with distant related sequences NJ tree was constructed. Maximum Likelihood is a tree model for nucleotide substitutions, which finds a tree based on probability calculations that best accounts for the large amount of variations of the data i.e. sequences set. From the dendogram it has been observed that the Genbank sequence of *R. unicornis* (X97336 and NC001779) revealed closeness to the Hap01 which is second most available haplotype of rhino population in Assam. The variations of the D-loop sequences are very less which was found from polymorphic site analysis and therefore the bootstrap values of the dendograms are below the significance level except in few samples. This is because D-loop is a rapidly evolving part of the mitochondrial genome and its

mutation rate is high (Saccone *et al.*, 1991; Lopez *et al.,* 1997; Hassan *et al.,* 2009) and perhaps the high diversity in D-loop region was observed in Rhino population. A high level of D-loop diversity has also been recorded in other vertebrates' species (Brown *et al.,* 1986). The genetic diversity is probably related to adaptation to living organism in harsh environmental conditions (Hirayama *et al.,* 2010) and this diversity may result of such. The mtDNA control region may not be neutral but under selection that operates at the point mutation, tandem repeat and the heteroplasmy levels (Munwes *et al.,* 2011). The genetic diversity has been always found to have positive implications to a population. The D-loop diversity observed in the rhino groups of three protected areas provides significant insight into the population structure of the Indian rhino and it provides considerable importance in the study population genetics of the species.

The mismatch distributions show one or two modes in populations having passed through a range expansion, depending on the population density and the amount of migrants exchanged with neighbouring populations. Mismatch distributions were originally developed to test for demographic stability in single populations (Slatkin & Hudson, 1991), but have since been used widely to distinguish between a stable distribution and recent range expansion across multiple populations (Ruegg and Smith, 2002; Toju and Sota, 2006). The distribution is usually bell-shape in populations having increased demographically in the past (Rogers and Harpending, 1992). But the rhino populations are unable to show such type of graphs. The past spatial range expansion might not display a molecular signature of a unimodal distribution characteristic of sudden expansions in un-subdivided populations, even though populations had expanded by several orders of magnitude after the past expansion. Goodness-of-fit of spatial expansion was assessed by calculating the significance of the raggedness index.

Raggedness indices were assumed to be smaller than 0.04 for expanding populations (Harpending, 1994). In Kaziranga rhino group the Raggedness index is 0.04638, which means that the population has been expanding.

The study of various directions from the D-loop haplotypes sequences obtained in the rhinoceros group of three habitats suggests that the three groups do not possess same type of genetic diversity. The genetic diversity is prominent in Kaziranga National Park and it contains a polymorphic population of rhino. But it is also to be mentioned that, the phylogenetic analyses based on different criterion showed that though the rhino population has genetic diversity but their differentiation do not reach to a level to categorized as a sub population. They cannot be considered as separate clade until more analysis on different nuclear DNA is done. This analysis has proved about the presence of more maternal lineage of rhinoceros in three habitats in Assam. The presence of same haplotypes in the three habitats also shows that there exist gene flow between the groups and movement of rhinoceros must be occurred among the different habitats. The movement is possible because rhinoceros is a highly mobile species and stray out is a common behavior of rhino. As the rhinoceros are good swimmer they can even cross the river Brahmaputra and moved from Kaziranga to Orang and vice versa. The patterns of demography and hierarchical genetic structure of rhinoceros was studied because for species with limited geographic ranges, such studies are important elements not only in determining the population structure, but also to be considered in the development of an effective and sustainable management plan (McCracken *et al.*, 2001). The genetic diversity database of rhinoceros population will help in various conservation efforts such as translocation of individuals and population viability assessments (Florescu *et al.,* 2003; Talbot *et al.,* 2003; Harley *et al.,* 2005). The present study has covered only a

few samples from huge 2700 rhino population. Moreover, sampling was not possible in few locations of the protected areas. Extensive sampling from the all rhino bearing places of the protected areas particularly in the Kaziranga national park has possibility to obtain more D-loop haplotypes. The findings of the present study did not correspond to the result of Zschokke and Baur (2003); where Kaziranga rhino group was showed as genetically monomorphic. However, recent studies by Zschokke *et al.* (2011) on captive rhino population from different zoos have demonstrated that *R. unicornis* population is genetically diverged. In another explanation it can be inferred that as there were a large number (24) of D-loop sequence haplotypes for the mtDNA was found in the rhino population in Assam there may be recent population expansion. Out of 21 polymorphic sites detected in 24 haplotypes 8 were "Singleton" variable sites. Moreover, for mtDNA haplotypes, Fu's *Fs* was found negative in both Kaziranga and Orang rhino group and is significant, indicating that there is an excess of low frequency alleles in the population and suggesting a recent post-bottleneck population expansion (Tajima, 1989; Fu, 1997). One possible reason for the high level of diversity of *R. unicornis* found in the protected areas of Assam because these three areas, particularly the KNP has harbor the remaining population in the late 19th and early 20th century that had to moved into the Park from nearby forest areas after losing habitat in the historic range of Brahmaputra River basins.

Wildlife management and conservation initiatives are only possible with the appropriate information on the genetic diversity of wild animals. Managing genetic diversity is one of the primary goals in various conservation efforts (Soltis and Gitzendanner, 1999). To develop effective conservation strategies for the Indian rhinoceros, genetic studies are necessary in order to know the history of partition and

genetic differentiation. Genetic diversity plays an important role for the persistence of a wild population and conservation of genetic diversity is essential for the future management of a species. The greater the genetic diversity within a population, the better it is for the survival of the species (Frankham *et al.*, 2002; Kierstein *et al.*, 2004). The present genetic study of this endangered population will facilitate conservation and key management decisions. Once widely distributed *Rhinoceros unicornis* population is now found only in few protected areas of India and Nepal. In India, the Kaziranga National Park holds the largest population of the Rhinoceros *unicornis* and the park has achieved success in conservation of wild animals particularly *Rhinoceros unicornis* in the last few decades. Protection of suitable rhino habitat seems to be important aspect of rhino conservation (Das and Goswami, 2012a). The population of the greater one-horned rhinos in Kaziranga National Park has increased to a suitable level according to official sources. But still there need of a special management programme for the future viability of the species. The present mitochondrial D-loop study indicates that the rhino population of Kaziranga National Park of Assam contains more than one lineage. It does not mean that the population variation is prominent because the D-loop variability is common in almost all animals. But it is clear that rhinoceros population in Assam have genetic diversity which has a positive effect on population viability. Hence an effective conservation strategy should be implemented to ensure the future of the population.

In last few decades the population of Indian rhinoceros has revived in the Kaziranga National Park though they went to an event of bottleneck during the beginning of 20^{th} century (Laurie *et al.*, 1983). Now the Kaziranga National Park holds the largest free-ranging wild populations of Indian rhinoceros which is more than 2500 individuals. The high level of genetic variation found in Indian rhinoceros populations

has ample scope for evolution to occur. The species is now once again in a critical condition in last two years due to increase rate of poaching which is the greatest threat to the rhinoceros population in Assam. Besides poaching, decline of or loss of suitable alluvial plain grasslands is regarded another threat to the species. The current D-loop diversity study would provide considerable help in the management and translocation of the rhino population to new habitat. The ongoing translocation programme of rhino from Kaziranga and Pobitora and reintroduction it to the former habitat in Manas National Park of Assam should also follow the genetic guideline for better success in the project.

4.9 SUMMARY

1. The partial sequencing and analysis of mitochondrial D-loop region of *Rhinoceros unicornis* population in the three protected areas of Assam showed that the rhino groups of these habitats are genetically diverse, comprising several haplotypes (24 haplotypes found).
2. In Kaziranga National Park the rhino group is more diverged and comprising many haplotypes.
3. Most of the haplotypes obtained in Kaziranga national park are also found in Orang national park and Pobitora WLS. But in Orang national park two different haplotypes were obtained which are not recorded in other two habitats.
4. The haplotypes obtained from in the three habitats namely Kaziranga, Pobitora and Orang however are not much more diverged from each other except two haplotypes found in Orang National park.
5. The population expansion model does not support very recent expansion in the rhino population of Assam.
6. Parsimony network analysis showed that the all three rhino populations have derived from single historical population in which all haplotypes found to connect by each other.
7. The genetic variability and divergence recorded within population is high and between populations is low.
8. Mismatch Statistical analysis indicated that Kaziranga rhino group is more stable than other two.

9. Fu's *Fs* in indicating an excess of low frequency alleles in the Kaziranga and Orang rhino group and suggesting a recent post-bottleneck population expansion.
10. The AMOVA analysis revealed low percentage of variation among the groups (populations) of rhino in Assam.
11. The Fst value showed that there is no differentiation between the Kaziranga and Pobitora rhino groups but Orang rhino group was found somewhat different.

CHAPTER–5

Genetic diversity and population structure analysis of *Rhinoceros unicornis* based on microsatellite analysis from three habitats of Assam

5.1 INTRODUCTION

The understanding of the genetic structure of a population or differentiation between populations is important because it reflects the number of alleles exchanged between populations that influence the genetic composition of individuals. A diverged genetic constitution of a species is produced through exchange of genetic material among populations of that species. Gene flow between populations determines the effects of selection and genetic drift. It also generates new polymorphisms and increases the local effective population size (Balloux and Lugon-Moulin, 2002). In most cases gene flow has positive implications to a population. Gene flow is found more in highly mobile species and this creates genetic divergence in a population. The genetic diversity measure covers the genome and they are usually not within the coding regions of genes. In eukaryotes, throughout the genome there are many regions comprised of tandemly repeated simple sequences. These repeat sequences vary in number and hence in length and are generally called variable number of tandem repeats (VNTRs), although the terms minisatellites and microsatellite are used depending on the size of the repeated sequence of base pairs. Microsatellites usually called Simple Tandem Repeats (STRs), repeated sequences of only 2 to 5 bp long are highly polymorphic class of genetic markers and minisatellites region are characterized by randomly repeating oligonucleotide units ranging from 10 to 60 bp in length. The microsatellites consist of

short sequence repeat motifs of tandemly repeated di, tri, tetra or penta nucleotide sequences and are occurring at large number of loci throughout the eukaryotic genome (Tauz, 1989; Weber and May, 1989; Haberfeld *et al*., 1991). The dinucleotide repeats of microsatellites, cytosine-adenine (CA) or Guanine-thymine (GT) is the most common in mammalian genomes (Meghen *et al*., 1994). The microsatellite loci are randomly distributed and subject to replication slippage while minisatellites loci tend to be concentrated near telomeres (Jeffreys *et al*., 1985; Weber and May, 1989). Polymorphism of microsatellite markers takes the form of variation in the number of repeats at any given locus. According to Weber (1990) microsatellites with more than ten dinucleotide repeats tend to be highly informative. He suggested that in haploid human genome there are at least 35,000 CA repeats which are found every 100,000 bp. The Tri- and tetra-nucleotides of microsatellite have been shown to occur at a frequency of 1 every 300 – 500 kb on X-chromosome (Edwards *et al*., 1991). Due to exceptionally high rate of mutation, the majority of microsatellite loci are highly polymorphic in most mammalian species (Weber, 1990). Microsatellites are conserved across related species. Moreover microsatellite variation is independent of age, sex and environmental changes and hence can be detected at the early stage of development. The microsatellites are found mainly in non-coding regions of genome. However Morin *et al.* (1994) reported the presence of microsatellite in protein coding region exhibiting regulatory role in gene expression and trinucleotide repeats (Richards and Sutherland, 1994). Generally the alleles of microsatellite loci conform to Hardy-Weinberg principle and segregate in a Mendelian fashion. Most microsatellite loci are selectively neutral. This makes them compatible with the assumption of the neutral theory of population genetics (Kimura

and Crow, 1964). To study genetic diversity of a genome, microsatellite loci provide unbiased information about the level of genetic diversity (Jobling *et al*., 2004).

5.2 Microsatellite analysis for population studies

Analysis of genotypic data from neutral loci is an important method for describing the patterns of genetic variation within species and inferring the evolutionary processes. Analysis of highly polymorphic microsatellite loci has provided new opportunities for population geneticists because microsatellite data have unprecedented power to detect and describe small genetic differences between populations but while interpreting a subject utmost care must be taken (Kalinowski, 2002).

The advantages of analysis of microsatellite polymorphism over other techniques are that it is a very reliable, highly accurate and repeatable method. Moreover, microsatellite loci have a much higher mutation rate than allozyme or mitochondrial loci. The greatest advantage of microsatellite is that a single loci can be amplified using PCR and there is ample potential for studying allele frequencies at single hypervariable loci in a population. The speed and accuracy are another advantage if once the appropriate PCR primers are known by which a great deal of polymorphism can be determined. Microsatellite loci are usually examined one at a time via PCR and several loci can be examined simultaneously by multiplexing. The PCR amplification products can be visualized using radioactive or florescence methods. Under radioactive methods, PCR amplified microsatellites can be detected either by direct incorporation of a single labelled dioxynucleotide triphosphate (dNTP) during thermal cycling (internal labeling) or a single dATP end-labelled primer in the PCR mix (end labelling). The products are resolved on acrylamide gels, fixed, dried and autoradiographed. Under the fluorescence methods, fluorescently labelled dNTPs are used for internal labelling in the PCR.

Alternatively and more commonly one of the PCR primers is fluorescently labelled. The fluorescently labelled PCR products are separated on polyacrylamide gel and detected when excited to fluorescence by a laser and analyzed by computer software (Sambrook *et al*., 1989).

The advantage of microsatellites over RFLPs and RAPDs is that their genetic basis of variability is readily apparent. The microsatellites are amplified through unique primers of a genomic region including a well-defined repeat structure that is responsible for the observed variation. Microsatellite markers are currently used for a wide range of molecular genetic studies such as establishing genetic linkage maps, analysis of mating system and population structures and reconstruction of phylogenetic relationships among populations (Queller *et al*., 1993; Roy *et al.*, 1994; Kappes et *al*., 1997). In comparison to minisatellites, microsatellites are found throughout the genome while minisatellites tend to be found more frequently near the ends of chromosomes. The disadvantage of microsatellite is that, the work required to develop primers for each new species examined and that only a few allelic states are possible, hence, increasing the chance of parallel evolution of a particular sequence repeat. However, with the increasing number of microsatellite maps for economically important species and due to the fact that primers developed for one particular species can be sometimes applied across a wide range of related taxa (Moore *et al*., 1991). Microsatellites have proven useful in the analysis of paternity and kinship, effective population sizes and inbreeding, the population levels and to study the amount of hybridization between closely related species (Edwards *et al*., 1992; Queller *et al*., 1993; Roy *et al*., 1994; Paetkau *et al.*, 1995). Microsatellites have also been increasingly used for the study of genetic variation between and within animal populations. They have been successfully applied

in the study of genetic variation in vertebrates (Kashi *et al.*, 1990). Besides, microsatellite markers are suitable for forensic applications and population genetics studies (Roy *et al*., 1994; Ganai and Yadav, 2001).

5.3 Genetic diversity and genetic distances

Genetic diversity is the variation at the level of individual genes, i.e. polymorphism which provides a mechanism for populations to adapt to their ever-changing environment. The more variation the better the chance that at least some of the individuals will have an allelic variant that is suited for the new environment, and they will produce offspring with the variant that will able to reproduce and continue the population in future. Genetic distance is a measurement of genetic relatedness of samples of populations. The estimate is based on the number of allelic substitutions per locus that have occurred during the separate evolution of two populations. This difference measured between two populations provides a good estimate of how divergent they are genetically (Avise, 1994). Genetic distances are used either for estimating divergent time or for construction of phylogenies. Many genetic distances have been developed, of which a few remain in regular use. Each of these genetic distances has unique evolutionary and statistical properties (Nei, 1996, 1987). Many of the earlier measures of genetic distances are geometric distances, which are based explicitly on geometric representations of gene frequencies rather than on any particular population genetic models. These distance measures obey the axioms of Euclidean geometry. The most commonly used of geometric distance is Cavalli- Sforza and Edwards's (1967) chord distance (*D*C). In this distance measure, populations are represented as points on a multidimensional hypersphere with dimension equal to the number of alleles. *D*C is the chord distance between two points representing the gene

frequencies in two different populations. This distance incorporates an angular transformation of gene frequencies and the principle of triangle inequality is fulfilled. Another widely used measure of genetic distance, which is close to *D*C*,* is the Nei *et al*.'s (1983) angular distance (*D*A). This is a modified Cavalli-Sforza and Edwards distance measure. It is based on genetic drift model and is more efficient in determining the true topology of phylogenetic trees especially for closely related populations. It is the most widely used distance measure since it reportedly increase more slowly with time and maintains a linear relationship for longer periods of time (Nei, 1984, Kalinowski, 2002). The most commonly used genetic distances are based on population genetic models. There are two most important genetic models, which have been proposed to explain the mode of mutation as cause of evolutionary divergence between copies of homologous genes since the time of their common ancestor. The models are infinite alleles model (IAM) and stepwise mutational model (SMM). The IAM model assumes that every new mutation gives rise to an allele that does not already exist in the population while the SMM assumes that mutations increase or decrease allele size by single unit. IAM seems to apply to classical genetic markers such as protein and blood polymorphisms (Nei, 1987) and SMM apply to microsatellite loci (Goldstein *et al.*, 1995) since alleles at microsatellites are thought to evolve by a stepwise mutation process. Because the majority of mutations at microsatellite loci are stepwise in nature, changing allelic sizes up or down by one or very few number of repeats. Thus, distance measures, which apply to microsatellites generally, assume the SMM model. However, Edwards *et al*. (1992) has shown that the pattern of mutation for microsatellite loci follow a kind of a stepwise mutation model which is close to the IAM of mutation when a relatively short evolutionary time is considered. Thus microsatellite data can be

analyzed similar to that of protein polymorphisms. The most widely used distance measure based on IAM is Nei (1972) standard genetic distance (*D*S). *D*S is intended to measure the number of codon substitutions per locus that have occurred after divergence between a pair of populations and is expected to increase linearly with time (Nei and Takezaki, 1994). *D*S assumes that the rate of gene substitution per locus is uniform across loci and lineages. This assumption is violated under microsatellite loci. According to Farris (1981) *D*S is not appropriate for making a phylogenetic tree because it is not a metric measurement and does not obey the triangle inequalities. Another widely used distance measure under IAM is Rogers' (1972) distance (*D*R). This measure has the virtues of simplicity and it satisfies the principle of triangle inequality. However, *D*S is better than *D*R with respect to the linear relationship with time. Both DS and *D*R have the undesirable property of being influenced by within-taxon heterozygosity. That is the distance between two taxa that are fixed for alternate alleles exceeds that between two taxa in which one or both are heteroallelic but have no alleles in common. Other widely used measures of genetic distances are various estimators of population subdivision (F_{ST}) such as G_{ST} (Nei, 1972; Weir and Cockerham, 1984) and *R*ST (Slatkin, 1995). Nei and Takezaki (1994) and Takezaki and Nei (1996) have shown that *D*C and *D*A are more efficient in obtaining the correct tree topology than other distances.

Microsatellites have emerged as one of the most powerful method for studies of population genetics, migration rates, population size, bottlenecks etc. They can be used to explain the mode of mutation on the basis of different genetic models and various genetic distances which makes them suitable for assessment of evolutionary divergence and genetic structuring at the species or lower level.

5.4 MATERIALS AND METHODS

5.4.1 Sample selection

In the previous study a large number of faecal samples were analysed for partial sequencing of mitochondrial D-loop region. The D-loop analysis results 24 different haplotypes from three habitats. The samples showing variations (D-loop haplotypes) were further analysed for any differentiation through nuclear microsatellite DNA.

Sample taken for Microsatellite analysis

1. All 22 D-loop haplotypes from Kaziranga Rhino group (three samples for each haplotype) =27

2. All 9 D-loop haplotypes from Pobitora Rhino group (three samples for each haplotype, except PH15) =10

3. All 7 D-loop haplotypes from Orang Rhino group (three samples for each haplotype) =10

5.4.2 DNA Extraction, PCR standardization

The genomic DNA was isolated from faecal samples by using QIAmp DNA stool mini kit (described in Chapter 2 and 3). The isolated DNA was amplified using 6 microsatellite loci: Rh1, Rh3, Rh5, Rh7, Rh9 and IR10 (Zschokke *et al*., 2003; Scott, 2008). The detail of primers source, sequence and annealing temperature for each locus is given in Table 5.1. The optimal annealing temperature for each primer was standardized through a gradient PCR. All PCR were confirmed by at least three replicates (more in few samples) at each locus for each sample. Negative control reactions (only sterile water and PCR reagents) were included in each PCR run. For all the microsatellite primer standardizations, amplification was carried out in 10 µl reaction volumes containing 2 µl of the DNA extract, 2 µL double-distilled water, 0.5

μM of each forward labelled with a fluorescent dye, either FAM (blue) or TET (green) or HEX (yellow) and reverse (unlabelled) primer, and 5 μL HotstarTaq master mix (Qiagen). (The HotstarTaq master mix contains 400 μm dNTP each, 0.5 units of HotStar*Taq* DNA Polymerase (Qiagen), and 2X PCR buffer (Qiagen), consisting of Tris-Cl, KCl, and $(NH_4)_2SO_4$, with a final concentration of 1.5 mm $MgCl_2$.)

5.4.3 Primer Selection

The primers used for the analysis of microsatellite for rhino samples were selected from Zschokke *et al*. (2003) and Scott (2008). The primers were then optimized for PCR reactions.

Table 5.1 Primers used to amplify 6 microsatellite loci in *Rhinoceros unicornis* groups from three habitats of Assam

Locus	**Repeat motif**	**ABI (colour)**	**Primer sequence (5/-3/)**	**Annealing temperature (*Tm* °C)**
*Rh1	(TG)13	FAM	F: GTGCCATTATTATCCCAGGTC R: CGTAAGACCTCAAGGGATGC	60
*Rh3	(TC)8TG(TC)7CCTG(TC)4 TG(TC)16	TET	F: TGTGTGGAGCACATCAGTCTTC R: CCAGGGACCCGTGAGGAT	62
*Rh5	(TG)15	HEX	F: CCATTAGAGGCTGTAGAGTAATATC R: GGACTCTAAACTCCAGGGTCAC	58
*Rh7	(TG)17	FAM	F: CCGTCACATATGACAGTGTGC R: GGGCAGCTTATGCTCAAGTC	62
*Rh9	(TG)4TT(TG)17TA(TG)5	HEX	F: TCTGGTACCACCAAATGTAGC R: ACGATTACGTCTTTCAGTTGC	60
**IR 10	(CA)22	TET	F:CAGTGAGGAAGATTGGTTGC R: CCTGACTCACACATCACCAG	62

*Zschokke *et al*. 2003; **Scott, 2008

5.4.4 PCR amplification and genotyping

The PCR conditions included an initial denaturation (95°C for 15 min); 40 (30 for one tissue sample used as positive reference) cycles of denaturation (94°C for 30 secs), annealing for 45 seconds (annealing temperatures for each primers are shown in the Table 5.1) and extension (72°C for 30 secs); followed by a final extension (72°C for 20 min) then stored at 4^{o}C in an Eppendorf thermocycler. The PCR amplified products were separated on 2% agarose gel. Depending on signal intensity of bands under UV light,1-2 μL of the amplified product was added into 10 μLof formamide and 0.5 μL of ROX 500 size standard, placed at 95°C for three minutes and in ice for 10 minutes and then run into an automated sequencer ABI3100XL (Applied Biosystems). Microsatellite alleles were scored with GENEMAPPER version 4.0 (Applied Biosystems). Formamide is used for the denaturation of nucleic acids in sequencing gel electrophoresis

5.4.5 Data Validation

Amplification success for microsatellites from fecal samples was high in comparison to D-loop amplification. Samples producing identical genotypes for at least three independent amplifications for each of the loci were considered reliable. All uncertain genotypes and unamplified samples were further amplified twice. Samples that did not amplify for all loci were removed from the data set. Moreover, samples showing more null allele or allelic dropout were discarded. The potential genotyping errors in the data set were checked using the software MICROCHECKER 2.2.1 (Van Oosterhout *et al*., 2004) based on 10 000 randomizations in each of the 3 analyzed rhino groups.

5.5 Analysis of data

The diploid data conversion was done using the computer program the EXCEL MICROSATELLITE TOOLKIT version 3.1. The various data format were prepared by using CONVERT program freely available in internet. The program CONVERT facilitates the conversion of diploid genotypic data files into formats that can be directly read by a number of commonly used population genetic computer programs such as GENEPOP, ARLEQUIN, STRUCTURE etc. Hardy-Weinberg Equilibrium (HWE) (non-random association of alleles within diploid individuals), linkage disequilibrium (non-random association of alleles at different loci) and heterozygote excess and deficiency were estimated using GENEPOP program available at the web address http://wbiomed.curtin.edu.au/genepop/ freely (Raymond and Rousset, 1995). Deviations from Hardy-Weinberg Equilibrium were tested with GENEPOP with exact *P* values being estimated using the Markov chain algorithm with 10,000 dememorization steps 100 batches and 1,000 iterations. Allelic richness as a standardized measure of the number of alleles per locus independent of the sample size was calculated by FSTAT version 2.9.3.2 (Goudet, 1995, 2001). The fixation index F_{ST} is the most inclusive measure of population substructure, used to analyse the genetic divergence among subpopulations of a total population (Hartl and Clark, 1997) was calculated by ARLEQUIN ver. 3.0 program (Excoffier *et al.,* 2005). The other values of F-statistics such as F_{IS} and F_{IT} were also tested in ARLEQUIN. F_{IS} and F_{IT} are inbreeding coefficients that give deviations from Hardy Weinberg equilibrium within subpopulations and within the total population respectively. Positive values indicate a deficit and negative values indicate an excess of heterozygote individuals. F_{ST}, F_{IS} and F_{IT} measures of the 3 groups of rhino for each of 6 microsatellite loci were also

calculated according to Weir and Cockerham (1984) using GENEPOP software (Raymond and Rousset, 1995). The absolute differentiation, relative differentiation and actual differentiation of all three groups for 6 loci are calculated with web version of SMOGD (Crawford, 2010). The Factorial Correspondence Analysis (FCA) was used to visualize the individuals in multidimensional space and to discover the relationships within and among the populations by using the program GENETIX v. 4.05 (Belkhir *et al*., 1996–2004). Principal co-ordinate analysis (PCoA) was done in GenAlEx 6.0 (Peakall and Smouse, 2006) to examine further the genetic relationships among groups on the basis of the microsatellite data. Admixture analysis of the 3 rhino groups of Assam was performed using the program STRUCTURE 2.3.4 (Pritchard *et al*., 2000). The probable occurrence of bottlenecks of Rhinoceros population in Assam was examined using the software BOTTLENECK version 1.2.02 (Piry *et al*., 1999). The recent bottleneck effect was inferred for each group using a Wilcoxon's signed rank test (Cornuet and Luikart, 1996).

5.6 RESULTS

5.6.1 Microsatellite diversity among three groups of *R. unicornis* in Assam

All the 6 microsatellite loci subjected to PCR amplification were found to be polymorphic. In the locus Rh1, 3 alleles were found for all 3 groups of rhino. The size of the alleles ranged from 148 to 252bp (Table 5.2). In Rh3, 14 alleles were recorded from Kaziranga and 8 from each of Pobitora and Orang. The sizes of the alleles ranged from 114 to 146bp. The locus Rh5 consists of 9, 6 and 6 alleles for Kaziranga, Pobitora and Orang group whose size ranges from 190 to 198. In Rh7 locus, the all 3 rhino groups have 3 alleles which ranged from 200 to 204bp. Another locus Rh9 consists of 13, 7 and 9 alleles for Kaziranga, Pobitora and Orang group respectively. The size of the locus Rh9 is in between 136 to 168bp. The 6^{th} locus "IR10" is found to have less variability than other loci and consists of 2 alleles for all populations and size ranged from 200 to 202bp. The Rh3 locus had the highest number of alleles (14) while the IR10 locus had the least (2). The frequencies of all 6 alleles are graphically presented in figure 5.1. The allelic patterns of 3 rhino groups are presented in figure 5.2. In the 3 rhino groups, the number of alleles per polymorphic loci varied from 2 to 14 and the values of observed heterozygosity and expected heterozygosity ranged from 0.37037 to 0.92593 and from 0.39474 to 0.91579 respectively (Table 5.3, 5.4, 5.5). The number of alleles per locus, size range of each locus, observed heterozygosity, expected heterozygosity, P-value and Garza-Williamson index for Kaziranga, Pobitora and Orang rhino groups are presented in table 5.3, 5.4, 5.5. Mean P value for Hardy–Weinberg equilibrium (HWE) estimates showed that all three group of rhino conformed to Hardy–Weinberg Equilibrium in the population ($P>0.05$). But few loci showed insignificant value ($P<0.05$) which are exception to the Hardy–Weinberg equilibrium. In table 5.6,

mean value of assignments from of all loci of rhino population of Assam for Hardy Weinberg test (heterozygote excess) is presented. No significant deviations from Hardy–Weinberg equilibrium were detected within either each of the three groups of *R. unicornis* in Assam or overall rhino population in Assam. The mean values of of all loci from three groups are shown in table 5. 6. In Hardy-Weinberg tests of Markov chain parameters the P-value of three rhino groups are found 0.9805 ± 0.0055, 0.9977 ± 0.0006 and 0.7395 ± 0.0137 for Kaziranga, Pobitora and Orang rhino group respectively. The significant deviations from Hardy-Weinberg equilibrium in all three groups and all 6 loci are presented in table 5.7. The two loci of Kaziranga rhino groupn showed highly significant deviations from HWE. The Pobitora rhino group and Orang rhino group showed less significant deviations from HWE. Allelic richness calculated for each locus in each group, the mean number of alleles observed for each group and for each locus is given in the table 5.8. The Significant linkage disequilibrium between the loci is presented in table 5.9. The mean p-value of the overall rhino population of Assam is 0.9974 ± 0.0011. The pairwise F_{ST} (based on Kimura 2P) of three Rhino group (below diagonal) and corresponding F_{ST} *p*-values are presented in the table 5.10. In table 5.11, P-value (Fisher's method) for each group pair across all loci is presented. The gene diversity per locus and mean of all loci of all 3 rhino groups is given in table 5.12. The locus wise p-value of Markov chain parameters of 6 loci are shown in table 5.13. The absolute differentiation, relative differentiation and actual differentiation of all three rhino groups for 6 loci are shown in table 5.14. Assignment tests were performed in order to see likelihoods of individuals belonging to different rhino groups for each individual. In the population assignment test from 3 rhino group showed almost same source populations with probable gene flow among the groups (Figure 5.3). The F_{ST} *p*-

values showed that differentiation between rhino group of Kaziranga and Pobitora is not significant. But the poplation of rhino from ONP is found to significantly different from Kaziranga and Pobitora. Fixation indices of all 6 loci of 3 Rhino groups are summarized in the table 5.14. The overall F_{IS} of three rhino groups are 0.079, 0.126 and 0.018 for Kaziranga, Pobitora and Orang respectively. The AMOVA revealed that 87.75 % of the variation was observed within individuals and 5.50 % among populations. The AMOVA result for all loci among groups and among populations is presented in table 5.16 and within population and within individual is given in the table 5.17. The percentages of within population and among population molecular variation in the three rhino groups are shown in figure 5.4. While examining the gene diversity of all 6 loci of all 3 rhino groups; it has been observed that the Kaziranga rhino group more diverged than other two groups. The various genetic distances estimated from Nei's estimation of heterozygosity for all 6 loci are presented in the table 5.18. In table 5.19, p-value and Chi-square test value of linkage disequilibrium calculated in Arlequine is presented. The PCoA analysis performed in two-dimensional plot did not clearly separate the individuals of three rhino groups and a mixing of individuals of all three groups was observed (Figure 5.5).

Table 5.2 Six microsatellite allele and their frequencies in *R. unicornis* from 3 groups and overall population

Locus	Size range	Kaziranga	Pobitora	Orang	Overall	Private alleles
Rh1	148	0.6667	0.7	0.25	0.5851	
	150	0.1296	0.25	0.35	0.2021	
	152	0.05	0.05	0.4	0.2128	
Rh3	114	0.037	0	0	0.0213	Pop 1
	116	0.037	0	0	0.0213	Pop 1
	118	0.037	0	0	0.0213	Pop 1
	122	0.037	0	0	0.0213	Pop 1
	124	0.0926	0	0	0.0532	Pop 1
	126	0.0741	0	0.15	0.0745	
	128	0	0	0.05	0.0106	Pop 3
	130	0.037	0.05	0	0.0319	
	132	0.1111	0.1	0	0.0851	
	132	0.1111	0.1	0	0.0851	
	134	0.0185	0.1	0	0.0319	
	136	0.0556	0	0.05	0.0426	
	138	0.0556	0.1	0.1	0.0745	
	140	0.0741	0.15	0.15	0.1064	
	142	0	0.15	0.15	0.0638	
	144	0.1111	0.2	0.25	0.1596	

Table 5.2 contd.

Locus	Size range	Kaziranga	Pobitora	Orang	Overall	Private alleles
	146	0.2222	0.15	0.1	0.1809	
Rh5	190	0.0185	0	0	0.0106	Pop 1
	192	0.0926	0	0.1	0.0745	
	194	0.1481	0	0	0.0851	Pop 1
Rh5	196	0.1481	0.25	0.15	0.1702	
	198	0.0926	0.05	0.1	0.0851	
	200	0.2407	0.4	0.45	0.3191	
	202	0.0741	0.05	0.1	0.0745	
	204	0.0741	0.1	0	0.0638	
	206	0.1111	0.15	0.1	0.117	
Rh7	200	0.7222	0.7	0.4	0.6489	
	202	0.1852	0.2	0.5	0.2553	
	204	0.0926	0.1	0.1	0.0957	
Rh9	136	0.0185	0	0	0.0106	Pop 1
	140	0.037	0	0	0.0213	Pop 1
	144	0	0.1	0.15	0.0532	
	146	0.0926	0.1	0.05	0.0851	
	148	0.2407	0.4	0.2	0.266	
	150	0.037	0.1	0.15	0.0745	
	152	0.1111	0	0.05	0.0745	

Table 5.2 contd.

Locus	Size range	Kaziranga	Pobitora	Orang	Overall	Private alleles
	154	0.0556	0	0	0.0319	Pop 1
	156	0.0556	0	0	0.0319	Pop 1
	158	0.0556	0.15	0	0.0638	
	160	0.0556	0.05	0.1	0.0638	
	162	0.1111	0.1	0.1	0.1064	
	164	0.0185	0	0.1	0.0319	
	168	0.1111	0	0.1	0.0851	
IR10	200	0.4444	0.5	0.25	0.4149	
	202	0.5556	0.5	0.75	0.5851	

Pop 1= Kaziranga rhino group, Pop 2= Pobitora rhino group, Pop 3= Orang rhino group

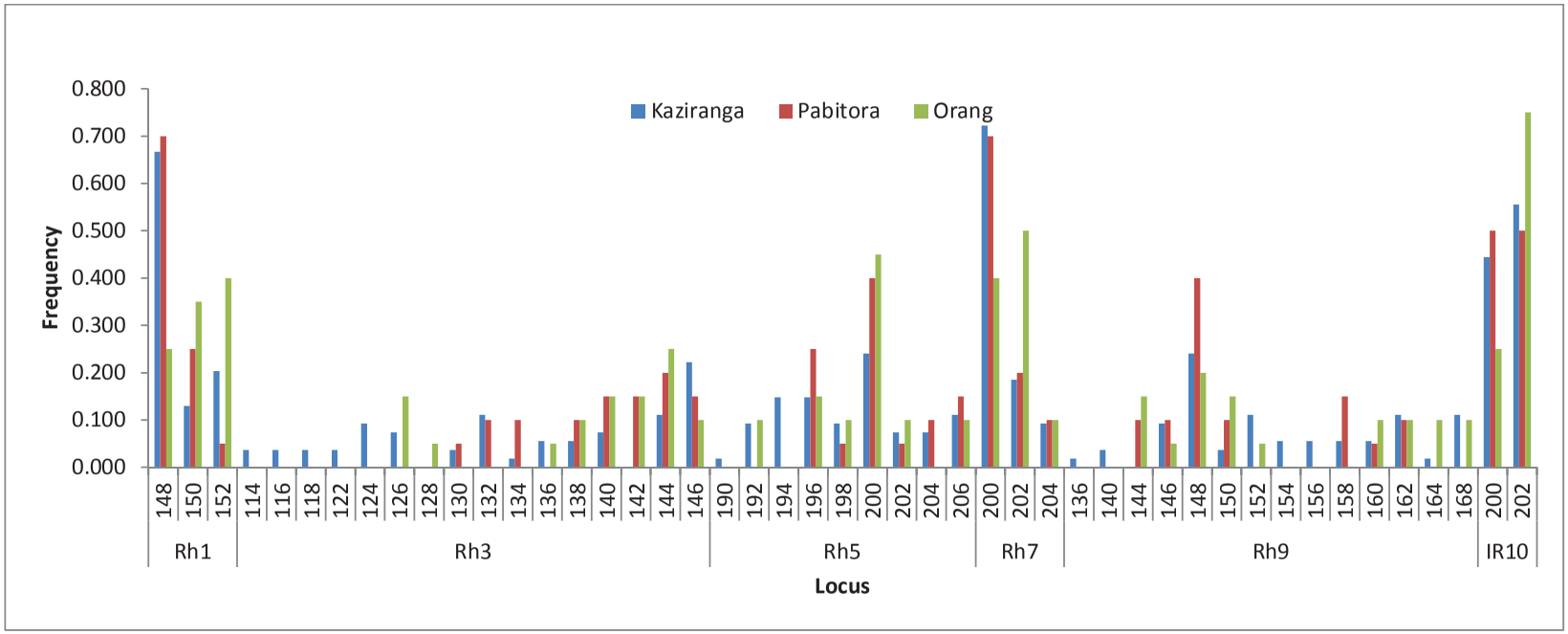


Figure 5.1 Allele frequencies of 6 microsatellite loci in three rhino rhino groups. The three rhino rhino groups namely Kaziranga, Pobitora and Orang are depicted with 3 different colour.

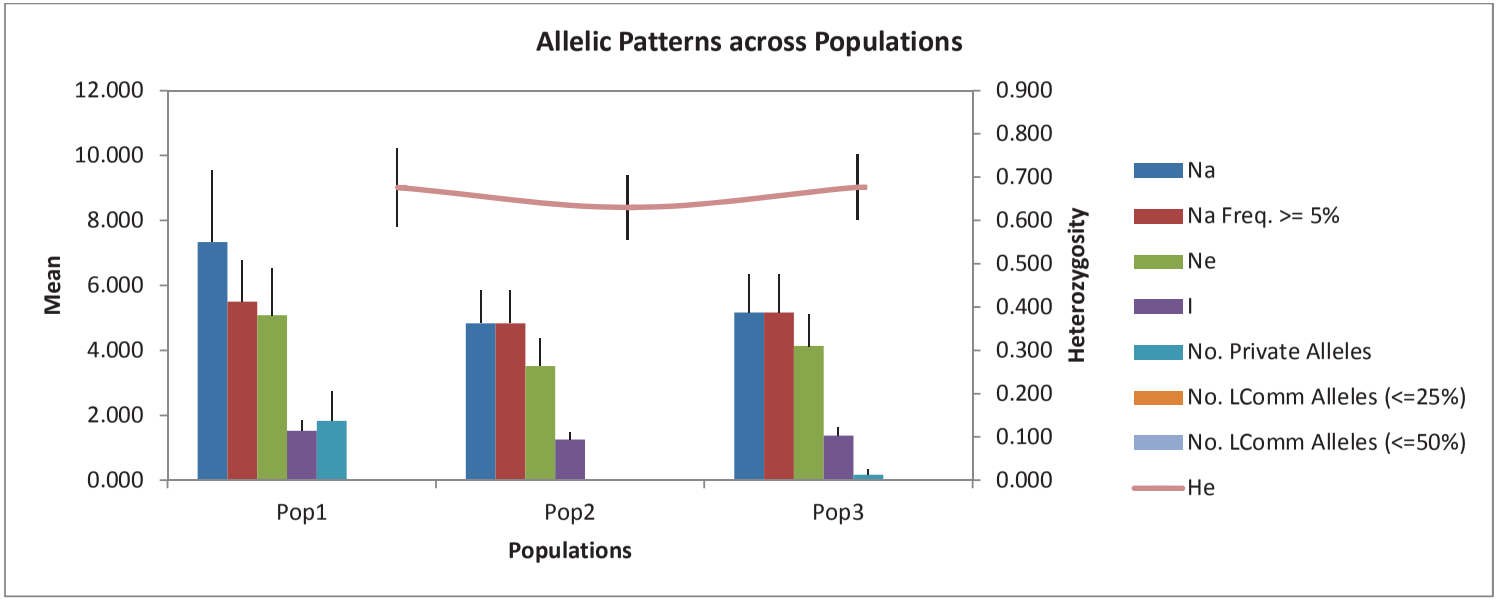


Figure 5.2 Allelic pattern of three rhino groups. [Na = No. of Different Alleles, Na Freq >= 5% = No. of Different Alleles with a Frequency >= 5%, Ne = No. of Effective Alleles, I = Shannon's Information Index, No. Private Alleles = No. of Alleles Unique to a Single Population, No. LComm Alleles (<=25%) = No. of Locally Common Alleles (Freq. >= 5%) Found in 25% or Fewer Populations, No. LComm Alleles (<=50%) = No. of Locally Common Alleles (Freq. >= 5%) Found in 50% or Fewer Populations, He = Expected Heterozygosity. Pop 1= Kaziranga rhino group, Pop 2= Pobitora rhino group, Pop 3= Orang rhino group

Table 5.3 Parameters of Hardy-Weinberg equilibrium of Kaziranga rhino group

Loci	Number of alleles	Obs.Het.	Exp.Het.	P-value	Allelic range	Garza-Williamson index
Rh1	3	0.44444	0.50664	0.20803	4	0.60000
Rh3	14	0.92593	0.90985	0	32	0.42424
Rh5	9	0.77778	0.87352	0.00004	16	0.52941
Rh7	3	0.40741	0.44375	0.00004	4	0.60000
Rh9	13	0.88889	0.89727	0.08285	32	0.39394
IR10	2	0.37037	0.50314	0.24269	2	0.66667
Mean (SD)	7.33 (5.39)	0.63580 (0.25598)	0.68903 (0.22545)	0.08894 (0.01273)	15.00 (14.07)	0.53571 (0.10768)

Table 5.4 Parameters of Hardy-Weinberg equilibrium of Pobitora rhino group

Loci	Number of alleles	Obs.Het.	Exp.Het.	P-value	Allelic range	Garza-Williamson index
Rh1	3	0.60000	0.46842	1.00000	4	0.60000
Rh3	8	0.80000	0.90526	0.06222	16	0.47059
Rh5	6	0.50000	0.77895	0.00848	10	0.54545
Rh7	3	0.40000	0.48421	0.09873	4	0.60000
Rh9	7	0.80000	0.81579	0.09873	18	0.36842
IR10	2	0.40000	0.52632	0.56379	2	0.66667
Mean (SD)	4.833 (2.483)	0.58333 (0.18348)	0.66316 (0.19183)	0.30532 (0.04342)	9.000 (6.782)	0.54186 (0.10725)

SD= Standard deviation

Table 5.5 Parameters of Hardy-Weinberg equilibrium of Orang rhino group

Loci	Number of alleles	Obs.Het.	Exp.Het.	P-value	Allelic range	Garza-Williamson index
Rh1	3	0.60000	0.68947	0.01416	4	0.60000
Rh3	8	0.70000	0.88947	0.02278	20	0.38095
Rh5	6	0.70000	0.77368	0.09849	14	0.40000
Rh7	3	0.80000	0.61053	0.16094	4	0.60000
Rh9	9	0.90000	0.91579	0.03172	24	0.36000
IR10	2	0.50000	0.39474	1.00000	2	0.66667
Mean (SD)	5.167 (2.927)	0.70000 (0.14142)	0.71228 (0.19406)	0.22134 (0.06357)	11.333 (9.352)	0.50127 (0.13531)

Table 5.6 Mean value of assignments from all three groups and of all loci

Loci	Hardy Weinberg test (heterozygote excess) P-value (S.E.)	Mean G.W. index	Mean Exp. Het.
Rh1	0.8299 (0.0039)	0.60000	0.55484
Rh3	0.9870 (0.0056)	0.42526	0.90153
Rh5	0.9896 (0.0023)	0.49162	0.80872
Rh7	0.8411 (0.0030)	0.60000	0.51283
Rh9	0.5484 (0.0272)	0.37412	0.87628
IR10	0.8395 (0.0023)	0.66667	0.47473
	0.8392 (0.0334)	0.52628 (0.11675)	0.68816 (0.20378)

SD= Standard error

Table 5.7 Significant deviations from Hardy-Weinberg equilibrium in all three groups and all 6 loci

Population	Locus	DF	Chi-square	Probability	Significance
Pop1	Rh1	3	2.813	0.421	ns
Pop1	Rh3	91	184.800	0.000	***
Pop1	Rh5	36	81.277	0.000	***
Pop1	Rh7	3	6.755	0.080	ns
Pop1	Rh9	78	88.149	0.203	ns
Pop1	IR10	1	1.688	0.194	ns
Pop2	Rh1	3	1.837	0.607	ns
Pop2	Rh3	28	41.111	0.052	ns
Pop2	Rh5	15	22.900	0.086	ns
Pop2	Rh7	3	10.816	0.013	*
Pop2	Rh9	21	27.292	0.161	ns
Pop2	IR10	1	0.400	0.527	ns
Pop3	Rh1	3	10.829	0.013	*
Pop3	Rh3	28	36.489	0.131	ns
Pop3	Rh5	15	26.049	0.038	*
Pop3	Rh7	3	5.600	0.133	ns
Pop3	Rh9	36	54.722	0.024	*
Pop3	IR10	1	1.111	0.292	ns

Pop1= Kaziranga, Pop2=Pobitora, Pop3=Orang, ns=not significant

* P<0.05, ** P<0.01, *** P<0.001

Table 5.8 Allelic richness calculated for each locus in each rhino group, the mean number of alleles observed for each group and for each locus

Locus	Kaziranga	Pobitora	Orang	Average
Rh1	2.967	3.000	3.000	2.990
Rh3	10.441	8.000	10.000	9.480
Rh5	7.813	6.000	6.000	7.287
Rh7	2.907	3.000	3.000	2.895
Rh9	9.747	7.000	9.000	8.582
IR10	2.000	2.000	2.000	2.000

Table 5.9 Significant linkage disequilibrium between the loci (significance level=0.0500)

	Rh1	Rh3	Rh5	Rh7	Rh9	IR10
Rh1	*	+	+	-	+	+
Rh3	+	*	+	+	+	+
Rh5	+	+	*	+	+	+
Rh7	-	+	+	*	+	-
Rh9	+	+	+	+	*	+
IR10	+	+	+	-	+	*

Table 5.10 Population pairwise F_{ST} (based on Kimura 2P) of three Rhino groups (below diagonal) and corresponding F_{ST} *p*-values (above diagonal)

	Rhino Kaziranga	Rhino Pobitora	Rhino Orang
Rhino Kaziranga	0	0.54054 (0.0470)	0
Rhino Pobitora	-0.0011	0	0.0091(0.0091)
Rhino Orang	0.0554	0.0481	0

Table 5.11 The Chi-square test and P-value (Fisher's method) for each group pair across all loci

Rhino Group pair	Chi^2	P-Value
Kaziranga and Pobitora rhino group	14.20042	0.288094*
Kaziranga and Orang rhino group	28.21219	0.005150
Pobitora and Orang rhino group	20.06171	0.065928*

*reject null hypothesis i.e. groups are not equal

Table 5.12 Gene diversity per locus/all loci of all 3 groups of rhino

Population/groups	Loci						Mean
	Rh1	Rh3	Rh5	Rh7	Rh9	IR10	
Kaziranga	0.508	0.91	0.875	0.444	0.897	0.506	0.690
Pobitora	0.461	0.911	0.794	0.489	0.817	0.533	0.667
Orang	0.694	0.9	0.778	0.6	0.533	0.389	0.649

Table 5.13 Locus wise p-value of Markov chain parameters

Locus	P-val	S.E.
Rh1	0.8299	0.0039
Rh3	0.9870	0.0056
Rh5	0.9896	0.0023
Rh7	0.8411	0.0030
Rh9	0.5484	0.0272
IR10	0.8395	0.0023

Table 5.14 Genetic diversity parameters of all three rhino groups for 6 loci

Locus ID	n	H_S	H_T	D_{ST}	G_{ST}	H_{ST}	Δ_{ST}	D	H_S/H_T	Δ_S/Δ_T
Rh1	3.000	0.532	0.603	0.071	0.117	0.151	1.178	0.226	0.883	0.849
Rh3	3.000	0.866	0.891	0.025	0.029	0.190	1.234	0.285	0.971	0.810
Rh5	3.000	0.777	0.798	0.020	0.026	0.092	1.101	0.138	0.974	0.908
Rh7	3.000	0.492	0.534	0.043	0.080	0.084	1.092	0.126	0.920	0.916
Rh9	3.000	0.842	0.868	0.027	0.031	0.168	1.202	0.252	0.969	0.832
IR10	3.000	0.456	0.479	0.023	0.048	0.042	1.044	0.063	0.952	0.958

n–number of populations, D_{ST}–absolute differentiation, G_{ST}–relative differentiation, H_{ST}–between-subpopulation heterozygosity, Δ_{ST}–between-subpopulation component of diversity, or the effective number of distinct subpopulations, D–actual differentiation, H_S/H_T–proportion intra-population heterozygosity vs total heterozygosity, Δ_S/Δ_T–proportion of total diversity that is contained in the average subpopulation

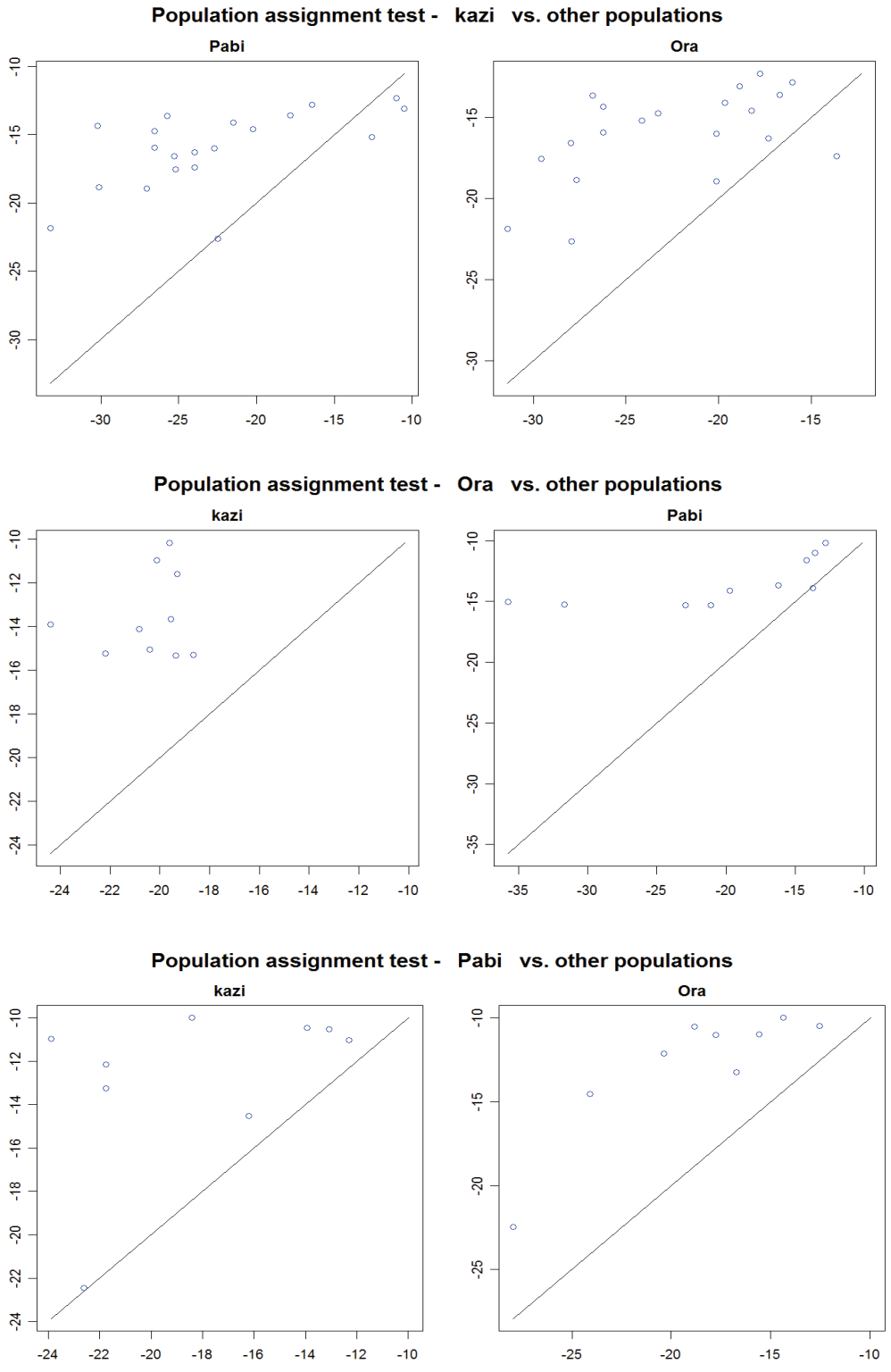


Figure 5.3 Population assignment of three habitats of *R. unicornis* of Assam based on microsatellite data for visual understanding the genetic structures of rhino groups.

Table 5.15 Fixation indices of all 6 loci of Rhino populations (FIS= inbreeding coefficient --measure of the extent of genetic inbreeding within subpopulations, FSC= the variance among subpopulations within groups, FCT = the variance among groups relative to the total variance, FIT = overall fixation index--the mean reduction in H (heterozygosity) of an individual relative to the total population

Loci	FIS	P-value	FSC	P-value	FCT	P-value	FIT	P-value
Rh1	0.0504	0.3695	0.2057	0.00391	-0.14094	1	0.13941	0.18964
Rh3	0.0611	0.1241	-0.0233	0.88368	0.03391	0.34604	0.07187	0.11828
Rh5	0.1615	0.0107	-0.0354	0.85337	0.03717	0.31965	0.16411	0.00293
Rh7	-0.0049	0.5845	0.119	0.03715	-0.0603	0.66667	0.06123	0.41153
Rh9	0.0137	0.4721	0.003	0.4086	0.00285	0.66276	0.01954	0.43206
IR10	0.1718	0.1935	0.0649	0.19941	-0.06759	1	0.17324	0.16325

Table 5.16 AMOVA Results for all 6 polymorphic loci of 3 rhino groups (among groups and among populations)

	among groups				among populations			
Loci	SSD	d.f.	Va	% variation	SSD	d.f.	Vb	% variation
Rh1	0.76078	1	-0.04181	-14.0938	1.675	1	0.06963	23.46964
Rh3	0.84048	1	0.01555	3.39077	0.275	1	-0.0103	-2.24583
Rh5	0.71036	1	0.01561	3.71725	0.2	1	-0.01432	-3.40868
Rh7	0.65351	1	-0.01572	-6.03015	0.9	1	0.03289	12.61793
Rh9	0.55254	1	0.00127	0.28501	0.475	1	0.00133	0.29943
IR10	0.11082	1	-0.01652	-6.75902	0.625	1	0.01695	6.93332

Table 5.17 AMOVA Results for all 6 polymorphic loci of 3 rhino groups (Within populations and within individuals)

	Within populations				Within individuals			
Loci	SSD	d.f.	Vc	% variation	SSD	d.f.	Vd	% variation
Rh1	12.42593	44	0.01354	4.56524	12	47	0.25532	86.05887
Rh3	21.16111	44	0.0277	6.04193	20	47	0.42553	92.81312
Rh5	21.39815	44	0.06763	16.10255	16.5	47	0.35106	83.58889
Rh7	10.65926	44	-0.00121	-0.4652	11.5	47	0.24468	93.87741
Rh9	19.72778	44	0.00609	1.3699	20.5	47	0.43617	98.04566
IR10	12.58333	44	0.04193	17.14994	9.5	47	0.20213	82.67575

Table 5.18 Nei's estimation of heterozygosity for all 6 loci of rhino population (Ho = Observed heterozygosity, Hs= mean expected heterozygosity, H_t = Global expected heterozygosity for all samples, Dst and Dst'= uncorrected and corrected genetic distance, Gst and Gst' = uncorrected and corrected relative genetic differentiation, Gis = deviations from Hardy-Weinberg equilibrium within species)

Loci	Ho	Hs	Ht	Dst	Dst'	Ht'	Gst	Gst'	Gis
Rh1	0.548	0.555	0.61	0.056	0.084	0.638	0.091	0.131	0.012
Rh3	0.809	0.906	0.905	-0.001	-0.001	0.904	-0.001	-0.002	0.107
Rh5	0.659	0.816	0.811	-0.005	-0.008	0.808	-0.006	-0.01	0.192
Rh7	0.536	0.511	0.541	0.03	0.045	0.556	0.055	0.081	-0.048
Rh9	0.863	0.877	0.88	0.003	0.005	0.882	0.003	0.005	0.016
IR10	0.423	0.477	0.486	0.009	0.013	0.491	0.018	0.027	0.113
Overall	0.64	0.69	0.705	0.015	0.023	0.713	0.022	0.032	0.073

Table 5.19 Chi-square test value and P values of linkage disequilibrium tests across all 6 loci and all three rhino groups (KNP-Kaziranga National Park, PWLS-Pobitora wildlife sanctuary, ONP-Orang National Park)

Locus Pair	Chi-square test value (χ^2)			DF			P value		
	KNP	PWLS	ONP	KNP	PWLS	ONP	KNP	PWLS	ONP
Rh1- Rh3	49.091	20.137	12.217	26	14	14	0.00403	0.12587	0.58886
Rh1- Rh5	36.415	20.137	5.084	16	10	10	0.00253	0.02798	0.88547
Rh3- Rh5	125.660	42.031	46.641	104	35	35	0.07295	0.19257	0.09023
Rh1- Rh7	5.423	14.318	5.789	4	4	4	0.24654	0.19257	0.21540
Rh3- Rh7	41.491	26.527	13.661	26	14	14	0.02767	0.02216	0.47522
Rh5- Rh7	31.833	26.527	13.317	16	10	10	0.01051	0.00309	0.20645
Rh1- Rh9	52.297	15.495	15.835	24	12	16	0.00072	0.21546	0.46453
Rh3- Rh9	131.996	44.942	57.620	156	42	56	0.91885	0.34966	0.41506
Rh5- Rh9	111.172	33.373	44.575	96	30	40	0.13794	0.30656	0.28529
Rh7- Rh9	39.630	26.527	20.758	24	12	16	0.02342	0.00903	0.18798
Rh1- IR10	11.641	3.931	2.043	2	2	2	0.00297	0.14005	0.35993
Rh3- IR10	52.690	17.682	11.944	13	7	7	0.00000	0.01349	0.10241
Rh5- IR10	23.056	17.176	6.877	8	5	5	0.00329	0.00418	0.22993
Rh7- IR10	1.05312	4.445	3.085	2	2	2	0.59063	0.10831	0.21382
Rh9- IR10	35.53980	12.476	11.742	12	6	8	0.00038	0.05214	0.16305

$p<0.05$

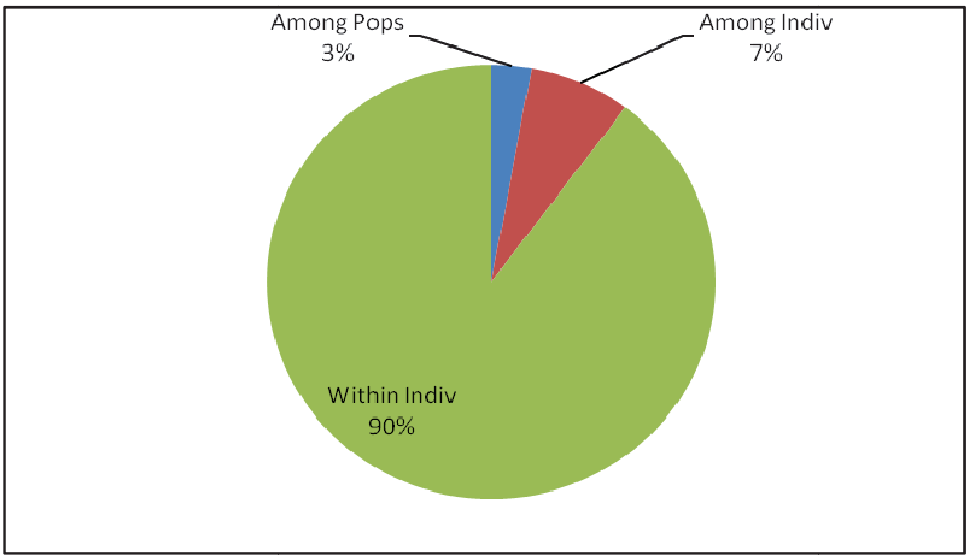


Figure 5.4 Percentage of within population and among population variation in the three rhino groups

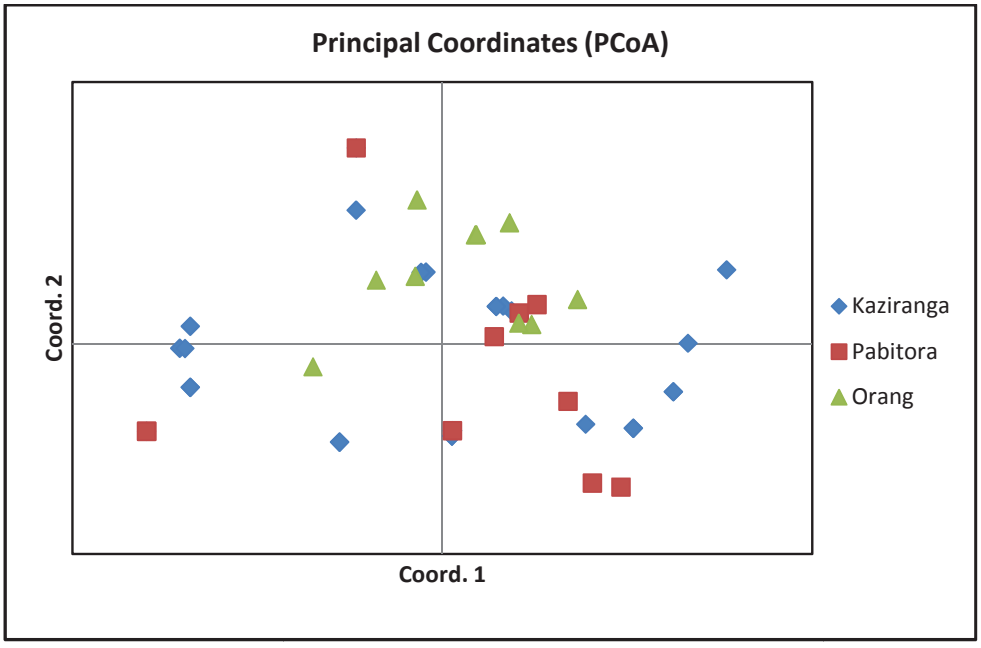


Figure 5.5 A two-dimensional plot of the Principal Coordinate Analysis (PCoA) of microsatellite data showing the clustering of individuals of three rhino groups.

5. 6.2 Factorial Correspondence Analysis (Individuals within Populations Based Analyses)

The Factorial Correspondence Analysis (FCA) was used to visualize the individuals in multidimensional space and to discover the relationships within and among the populations. GENETIX v. 4. 05 (Belkhir *et al*., 1996–2004) was used for the analysis and the samples are inspected on 3-Dimensional graphics with different triple combinations of first 4 factors (each represented by an axis) estimated by the software. The results of the analysis are shown in Figure 5.2. This graph showed that in the 6 loci studied; samples are dispersed and not overlapping and can be separated. Among all the rhino groups a certain degree of differentiations were observed but they do not fall apart from one another. It is also noticed that the individuals of three groups have less differences except two individuals differentiated separately. None of the group seems to have high degree of differentiation as observed in the graph.

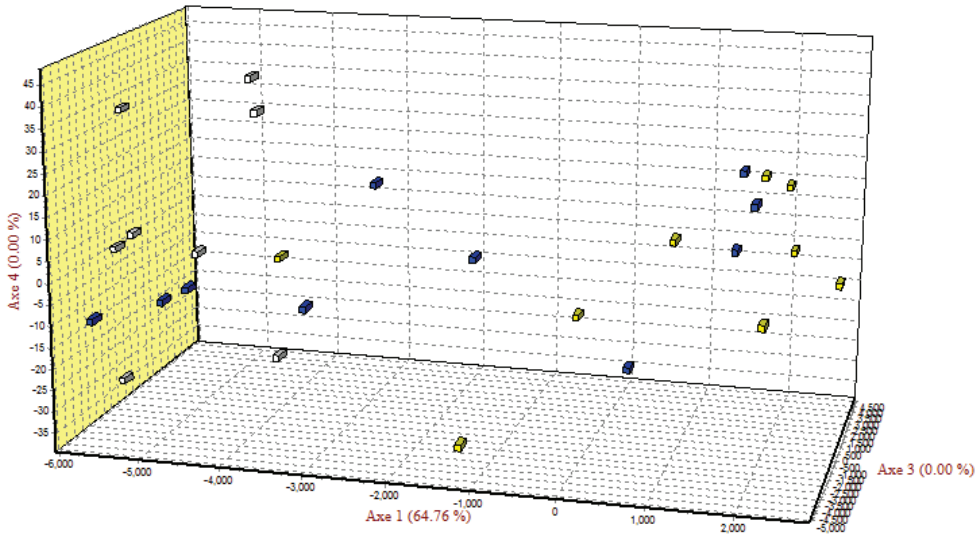


Figure 5.6 Factorial Correspondence Analysis result showing the relationship between all of the individuals analyzed in the study. The colour labels show the different rhino group on the graph.

5.6.3 Population structure analysis

The STRUCTURE software provides an effective way to illustrate the presence of population structure and to distinguish distinct genetic populations (Pritchard*, et al.,* 2000). Pritchard *et al*. (2000) developed a model-based clustering approach to infer population structure and assign individuals to populations using multilocus genotype data, which identifies subgroups that have distinctive allele frequencies. STRUCTURE requires the user to make two decisions regarding the model of population structure used. First, the user must specify an ancestry model, which specifies the degree of genetic isolation of modelled populations. The no-admixture model represents populations between which rates of gene flow are so low that individuals can be treated as having descended exclusively from one population or another. The admixture model, on the other hand, is appropriate for populations that have recently or are currently experiencing gene flow at sufficient rates that individuals may have recent ancestors from more than one population. In the second choice the user must make is whether the allele frequencies in the different populations should be treated as independent or correlated. The independence model assumes that the allele frequencies in one population are in no way related to allele frequencies in other populations. This implies that gene flow between the populations is effectively zero, and has been for quite a long time. The correlated model, in contrast, assumes that the populations diverged from a single ancestral population at some point in the past, and the differences in their allele frequencies are the result of drift that has occurred since their divergence. For this model, the degree of correlation between populations is an estimable parameter: populations that have diverged more recently or are experiencing a higher level of ongoing gene flow will have more similar allele frequencies than those that have

experienced a greater degree of isolation. It is important to note that both models have its own merits and demerits. The best model choices depend on the particular biology and history of the individuals studied.

STRUCTURE model based algorithm detailed in Pritchard *et al*. (2000) and Falush *et al*. (2003), places individuals in to K clusters. The underlying assumptions of the model in which there are K populations (where K may be unknown), each of which is characterized by a set of allele frequencies at each locus. If their genotypes indicate that they are admixed, the individuals in the sample are assigned jointly to two or more populations.

K is a parameter that is chosen in advance, but that can be varied in independent structure runs. Individuals are given 'membership coefficient' for each cluster, such that the estimated membership coefficient of an individual sum to 1 across the K clusters. The matrix of membership coefficients, where the number of individuals is the number of rows and K is the number of columns, is referred to as the individual Q-matrix. For each population, membership coefficients for each cluster can be averaged across individuals to create a population Q-matrix (Evanno, 2005). The most significant factors to determine for STRUCTURE analysis are the burn in length, the ancestry model and estimation of K (number of populations). Burn in length explains how long to run the simulation before collecting data to make sure that the simulated population reached to drift-mutation equilibrium which minimizes the starting configuration. Typically a burn in length 10,000 to 100,000 is more than adequate (Falush *et al.,* 2003, 2007). A burn-in period of 100,000 iterations of Markov chain Monte Carlo (MCMC) was used in this analysis and ten STRUCTURE runs for each value of K were carried out.

In this study, to get the best result of the microsatellite data were subjected to Bayesian cluster analyses of STRUCTURE, the ancestry of individuals, admixture model was performed. This model is reasonably flexible for many of the complexities of real populations (Falush *et al.,* 2003). It assumes that individuals may have mixed ancestry.

There are several methods suggested to estimate K (number of populations). For the true K, the distribution of Ln P(D) (or L(K), according to Evanno *et al*. (2005), do not indicate a clear mode, but at the true value of K the second order rate of change of the likelihood function (ΔK = m|L(K)|/ s[L(K)]) with respect to K ('K) does show a clear peak. In comprehensive simulation testing, Evanno *et al*. found that "in most cases the estimated 'log probability of data' does not provide a correct estimation of the number of clusters, K". They developed an alternative measure, denoted ΔK, which has a mode at the true K for most of the situations investigated.

Allele frequencies were correlated among populations and assumed different values of F_{ST} for the different subpopulations. STRUCTURE HARVESTER ver. A.1 (Earl and vonHoldt, 2012) which applies the Evanno method (Evanno *et al*., 2005) was used to visualize STRUCTURE output.

Figure 5.3 shows the population structure, displayed with population Q-matrix and individual Q-matrix, respectively, inferred from allelic frequencies at 6 microsatellite loci. Q is the estimated membership coefficients for each individual, in each cluster. When K = 2 was applied, all the three rhino groups did not showed separate cluster and all k colour are available in all 3 groups. Thereafter K = 3, 4 and 5 simulations were conducted which also did not reveal any clear differentiation of the three rhino groups. When K = 3 was applied, the ΔK value was highest among the 4

runs (Table 5.20), but the value is very small one unable to yield clear population clustering. Therefore it can be said that the rhinoceros group of three habitats in Assam are not genetically separate population and there exist a mixed allele in all three populations.

In order to know rhino population of three habitats the ancestry of individuals' plots everybody into a triangle the second type of plot was the triangle plot of Q model was tested (Figure 5.4). This type of plot is useful for visualizing the data for K = 3 (Pritchard *et al*., 2000). Therefore plot was done assuming K=3. From the triangle plot it can be assumed that the three rhino groups have genetic diversity but they cannot be separated from each other though they possess microsatellite allelic variation in the whole populations.

Table 5.20 Evanno table showing Delta K value. Evanno *et al*. (2005) proposed a new criteria ΔK, a measure of the second order rate of change in the likelihood of K, to select the most likely number of clusters K. The modal value of the distribution ΔK was found to be more similar to the real K number of populations in the simulation study.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
2	10	-856.920000	2.304970	—	—	—
3	10	-845.410000	4.504923	11.510000	13.520000	3.001161
4	10	-820.380000	33.150324	25.030000	50.460000	1.522157
5	10	-845.810000	63.997699	-25.430000	—	—

LnP(K)= posterior probability of the data for a given K, Ln'(K)= Log probability of data, Delta K =change of the likelihood function with respect to K

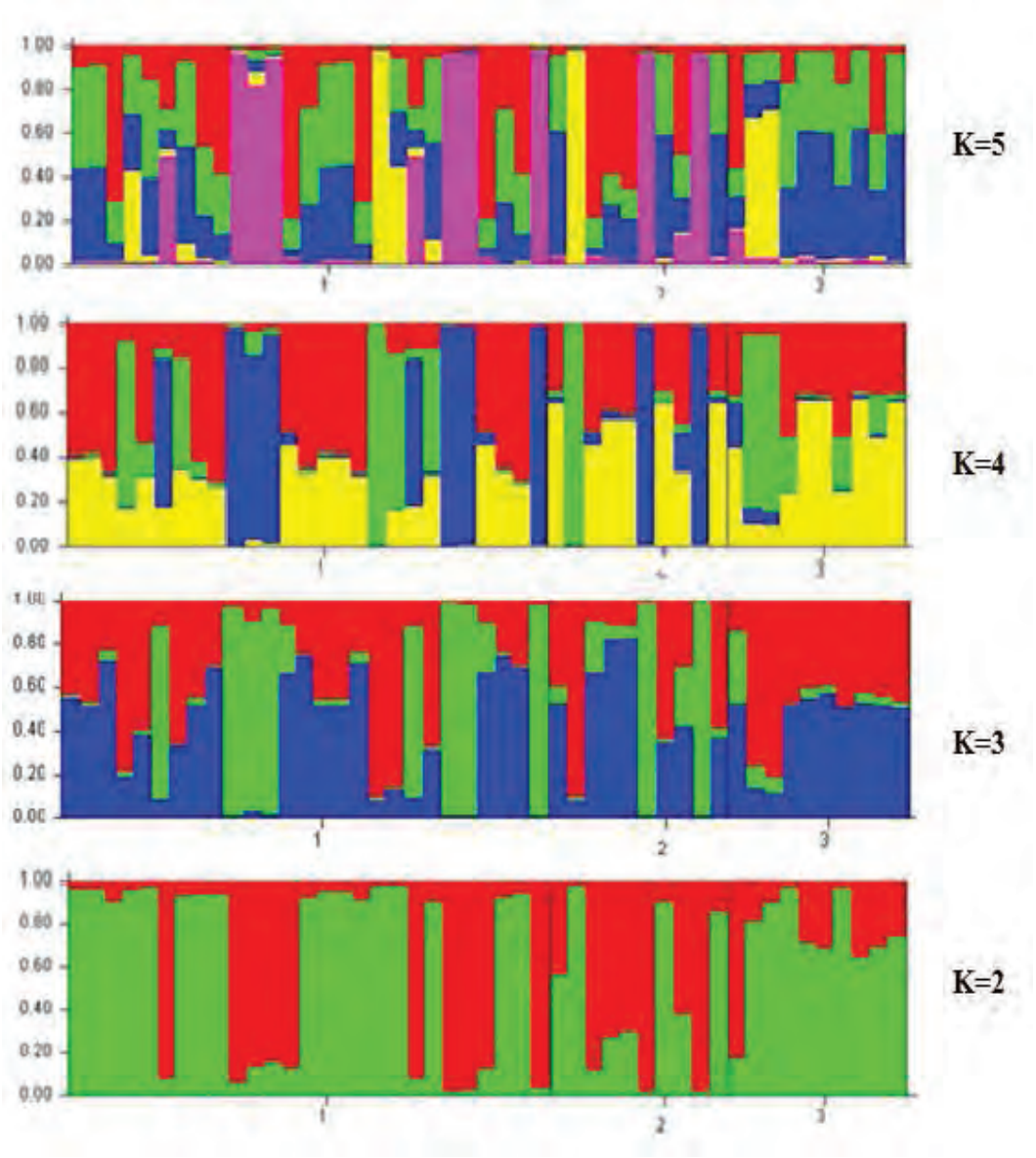


Figure 5.7 Bar plot for Bayesian clustering using ancestral model of three rhino groups' structure displayed obtained from STRUCTURE 2.3.4. The models were tested assuming K=2 to 5. Groups were partitioned in to K-coloured segments, which represent population's estimated average membership fractions in the K clusters. The black lines separate the different groups used for the study. The group 1 contains 27 individuals; the groups 2 and 3 represent 10 individuals each.

Findings from the Bayesian clustering analysis suggest low genetic structuring in the rhino population from three habitats: Kaziranga NP, Pobitora WLS and Orang NP The analyses unable to make clear distinctions between the three rhino groups and neither the group is distinctly different from one another.

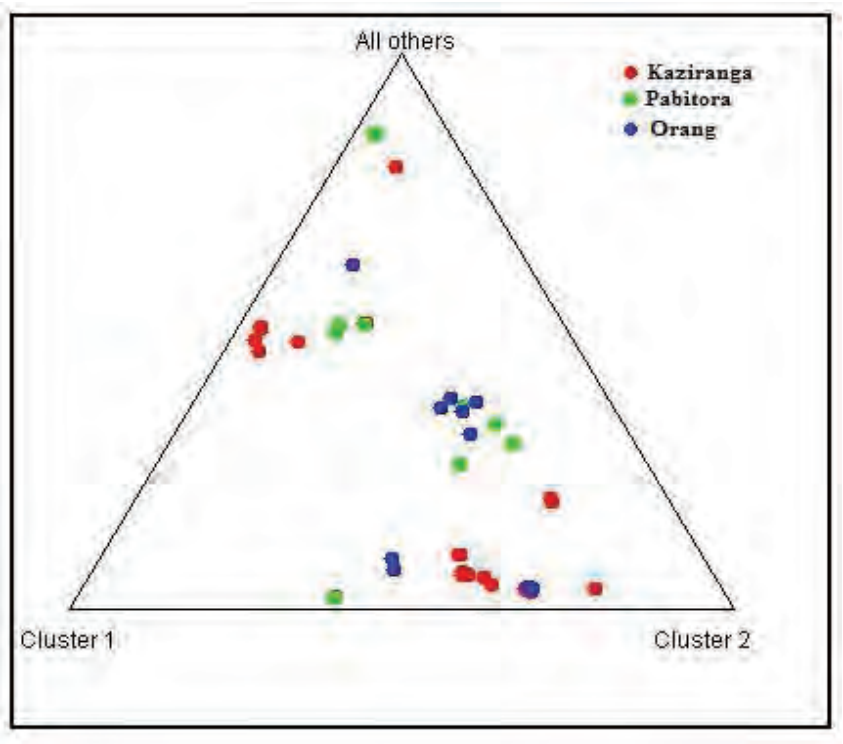


Figure 5.8 The triangle plot of Q obtained from three groups of rhino when K = 3. Each individual is represented by a coloured point. The colours correspond to the prior population labels. Each of the three components is given by the distance to one edge of the triangle. No individuals are in one of the corners and therefore cannot assign as separate population.

To know the population cluster of rhino from three habitat the triangle plot of Q was analysed when K=3 (Figure 5.4), because the delta K value showed as the most conclusive information about the three groups. But Triangle plot analysis was not able to separate the three groups and there exist a mixed type of population contained in the three habitats and neither the group(s) is distinctly different from other. From the above analysis of population structure of *Rhinoceros unicornis* in Assam it has been found that rhino group from the three different habitats are not distinctly separate from one another though they have genetic differences.

5.6.4 Bottleneck Tests for Rhinoceros population in Assam

The microsatellite data were also subjected to statistical analysis to test whether the rhinoceros populations in Assam have undergone recent genetic bottleneck. The bottleneck is an event of a population which causes the decline in fitness of the individuals in the population. A demographic bottleneck occurs when a large population experiences a severe, temporary reduction in size due to environmental or demographic events. The various events of bottleneck include drought, disease outbreak and war. These events may kill a certain percentage of a population and therefore reduce the effective population size. The genetic variability of all subsequent generations is contained in the few individuals that survive the bottleneck and reproduce. Therefore, some genetic diversity is lost in this process. The magnitude of the loss in diversity depends on the size of the bottleneck and the growth rate of the population afterward (Hunter, 1996). The founder effect event is another demographic event of bottleneck. A founder event occurs when a few individuals of a population establish a new population. The genetic constitution of the new population depends up on the genetics of the founder animals. The genetic diversity of the original larger populations is reduced because the sample of genes in the few founder animals is not likely to be representative of the original gene pool. According to Carson (1983) a genetic bottleneck in a population can create two problems: a loss of certain alleles, especially rare alleles, if no individuals possessing those alleles survive, and a reduction in the amount of variation in genetically determined characteristics due to the presence of fewer alleles and decline in heterozygosity. According to Leberg (1992), bottlenecks results to reduction of allozyme heterozygosity

According to Cavalli- Sforza *et al*. (1994) phylogeny construction and calculation

of genetic distances have the disadvantage of not being able to detect the effects of past bottleneck effects on populations and any admixture that has taken place between populations. Cornuet and Luikart (1996) developed a statistical test (a sign test for heterozygosity excess) to detect recent historical bottlenecks using allele frequency data. The test requires no data on historical population size or levels of genetic variation; it requires only measurement of allele frequencies from 5 to 20 polymorphic loci in a sample of approximately 20 – 30 individuals. The test has reasonable statistical power when applied to allele frequency data sets generated by computer simulations.

In natural populations, allele number and heterozygosity at selectively neutral loci result from an equilibrium between mutation and genetic drift. The heterozygosity expected at a locus in an equilibrium population (*Heq*) can be calculated from the number of alleles observed and the sample size of individuals, assuming neutrality and mutation-drift equilibrium. In non-bottlenecked population defined as not has been recently bottlenecked and is therefore likely to be near mutation-drift equilibrium, the expected heterozygosity (*Heq*) will equal the measured Hardy-Weinberg equilibrium heterozygosity (*H*E). However, if a population has suffered a recent bottleneck, the mutation-drift equilibrium is transiently disrupted and the heterozygosity measured at a locus (*H*E) will exceed the heterozygosity (*Heq*) computed from the number of alleles' sampled (Luikart and Cornuet, 1998).

Identifying recently bottlenecked populations (populations severely reduced in size) is important as bottlenecks can increase demographic stochasticity, rate of inbreeding, loss of genetic variation, and fixation of deleterious alleles and, thereby reduce adaptive potential and increase the probability of population extinction (Luikart and Cornuet, 1998). The probable occurrence of bottlenecks in Rhinoceros population

of Assam was examined using the software BOTTLENECK version 1.2.02 (Piry *et al*., 1999). A number of analyses were performed using allelic variation obtained at 6 microsatellite loci in the rhino population. BOTTLENECK software was applied to test for heterozygosity excess, which can be interpreted as a evidence of a bottleneck event. This approach is based on a faster allelic diversity reduction compared to the heterozygosity decrease, and a past bottleneck can consequently be detected when the observed heterozygosity is larger than that the heterozygosity expected (Piry *et al*., 1999). To determine whether a population exhibits a significant number of loci with gene diversity excess, there are three tests, namely a "sign test", a "standardized differences test" (Cornuet and Luikart, 1996), and a "Wilcoxon sign-rank test" (Luikart *et al*., 1998). All the three models of microsatellite evaluation Infinite Allele Model (IAM), Stepwise Mutation Model (SPM) and Two Phase Model (TPM) were utilized for the purpose. The Wilcoxon's test (although this test is analogous to the sign test) is generally the most useful of all the tests because it is the most powerful (along with the standardized differences test) and robust (like the sign test) when used with few (< 20) polymorphic loci. In testing for bottlenecks, the null hypothesis of Wilcoxon's test has no significant heterozygosity excess (on average across loci). Thus the alternative hypothesis is significant heterozygosity excess (and thus evidence of a recent bottleneck). This is a one tailed test that requires at least four polymorphic loci to have many possibility of obtaining a significant ($P < 0.05$) test result. The sign test (Cornuet and Luikart, 1996) determines if a significant majority of loci in a population have a heterozygosity excess, and thus if a population appears to have been recently bottlenecked. The test for heterozygosity excess is different from test for Hardy-Weinberg proportions. Tests for Hardy-Weinberg proportions compare the observed

proportion of heterozygosity (*H*O) to the heterozygosity expected (*H*E) when a population is in Hardy- Weinberg proportions. The test for Heterozygosity excess compares *H*E to the heterozygosity (*Heq*) expected at mutation drift equilibrium in a sample that has the same size and the same number of alleles as the sample used to measure heterozygosity expected (*H*E*)*. The standardized differences test requires at least 20 loci. In this analysis 6 polymorphic loci were used, therefore this test is not very useful.

All 6 loci showed significant heterozygosity excess (Table 5.21). The data showed that heterozygosity measures of all loci (HE) have exceeded the heterozygosity (*Heq*) in all three models from which it can be inferred that population has suffered a recent bottleneck. The recent bottleneck effect was also inferred for each group by using Wilcoxon's test, and the results are presented in Table 5.22 to Table 5.25. According to Wilcoxon's test the rhino group of Kaziranga NP have suffered from bottleneck event as significant results were obtained in IAM and TPM model ($P < 0.05$). In Kaziranga rhino, under Sign test, the expected numbers of loci with heterozygosity excess were 3.29 (IAM), 3.38 (TPM) and 3.40 (SMM) which were lower than the observed numbers of loci 6 (IAM), 6 (TPM) and 5 (SMM) with heterozygosity excess (Table 5.22). So the null hypothesis that as the rhino group is under Mutation-drift equilibrium was accepted. The Standard difference test (T2 statistics) in this group provided the significant ($p<0.01$) gene diversity excess IAM (2.684). In TPM and SMM there were heterozygosity excess (2.080 and 1.081) and non significant ($p>0.01$). Under Wilcoxon rank test, probability values of 0.00781 for IAM and TPM were significant ($p<0.01$). (Table 5.22 to 5.25). In the analysis it has been observed that, there was an event of

recent bottleneck effect detected in rhino population. Interestingly, the population did not show a significant deficiency of heterozygosity.

The Mode-shift indicator test was also utilized as a second method to detect potential bottlenecks, as the non bottleneck populations that are near mutation-drift equilibrium are expected to have a large proportion of alleles with low frequency. This test discriminates many bottlenecked populations from stable populations (Luikart and Cornuet, 1998). The allele frequencies (0.01–0.5) are the most numerous and proportion of alleles showed a normal "L" shaped distribution in Kaziranga rhino group (figure 5.9A) and shifted mode distribution in Pobitora and Orang rhino groups. However the three rhino groups when considered as one population the graph become shifted mode (figure 5.9B and 5.9C). These distributions clearly show that the studied populations have experienced a recent bottleneck which was not serious (Figure 5.9).

Table 5.21 Data on probable bottlenecking of Rhino population assuming different mutation models calculated in Bottleneck Programme. (H*e* - Expected heterozygosity, H*eq* - Heterozygosity equilibrium, *Prob* –probability, IAM - Infinite Allele Model, TPM - Two Phase Model, SMM -Stepwise Mutation Model).

Loci	Observed (He)	IAM		TPM		SMM	
		H*eq*	*Prob*	H*eq*	*Prob*	H*eq*	*Prob*
Rh1	0.577	0.333	0.089	0.392	0.128	0.462	0.190
Rh3	0.630	0.429	0.140	0.502	0.216	0.584	0.411
Rh5	0.755	0.435	0	0.507	0	0.581	0
Rh7	0.505	0.192	0.022	0.214	0.026	0.234	0.023
Rh9	0.643	0.427	0.097	0.506	0.188	0.590	0.391
IR10	0.505	0.192	0.018	0.214	0.028	0.231	0.024
Average	0.602	0.335		0.389		0.447	

Table 5.22 Mutation-drift equilibrium, heterozygosity excess and heterozygosity deficiency under different mutation models of Kaziranga rhino group

Models	Sign Test	Standardized	Wilcoxon test
IAM	Hee = 3.29	T2= 2.684	P (one tail for H deficiency)= 1.00000
	Hd =0	P= 0.00364	P (one tail for H excess)= 0.00781
	He = 6		P (two tails for H excess and deficiency)= 0.01563
	P = 0.02605		
TPM	Hee = 3.38	T2= 2.080	P (one tail for H deficiency)= 1.00000
	Hd =0	P= 0.01875	P (one tail for H excess)= 0.00781
	He = 6		P (two tails for H excess and deficiency)= 0.01563
	P = 0.03108		
SMM	Hee = 3.40	T2= 1.081	P (one tail for H deficiency)= 0.96094
	Hd =1	P= 0.13994	P (one tail for H excess)= 0.05469
	He = 5		P (two tails for H excess and deficiency)= 0.10938
	P = 0.18310		

Parameters for T.P.M: Variance = 30.00, Proportion of SMM in TPM = 70.00%; Estimation based on 1,000 replications; Hee: Heterozygosity excess expected; Hd: Heterozygosity deficiency; He: Heterozygosity excess; P: Probability; IAM: Infinite allele model, TPM: Two phase model, SMM: Stepwise mutation model.

Table 5.23 Mutation-drift equilibrium, heterozygosity excess and heterozygosity deficiency under different mutation models of Pobitora rhino group

Models	Sign Test	Standardized	Wilcoxon test
IAM	Hee = 3.39	T2= 1.670	P (one tail for H deficiency)= 1.00000
	Hd =0	P= 0.04751	P (one tail for H excess)= 0.00781
	He = 6		P (two tails for H excess and deficiency)= 0.01563
	P = 0.03060		
TPM	Hee = 3.40	T2= 1.051	P (one tail for H deficiency)= 0.78125
	Hd =3	P= 0.14659	P (one tail for H excess)= 0.28125
	He = 3		P (two tails for H excess and deficiency)= 0.56250
	P = 0.52506		
SMM	Hee = 3.59	T2= 0.303	P (one tail for H deficiency)= 0.57813
	Hd =4	P= 0.38099	P (one tail for H excess)= 0.50000
	He = 2		P (two tails for H excess and deficiency)= 1.00000
	P = 0.18063		

Parameters for T.P.M: Variance = 30.00, Proportion of SMM in TPM = 70.00%; Estimation based on 1,000 replications; Hee: Heterozygosity excess expected; Hd: Heterozygosity deficiency; He: Heterozygosity excess; P: Probability; IAM: Infinite allele model, TPM: Two phase model, SMM: Stepwise mutation model.

Table 5.24 Mutation-drift equilibrium, heterozygosity excess and heterozygosity deficiency under different mutation models of Orang rhino group

Models	Sign Test	Standardized	Wilcoxon test
IAM	Hee = 3.39	T2= 2.569	P (one tail for H deficiency)= 1.00000
	Hd =0	P= 0.00510	P (one tail for H excess)= 0.00781
	He = 6		P (two tails for H excess and deficiency)= 0.01563
	P = 0.03091		
TPM	Hee = 3.42	T2= 2.023	P (one tail for H deficiency)= 1.00000
	Hd =0	P= 0.02154	P (one tail for H excess)= 0.00781
	He = 6		P (two tails for H excess and deficiency)= 0.01563
	P = 0.03323		
SMM	Hee = 3.58	T2= 1.604	P (one tail for H deficiency)= 0.99219
	Hd =1	P= 0.05436	P (one tail for H excess)= 0.01563
	He = 5		P (two tails for H excess and deficiency)= 0.03125
	P = 0.22739		

Parameters for T.P.M: Variance = 30.00, Proportion of SMM in TPM = 70.00%; Estimation based on 1,000 replications; Hee: Heterozygosity excess expected; Hd: Heterozygosity deficiency; He: Heterozygosity excess; P: Probability; IAM: Infinite allele model, TPM: Two phase model, SMM: Stepwise mutation model.

Table 5.25 Mutation-drift equilibrium, heterozygosity excess and heterozygosity deficiency under different mutation models of all three rhino groups combined as one population.

Models	Sign Test	Standardized	Wilcoxon test
IAM	Hee = 3.01	T2= 3.792	P (one tail for H deficiency)= 1.00000
	Hd =0	P= 0.00007	P (one tail for H excess)= 0.00781
	He = 6		P (two tails for H excess and deficiency)= 0.01563
	P = 0.01509		
TPM	Hee = 3.22	T2= 3.251	P (one tail for H deficiency)= 1.00000
	Hd =0	P= 0.00057	P (one tail for H excess)= 0.00781
	He = 6		P (two tails for H excess and deficiency)= 0.01563
	P = 0.02255		
SMM	Hee = 3.22	T2= 2.746	P (one tail for H deficiency)= 1.00000
	Hd =0	P= 0.00302	P (one tail for H excess)= 0.00781
	He = 6		P (two tails for H excess and deficiency)= 0.01563
	P = 0.02301		

Parameters for T.P.M: Variance = 30.00, Proportion of SMM in TPM = 70.00%; Estimation based on 1,000 replications; Hee: Heterozygosity excess expected; Hd: Heterozygosity deficiency; He: Heterozygosity excess; P: Probability; IAM: Infinite allele model, TPM: Two phase model, SMM: Stepwise mutation model.

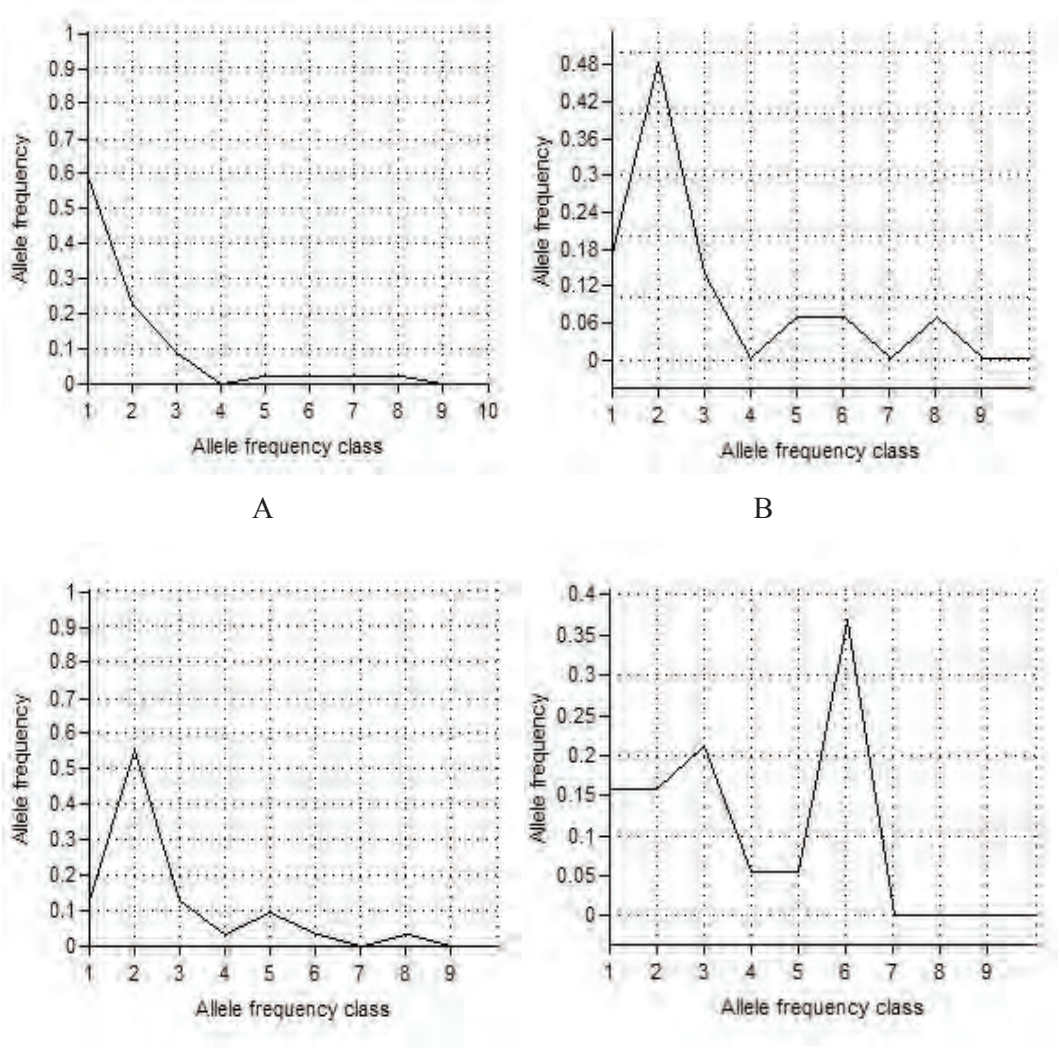


Figure 5.9 The Mode-shift indicator test for three rhino groups

A. L-shaped mode-shift graph showing lack of recent genetic bottleneck in Kaziranga rhino group.

B. Shifted mode graph showing evidence of genetic bottleneck in Pobitora rhino group.

C. Shifted mode graph showing evidence of genetic bottleneck in Orang rhino group.

D. Shifted mode graph showing evidence of genetic bottleneck in all three rhino groups combined as one population.

Bottlenecks generate 'heterozygosity excess' as alleles are generally lost faster than heterozygosity during a bottleneck. Any population that experienced a recent bottleneck will show higher than expected (equilibrium) heterozgosity for a large number of loci. During bottleneck rare alleles are lost rapidly since they have little effect on heterozygosity (Hedrick *et al.,* 1986). Thus, many alleles can be lost without much reduction in heterozygosity in a bottlenecked population. The bottleneck-induced heterozygosity excess is transient and is likely to be detected only for a short time, approximately 0.02 – 4.0 *Ne* generations (*N*e is the bottleneck effective size), until a new equilibrium between mutation and drift is reached at the new *N*e (Cornuet and Luikart, 1996). Thus, only bottlenecks that have occurred in the recent past (less than 4 *N*e generations ago) are likely to be detectable by the sign test for heterozygosity excess. It has been believed that the Rhino population in Assam has recovered and increased during the past 105 years with 8 or 9 generation. According to Cornuet and Luikart (1996), this depends not only on *N*e but also on factors such as the mutation rate and mutation model of the loci sampled. In a non-bottlenecked, equilibrium population, approximately 50% of the loci sampled are expected to have a slight excess of heterozygosity (*H*e > *H*eq), and 50% will have a slight deficiency of heterozygosity (*H*e < *Heq*), resulting from genetic drift and sampling error. However, in recently bottlenecked populations, a majority of loci will exhibit an excess of heterozygosity (Luikart and Cornuet, 1998). Positive values of the Bottleneck statistic T2 are indicative of gene diversity excess caused by a recent reduction in effective population size, while negative value are consistent with a recent population expansion without immigration or immigration of some private (unique) alleles in population. The Wilcoxon test provides relatively high power and it can be used with as few as four polymorphic loci and any

number of individuals (15-40 individuals and 10-15 polymorphic loci is recommend to achieve high power and according to Infinite allele model the rhino populations have suffered from recent bottleneck event. But the other two models do not support the bottleneck event and therefore it can be said that the bottleneck event that took place in Kaziranga rhino group is not a very recent one.

5.7 DISCUSSION

The assays of microsatellite loci showed that the Indian one horned rhino population found in three habitats of Assam have genetic variability as evidenced by the high mean heterozygosity, high average number of alleles per locus, and the high allelic richness. The rhino group of Kaziranga NP has more divergence than other two small populations. Among the allele of 6 microsatellite loci in three rhino groups, the two loci namely Rh3 and Rh9 have higher allelic range and IR10 has the lowest allelic range. The allelic range has been found high in the Kazirznga rhino group and the group has some private alleles which mean that few unique haplotypes are available in the park which are not available in other habitats. Private alleles are those alleles that are found at one locality only. In Pobitora rhino group no private allele was recorded where as in orang one private allele was found. This means that every allele that appears in the Pobitora group is also present in other group. Two alleles of the Kaziranga group showed significant deviations from Hardy-Weinberg equilibriums (HWE). In Kaziranga rhino group the high mutation of microsatellites was observed which help in determine population structure (Jarne and Lagoda, 1996) of the group. The microsatellite variability was also found in captive *R. unicornis* of various zoos of world (Zschokke *et al.,* 2011). The similar analysis on other rhino species has also indicated that microsatellite genetic variability also exists within the black and white rhino species (Nielsen *et al*., 2008; Anderson-Lederer *et al*., 2012). Generally, high genetic variation promises high genetic improvement of traits through selection, provided that the traits were determined mainly by additive genetic factors (Lacy, 1987). High genetic variation is also essential for the survival of populations because genetic variation within a certain range can allow adaptation to a changing environment. The 6 microsatellites used in this

study were polymorphic in all 3 groups. In majority of the loci, the 1 - *Ho*/*He* values were positive in all 3 rhino groups, indicating that those were deficient in heterozygosity at most of the loci. On the other hand, the 1 - *Ho*/*He* values for Kaziranga at locus Rh3 is negative, suggesting that the Kaziranga group showed an excess of heterozygosity at that locus. In Pobitora rhino group locus Rh1 and in Orang rhino group, locus Rh7 showed such condition of excess of heterozygosity. From the perspective of conserving an endangered population, if there is a significant reduction in the number of observed heterozygotes, compared with the number of expected heterozygotes under the assumption of Hardy-Weinberg, a loss of heterozygosity will cause the reduction in genetic diversity when measured at the individual level. Furthermore, a reduction in heterozygosity may also be associated with inbreeding Keller and Waller, 2002). Expected heterozygosity and gene diversity are related values and are calculated from observed allele frequencies. The difference between these two diversity statistics is the expected heterozygosity is equal to gene diversity but adjusted by a measure of sample size. For large samples there will be no difference between the two statistics; however, for populations with small samples and for inbreed populations with few heterozygotes, gene diversity is a more appropriate measure of genetic variability (Nei, 1987; Weir, 1996).

The population variability may be due to the fact that microsatellite markers are known to be more sensitive indicators of local genetic structure than mitochondrial DNA (Sunnucks, 2000). Microsatellite DNA regions are often referred to as neutral markers in that they do not code for fitness traits (Luikart and England, 1999; Neff and Gross, 2001) suggesting that they may not always reflect how events such as

bottlenecks or inbreeding may have impacted a population's genetic fitness (Hedrick, 1999; 2005).

The microsatellite genotypic data of rhino obtained from three habitats was also used to know the population differentiations in 3 rhino habitats because microsatellites are known to be more sensitive indicators of local genetic structure than mitochondrial DNA (Sunnucks, 2000). The population differentiation of the rhino from three habitats was studied through calculation of F_{ST} value from microsatellite genotype of three populations. Pair wise F_{ST} values were reported to be used as short-term genetic distances with a slight transformation (Slatkin, 1995). Pairwise F_{ST} values and their P values giving the proportion of the permutations (Distribution of F_{ST} values under the null hypothesis of no difference among populations is obtained by permutation of haplotypes between the populations) giving an F_{ST} greater or same with the observed one. Theoretically F_{ST} measures changes between 0 (no divergence) and 1(fixation of different alleles in different populations). However the F_{ST} levels are generally much lower than 1. According to Wright (Hartl and Clark, 1997) the F_{ST} levels between 0 and 0.05 indicate little genetic differentiation, between 0.05 and 0.15 indicate moderate level genetic differentiation, levels between 0.15 and 0.25 indicate great genetic differentiation and levels higher than 0.25 indicate very great genetic differentiation. In the present study the Orang rhino group found to somewhat different from other two groups, viz Kaziranga and Pobitora. No differences in rhino group were observed in between the Kaziranga and Pobitora. In another analysis of population differentiation molecular variance (AMOVA) was calculated. The AMOVA results of the 3 rhino groups genetic structuring generated by the microsatellite markers showed that Orang rhino group is somewhat genetically different from other two rhino group s.

To find out the differences in individuals of three rhino groups, two different analysis viz. Principal co-ordinate analysis and Factorial Correspondence Analysis was performed which showed a mix type of populations in three habitats and neither the group of 3 habitats can be separated as separate population. From the population structure analysis (done in STRUCTURE 2.3.4 program) it is once again proved that the *Rhinoceros unicornis* group found in the three habitats namely Kaziranga, Pobitora and Orang of Assam do not have clear population differentiation. The all three rhino groups are polymorphic but all groups have found to represent shared haplotypes.

The identification of population bottlenecks is critical in conservation because populations that have experienced significant reductions in abundance are subject to a variety of genetic and demographic processes that can hasten extinction. Genetic bottleneck tests constitute an appealing and popular approach for determining if a population decline has occurred because they only require sampling at a single point in time and reflect demographic history. The probable occurrence of bottlenecks in Indian Rhinoceros population of Assam was examined by using the genotypic data of microsatellite. Understanding the effect of population bottlenecks on genetic variation has become increasingly important in population genetics, speciation theory, and conservation biology. In order to identify conservation objectives properly, it is important to identify populations that have lost genetic variability recently, as they may be more susceptible to demographic stochasticity (Lande, 1988; Mills and Smouse, 1994). Genetic diversity, particularly reduced heterozygosity, has often been linked to their respective bottlenecks (Ashley *et al.,* 1990). According to England *et al*. (2003), a short, severe bottleneck will likely reduce allelic diversity but not heterozygosity. The reduced allelic richness may be an indicator of past bottlenecks or subsequent

inbreeding effects (Nei, 1987). The Garza-Williamson index calculated from microsatellite data of three rhino habitats showed that the rhino populations have suffered from bottleneck event or founder effect. The values are less than 0.68 which is regarded as unstable population and implies existence of bottleneck in the population (Garza and Williamson, 2001). Garza and Williamson (2001) showed that the mean ratio of the number of alleles to the range of allele size (M) is sensitive to population bottlenecks, dropping in size when a population is reduced. Further the result obtained from analysis of bottleneck effect in *R. unicornis* population in Assam suggests that the rhino population did not experience a severe recent bottleneck. The Wilcoxon test results an occurrences of mild bottleneck effect in the rhino population. But the bottleneck may not very severe as suggested by earlier researcher that nearly 20 surviving individuals were during 1908 (Ullrich, 1972; Laurie *et al*., 1983). The population has experienced a recent expansion in population size or perhaps a recent entry of some alleles due to movement of individual to Kaziranga National Park from nearby former rhino habitats. This result supports the findings of Zschokke *et al*. (2011) where they found that the occurrence of a bottleneck in the Assam population long before the reported bottleneck in 1908. The sustainable breeding in captivity of Indian rhino in various parts of the world also supports the absence of inbreeding depression in the rhino population. The Assam population of *R. unicornis* is one of the few known examples in captive breeding, in which inbreeding does not lead to an increased juvenile mortality. So, the worldwide captive population of *R. unicornis* is largely sustainable and does no longer depend on newly imported wild individuals from India/Nepal (Zschokke and Baur, 2002; von Houwald *et al*., 2010). That means the in the captive

bred individuals there is no ill effect such as inbreeding depression due to existence of genetic variability in the wild rhino population of Assam.

The finding of this study has revealed that a comparatively high genetic diversity has been observed in the rhino group of Kaziranga National Park. It has been suggested that to maintain the present genetic diversity in the rhino population, future translocations to safer places (Reintroduction in other protected areas) should consider. The patterns and profile of genetic differentiation among the donor populations should be maintained to maximize the genetic impact of translocations with an aim to maintain heterozygosity at as high a level as possible. Recently Poaching has once again become a greatest threat to rhino population in Assam which may lead to loss of alleles and overall genetic diversity of the rhino population. If this threat persists, the rhino population must be suffered from loss of genetic diversity and subsequent inbreeding and genetic drift which results genetic bottleneck and future viability of *R. unicornis* population in Assam.

5.8 SUMMARY

1. The microsatellite analysis showed that rhino population found in the three habitats of Assam has genetic divergence.
2. High mutation rate of microsatellites was observed in the rhino groups of all three habitats.
3. Among the 3 habitats, the Kaziranga rhino group is genetically more diverged.
4. The Garza-Williamson index showed that the rhino populations of Assam is not completely stable population and implies existence of bottleneck in the population.
5. The estimation of population F_{ST} value was unable to make genetic differentiation among the rhino groups from three wild habitats of Assam. However the F_{ST} value between Orang and other two groups of rhino viz. Kaziranga and Pobitora are somewhat significant.
6. The Kaziranga rhino group contain some unique genotypes which were not found in the other two habitats.
7. The AMOVA reveals that within population variation of rhino is high but among population (group) variation is low.
8. The population structure analysis denied the existence of more than one genetically different rhino population in Assam.
9. The bottleneck analysis showed that the rhino population of Assam had experienced a bottleneck effect in recent past which was not very much severed.

CHAPTER- 6

General Discussion and Conclusion

6.1 Genetic diversity and population differentiation of three groups of *Rhinoceros unicornis* present in three wild habitats of Assam

Genetic diversity is the raw material for evolutionary change within wildlife populations. It is generated by mutation and is lost from populations by genetic drift due to finite population size. Natural selection may either erode genetic variation by leading to fixation of alleles or promote its retention as a result of balancing or diversifying selection (Frankham, 1996). Genetic differences in a population are expressed as differences in the quantity and quality of alleles, genes, chromosomes, and gene arrangements on the chromosomes that are present within and among populations (Williamson, 2001; Okumus and Ciftci, 2003). In the present study, the genetic divergence of three groups of *Rhinoceros unicornis* was studied from the three wild habitats namely Kaziranga National Park, Rajiv Gandhi Orang National Park and Pobitora Wildlife Sanctuary of Assam through mitochondrial DNA and nuclear DNA markers which revealed genetic divergence in the rhino population. The partial sequencing of mitochondrial D-loop of *R. unicornis* population in Assam reveals 24 different haplotyptes. In Kaziranga NP 22 haplotypes were obtained from 241 sequences (samples) with 21 variable sites. Nine haplotypes were found in Pobitora WLS with 11 variable sites and 7 haplotypes from Orang National Park with 10 variable sites. The analysis of phylogenetic trees revealed a low differentiation between different

haplotypes of *R. unicornis* obtained from three different habitats. The F-statistics results showed some differentiation between rhino group of Kaziranga and Orang. The Analysis of molecular variance (AMOVA) also showed low population differentiation between the groups. Among the three habitats, Kaziranga rhino group is found to be more diverged than the other two groups. The Raggedness index of Kaziranga rhino group calculated from mitochondrial DNA indicates that the population has been expanding. The rhino groups of other two habitats showed less variability. The microsatellite data analysis in the 3 rhino groups showed probability of gene flow among the groups. The F_{ST} *p*-values showed that there is no differentiation between rhino group of Kaziranga and Pobitora. But the rhino group of Orang NP is found have some significant difference from Kaziranga and Pobitora rhino group. The AMOVA result of microsatellite data revealed that 87.75 % of the within individuals variation and 5.50 % among populations variation.

The genetic divergence that have been observed in the rhino population may be due presence of diverged ancient stock i.e. no serious bottleneck took place in recent past and presence of genetically diverged rhino population at post bottleneck period. The cause of low population differentiation in the three habitats may be due to movement of rhino among the habitats. The 3 habitats are located at a distance of more than 100 kilometers from each other and thus fragmented. Loss and fragmented habitat of large mobile mammal species contribute to decline of population due to lack of natural dispersal and population dynamics. Habitat fragmentation can also interrupt mate selection and effect juvenile survival (Bjornstad *et al.,* 1998; Boudjemadi *et al.,* 1999). To maintain genetic variability, stock viability and conservation of their gene pools the natural dispersal is important between fragmented populations (Waser and

Strobeck, 1998). Loss of genetic diversity within populations cause inbreeding depression, which in turn results in reduced fitness and ultimately jeopardizes the population persistence and future adaptation (Bjornstad *et al.,* 1998; Bonin *et al.,* 2007). Though natural dispersal is a common phenomenon in *R. unicornis,* but it is almost impossible due to fragmentation of habitats. Rhino migrate (stray out) to nearby forest for different reasons including searching for food, sex partners and suitable habitat. They are also good swimmer and they even cross big river like Brahmaputra. When migrated animal settled in new habitat it may be facilitated the intermixing of germplasm between the populations. Various studies showed that the rhino could move from Pobitora Wildlife Sanctuary to Orang National Park. There are enormous incidences of migration of rhino from Kaziranga National Park to Majuli (100 km away from Kaziranga NP), Pobitora WLS (150 Km away from Kaziranga NP), North Lakhimpur district (250 km away from the park) and other places of Assam (Bhattacharyya, 1991; Talukdar *et al*., 2007; Das and Goswami, 2012b).

Earlier it was believed that the rhino population of Assam is lacking of genetic diversity as no allozyme variation was found among 3 individuals examined by Merenlender *et al.* (1989) and reason was shown due to bottleneck in rhino population in Assam that took place in the beginning of 20th century. The population bottleneck is the pronounced reduction in population size often which affects a species' ability to adapt to environmental change and elevated extinction risk (Nei *et al.,* 1975; Tajima, 1996; O'Brien and Evermann 1988; Frankham *et al.,* 1999; Bouzat, 2010). But high genetic diversity observed in the rhino population through high resolution marker like Microsatellites and Mitochondrial DNA indicates that the bottleneck in rhino population in Assam is not recent one. The result of current study of mitochondrial DNA data is

found positively correlated between microsatellite data. But more divergence was found in Mitochondrial DNA study. It is also to be noted that the D-loop region is a rapidly evolving part of mitochondrial DNA and mere divergence in this DNA region does not qualify a population as separate one and addition of further molecular investigations can only ensure it as separate population. Microsatellites are fast evolving markers which are very suitable for intra-specific population genetic studies. The microsatellite loci have advantages of being mostly neutral, having high mutation rates and exhibiting codominant inheritance as population genetic study markers for closely related species and populations over the morphometric and electrophoretic markers which are subject to selection pressure (Freeman and Herron, 1998). However, Mitochondrial DNA is uniparentally inherited marker has drawbacks such as inheritance as a single allele without recombination. A randomly evolving DNA sequence contains mutations with no effect on the fitness and survival of an organism. The randomly evolving mutations are called "neutral", while mutations under selection are "non-neutral". In population genetic studies, analysis of polymorphic microsatellite loci is important. Increased number of microsatellites could compensate for the decreased polymorphism because of homoplasy (Estoup *et al.,* 2002). In the present genetic divergence study of *R. unicornis* it has been found that the rhino population distributed in the three wild habitats of Assam has genetic diversity which was revealed by mtDNA analysis and microsatellites genotyping and the results are found consistent with the similar studies done by Zschokke *et al.* (2011) in few captive rhino from different zoos of the world.

6.2 Summary

In the present study a comprehensive investigation of genetic divergence of *Rhinoceros unicornis* from three habitats of Assam (India) was conducted which gave

following results-

1. The mitochondrial DNA analysis showed that the rhino groups in three habitats of Assam are genetically diverged. The rhino group of Kaziranga National Park is more diverged and comprising many haplotypes.
2. The rhino group of other two habitats namely Orang national park and Pobitora WLS are also diverged. Most of the haplotypes obtained in Kaziranga national park were also found in both the habitats.
3. Significant mitochondrial and nuclear DNA diversity with high mutation rate of microsatellites was observed in the rhino population of all three habitats.
4. A High level of genetic diversity was found both from mitochondrial and microsatellite DNA analysis which implies that the rhino population in Assam did not suffer from serious recent population bottleneck.
5. The result of microsatellite and mitochondrial DNA data were positively correlated.
6. Population differentiation based on F_{ST} values of both microsatellite and mtDNA data indicate that Orang rhino group is slightly different from the other two groups.
7. The mitochondrial DNA analysis did not revealed any differentiation between Kaziranga and Pobitora rhino group.
8. The Kaziranga rhino group contain some private alleles which have been found deviated from Hardy-Weinberg equilibrium.
9. The bottleneck analysis showed that the rhino population of Assam has experienced a bottleneck effect in recent past which was not very much severed.
10. The high genetic diversity observed in the present study indicates that

population decline at the beginning of the 20th century (about 100 years ago) must not be as low as believed earlier (Laurie *et al.,* 1983).

11. The Garza-Williamson index showed that the rhino populations of Assam is not completely stable one and implies existence of bottleneck in the population.
12. The AMOVA result of mitochondrial and microsatellite DNA reveals that within population variation of all rhino is high but among population variation is low.
13. The population structure analysis based on Bayesian genotype clustering method was unable to differentiate the rhino groups from three habitats of Assam.
14. The bottleneck analysis showed that rhino population of Assam must have suffered a bottleneck event which was not very serious.

6.3 Implications of this study

The intra-species genetic diversity is now widely accepted as a key parameter to determine populations to prioritize for conservation and protection purposes. The genetic data found in the population of *R. unicornis* in three habitats of Assam may use for conservation of the species. Till now there has been no such effort has come into effect in the management program of Indian rhino. The findings of present study will provide invaluable data towards characterizing genetic architecture and social structures in wild rhino population. Further, Genetic study of wild animals is important in understanding evolutionary history, population structure and management of populations in their wild habitat. For successful conservation process manager must not only consider species demography but genetic factors as well. The conservation efforts for wild animal can be effectively enhanced by accurate and detailed estimation of

genetic variability. The current data may be used for demographic characterization and to know dispersal patterns of rhino and gene flow in fragmented populations of rhino in Assam which is important in maintaining the genetic diversity. Translocation of animals from one habitat to other may effectively be conducted with the knowledge of genetic diversity of the rhino population. For sustainability of wildlife, application of genetics is need of the hour. The findings of the present study will also help the policy makers and management of rhino to protect the animal in a more scientific manner, which will further accelerate the rhino conservation movement in the state of Assam.

6.4 Recommendations

Rhinoceros unicornis is the flagship species for conservation programs in Kaziranga NP, Pobitora WLS and Orang NP. They are flagships because they have the charisma to secure support for conservation. Conservation of genetic diversity is an essential aspect of the management of threatened and endangered species. It is well established that a decline in genetic variation reduces the ability of a population to adapt to environmental changes and thereby decreases individual fitness which affect on the long term survival of the species. The assessment of genetic diversity within and among the rhino groups would facilitate effective conservation and better management of this endangered species, besides providing information on its DNA sequence. The recommendations for the conservation of rhino are:

1. Genetic management should be carried out by moving individuals between groups to prevent further loss of genetic diversity.
2. To maintain genetic variability artificial dispersal/translocation of the species between the habitats may be done which helps in the selection of new mating

partners. This will also reduce inbreeding depression in the population because inbreeding reduces survival capacity of wild animals.

3. Population viability of wild animals is primarily affected due to anthropogenic threats and habitat fragmentation. These two factors directly correlated with the genetic diversity of wild animals. Population decline of wild animals results inbreeding. The effects of inbreeding can accumulate over many generations, as the frequency of slightly deleterious alleles can gradually increase over time due to genetic drift. So habitat protection should be done.
4. Immediate measures should be taken to prevent further loss of genetic diversity because reduction in population size will compromise the ability of rhino populations to adapt genetically to changing environments.
5. The wildlife managers should apply the knowledge of genetic diversity in conservation process.
6. The current data on microsatellites and mtDNA provide several useful perspectives for conservation biology of rhino
7. The captive breeding programs should be cautiously managed as it may create genetically non-viable animal.
8. To maintain genetically viable wild population captive programs need to be taken in natural condition (in situ) in a specially designed protected area.

6.5 Limitations of the study

It is important to note that complete genetic characterization of a population requires large numbers of samples. The limitation of this study is that a large number of dung samples did not give positive amplification during PCR. Therefore the sample

sizes become low. The DNA obtained from dung samples were degraded and had to be amplified more than once for same sample. In some samples gel extraction method had to be followed from PCR product before sending it to sequencing which was time consuming. Besides, appearance of non specific band during PCR is another problem of this study. Another limitation of this study is that the all amplified samples were not tested for microsatellite genotyping due to high cost.

6.6 Future research

The present study suggests that further investigation is needed to understand the genetic diversity in all three habitats and relationship between genetic diversity and demographic histories of rhinos. There is ample scope for further genetic/DNA studies on *R. unicornis* in India by collecting and analyzing non-invasive sample extensively from all wild habitats. In some cases neutral markers are unable to answer questions about the impact of an effective population size of a small endangered population. For better understanding of the world's most endangered species, analysis of some functional (coding) genes should be done which are under selection. Aanalyses of hyper-variable DNA regions such as the Major Histocompatibility Complex (MHC) can be done as the MHC genes have been connected with individual fitness, population viability and evolutionary potential in changing environments, which makes them ideal for studying adaptive genetic diversity (Strand, 2011). The future work is required to investigate by combination of different markers for better understanding of the species.

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REFERENCES

Adcock, K. (2003). Status and management of black rhino in Namibia and South Africa: January 1999 and December 2001. Rhino Management Group. www.rhinoresourcecenter.com.

Ali, S., Azfer, A., Bashamboo, A., Mathur, P.K., Malik, P.K., Mathur, V.B., Raha, A.K., and Ansari, S. (1999). Characterization of a species-specific repetitive DNA from a highly endangered wild animal, *Rhinoceros unicornis*, and assessment of genetic polymorphism by microsatellite associated sequence amplification (MASA). Gene, 228: 33-42.

Allendorf, F. W. (1986). Genetic drift and the loss of alleles versus heterozygosity. Zoo Biology, 5(2):181-190.

Allendorf, F. W. and Luikart, G. (2007). Conservation and the Genetics of Populations. First Edition, Wiley-Blackwell, MA, USA.

Amato, G., Ashley, M. and Gatesy, J. (1993). Molecular evolution in living species of rhinoceros: implications for conservation. Rhinoceros biology and conservation: Proceedings of an international conference. San Diego, USA. San Diego, Zoological Society, 114–122.

Anderson-Lederer R.M., Linklater, W.L. and Ritchie, P.A. (2012). Limited mitochondrial DNA variation within South Africa's black rhino (*D. b. minor*) population and implications for management. Afr J Ecol., 50 (4):404-413.

Ashley, M.V., Melnick, D.J. and Western, D. (1990). Conservation genetics of the black rhinoceros *Diceros bicornis*, I: evidence from the mtDNA of three populations. Conserv. Biol., 1: 71–77.

Avis, J. C. (1994). Molecular markers, Natural history and evolution. Chapman & Hall. New York, London. pp 511.

Ayala, F. J. (1982). Population and Evolutionary Genetics: A Primer. The Benjamin/Cummings Publishing Co., California, U.S.A.

Ballard, J. W. and Whitlock, M. C. (2004). The incomplete history of mitochondria. Mol. Ecol., 13:729–744.

Ballou, J. D. and Foose, T. J. (1994). Demographic and genetic management of captive populations. In: Kleiman, D. G., Allen, M., Thompson, K., Lumpkin, S. and Harris, H. (Eds.). Wild Mammals in Captivity, Chicago, IL: University of Chicago Press. pp 263-283.

Balloux, F., and Lugon-Moulin, N. (2002). The estimation of population differentiation with microsatellite markers. Mol Ecol., 11(2):155-65.

Bandelt H.J., Forster, P. and Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. Mol. Bio. Evol., 16(1):37-48.

Banerjee, S. and Chakravoorty, S. (1973). Remains of the Great One-horned Rhinoceros, *Rhinoceros uncornis* Linnaeus from Rajasthan. Sci. and Cult., 39(10): 430-431.

Belkhir, K., Borsa, P., Chikhi L., Raufaste, N. and Bonhomme, F. (1996–2004).

GENETIX 4.05, logiciel sous Windows pour la ge´ne´tique des populations. Universite´ de Montpellier II, Montpellier, France.

Bhattacharyya, B.K. (1991). Studies on certain aspect of the biology of the Greater Indian One-horned Rhinoceros. Ph.D thesis, Gauhati University, pp 1 – 287.

Bhatta, R. (2011). Ecology and Conservation of Great Indian One-Horned Rhino (*Rhinoceros unicornis*) in Pobitora Wildlife Sanctyary, Assam, India. Ph.D thesis, Gauhati University, pp 1 – 226.

Bjornstad, O. N., Andreassen, H. P. and Ims, R. A. (1998). Effects of habitat patchiness and connectivity on the spatial ecology of the root vole *Microtus oeconomus*. Journal of Animal Ecology, 67:127-140.

Blanford, W.T. (1891). The Fauna of British India, including Ceylon and Burma. Mammalia. Taylor and Francis, London.

Bollongino, R., Burger, J. and Hummel, S. (2003). DNA Typing from elephant tusks and rhinoceros horn – strategies and applications in wildlife conservation and animal forensics. Forensic Science International, 136: 374-375.

Bonin, A., Ehrich, D. and Manel, S. (2007). Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. Molecular Ecology, 16:3737–3758.

Bora, P. J. and Kumar, Y. (2003). Floristic Diversity of Assam, Study of Pobitora Wildlife Sanctuary. Daya Publishing house, New Delhi; 488p.

Boudjemadi, K., Lecomte, J. and Clobert, J. (1999). Influence of connectivity on

demography and dispersal in two contrasting habitats: an experimental approach. Journal of Animal Ecology, 68:1207-1224.

Borner. M. (1979). A field study of the Sumatran rhinoceros *Dicerorhinus sumatrensis* Fischer 1814. Ecology and behavior conservation situation in sumatra. Ph.D. Dissertation. University of Basel, Switzerland.

Bouzat, J.L. (2010). Conservation genetics of population bottlenecks: the role of chance, selection, and history. Conservation Genetics, 11:139–147.

Broquet, T., Menard, N. and Petit, E. (2007). Non-invasive population genetics: a review of sample source, diet, fragment length and microsatellite effects on amplification success and genotyping error rates. Conservation Genetics, 8: 249-260.

Brown, W.M., George, M. and Wilson, A.C. (1979). Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci., USA, 76:1967–1971.

Brown, G. G., Gadaleta, G., Pepe, G., Saccone, C. and Sbisaa, E. (1986). Structural conservation and variation in the D-loop- containing region of vertebrate Mitochondrial DNA. J. Mol Evol., 192: 503-511.

Brown, W.M. (1983). Evolution of animal mitochondrial DNA. In: Nei, M. and Koehn R.K. (eds). Evolution of Genes and Proteins. Sunderland, Mass: Sinauer, pp 62–88.

Brown, S.M. and Houlden, B.A. (1999). Isolation and characterization of microsatellite markers in the black rhinoceros (*Diceros bicornis*). Mol Ecol., 8 (9):1559-1561.

Brown, S.M. and Houlden, B.A. (2000). Conservation genetics of the black rhinoceros (*Diceros bicornis*). Con Genet., 1:365-370.

Cann, R.L. and Wilson, A.C. (1983). Length mutations in human mitochondrial DNA. Genetics, 104:699–711.

Carson, H. L. (1983). The genetics of the founder effect. In: Genetics and Conservation: A reference for managing wild animal and plant populations. (Eds) C.M. Schonewald Cox; S.M. Chambers; B. and L. Thomas). Benjamin/Cummings, menlopark, CA. pp 189-200.

Caughley, G. (1994). Directions in conservation biology. J Anim Ecol., 63 (2):215-244.

Cavalli Sforza, L. L., Menozzi, P. and Piazza, A. (1994). The history and geography of human genes, Princeton University Press, Princeton, New Jersey.

Cavalli-Sforza, L. L. and Edwards, A. W. (1967). Phylogenetic analysis. Models and estimation procedures. Am J Hum Genet., 19(3), Suppl 19:233-240.

Cerdeno, E. (1995). Cladistic analysis of the family Rhinocerotidae (Perissodactyla). American Museum Novitates, 3145: 1–25.

Champion, H.G. and Seth, S.K. (1968). "Revised survey of the forest types of India" Government of India Press.

Choudhury, A.U. (1985). Distribution of Indian one-horned Rhinoceros. Tiger paper, XII (2): 25-30.

Choudhury, A.U. (1989). Pabitora, Assam's Rhino reserve. The India Magazine, 9: 46-

54.

Clarke, T. H. (1973). The Iconography of the Rhinoceros from Durer to Stubbs, Part I: Durer's Ganda. The Connoisseur, 183: 1-39.

Clayton, D.A. (1982). Replication of animal mitochondrial DNA. Cell, 28:693–705.

Clayton, D.A. (1984). Transcription of the mammalian mitochondrial genome. Annu Rev Biochem., 53:573-594.

Clement, M., Posada, D. and Crandall, K. (2000). TCS: a computer program to estimate gene genealogies. Molecular Ecology, 9(10): 1657-1660.

Cornuet, J. M., and Luikart, G. (1996). Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics, 144(4)**:** 2001-14.

Crawford, N.G. (2010). SMOGD: software for the measurement of genetic diversity. Molecular Ecology Resources, 10, 556-557.

Cunningham, J., Harley, E.H. and O'Ryan, C. (1999). Isolation and characterization of microsatellite loci in black rhinoceros (*Diceros bicornis*). Electrophoresis, 20 (8):1778-1780.

Das, P. and Goswami, U. C. (2012a). Status, distribution and threats on five extant species of Rhinoceros. Proceedings of Zool. Soc., India, 11 (2):73-79.

Das, P. and Goswami, U. C. (2012b). Certain aspects of biology, threats and conservation policies of *Rhinoceros unicornis* In: Diversity and Physiological

Processes, Eds. Desai, P V & R. Roy, 1-16. Goa University.

Das, P., Choudhury, R. and Goswami, U. C. (2013). Genetic Conservation and Management of *Rhinoceros unicornis*. In: Bhattachrjya, D.K. and Ali, E. (Eds.). Environmental degradation & its impact on Biodiversity. EBH Publishers (INDIA), ISBN: 9789380261843, pp 92-98.

Dinerstein, E. (1979a). An ecological survey of the Royal Karnali-Bardia Wildlife Reserve, Nepal. Part I: Vegetation modifying factors and successional relationships. Biol. Conserv., 15:127-150.

Dinerstein, E. (1979b). An ecological survey of the Royal Karnali-Bardia Wildlife Reserve, Nepal. Part II: Habitat/ animal interactions. Biol. Conserv., 18: 5-38.

Dinerstein, E. and McCracken, G.F. (1990). Endangered greater one-horned rhinoceros carry high levels of genetic variation. Conservation Biology, 4: 417-422.

Dinerstein, E. (1991). Sexual dimorphism in the Greater One-horned Rhinoceros (*Rhinoceros unicornis*). J. Mamm., 72(3): 450- 457.

Dinerstein, E. and Price, L. (1991). Demography and habitat use by greater one horned rhinoceros in Nepal. Journal of wildlife management, 55: 401-411.

Dinerstein, E. (2003). The Return of the Unicorns: The Natural History and Conservation of the Greater One-Horned Rhinoceros. New York: Columbia University Press. ISBN 0231084501, pp 384.

Dominguez, M. T., Maranon, T., Murilli, J. M. and Schulin, R. (2007). Trace elements accumulation in woody plants of the Guadiamar valley,SW Spain: a large – scale

phytomanagement case study. Environmental Pollution, 152:50-59.

Du Toit, R., Fosse, T.J. and Cummings, D.H.M. (eds). (1987). Proceedings of African Rhino Workshop, Cincinnati, October 1986. Pachyderm. Special Issue no. 9.

Earl, D.A. and vonHoldt, B.M. (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Con Genet Res., 4 (2):359-361.

Edwards, A., Civitello, A., Hammond, H. A., and Caskey, C. T. (1991). DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am J Hum Genet., 49(4):746-56.

Efron, B. (1982). The Jackknife, the Bootstrap, and Other Resampling Plans, Society for Industrial and Applied Mathematics, Philadelphia.

Esmlie, R.H. and Brooks, M. (1999). African Rhino. Status Survey and Conservation Action Plan. IUCN/SSC African Rhino Specialist Group, IUCN, Gland, Switzerland and Cambridge, UK.

Emslie, R. (2011). *Ceratotherium simum*. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.2. www.iucnredlist.org

Emslie, R. H., Milliken, T. and Talukdar, B. (2012). African and Asian Rhinoceroses – Status, Conservation and Trade, A report from the IUCN Species Survival Commission (IUCN/SSC) African and Asian Rhino Specialist Groups and TRAFFIC to the CITES Secretariat pursuant to Resolution Conf. 9.14 (Rev. CoP15).

England, P.R., Osler, G.H.R., Woodworth, L.M., Montgomery, M.E., Briscoe, D.A. and Frankham, R. (2003). Effects of intense versus diffuse population bottlenecks on microsatellite genetic diversity and evolutionary potential. Conserv Genet., 4 (5):595-604.

Estoup, A., Jarne, P. and Cornuet, J.M. (2002). Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. Molecular Ecology, 11 (9): 1591-1604.

Evanno, G., Regnaut, S. and Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol., 14:2611–2620.

Excoffier, L., Laval, G. and Schneider, S. (2005). Arlequin ver3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1:47–50.

Falconer, D. S. and Mackey, T. F. C. (1996). Introduction to quantitative Genetics, Longman Group Ltd, Harlow.

Falush, D., Stephens, M. and Pritchard, J. K. (2003). Inference of population structure: Extensions to linked loci and correlated allele frequencies. Genetics, 164:1567–1587.

Falush, D., Stephens, M. and Pritchard, J. K. (2007). Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes, 7:574– 578.

Farris, J. S. (1981). Distance data in phylogenetic analysis. In: Advances in Cladistics. Proceedings of thr first meeting of the willi Henning Society (V.A. Funk and D.R. Brooks edition). New York Botanical garden, New York. pp 3-23.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol., 17(6):368-76.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39:783-791.

Fernando, P., Polet, G., Foead, N., Ng, L.S., Pastorini, J. and Melnick, D.J. (2006). Genetic diversity, phylogeny and conservation of the Javan rhinoceros (*Rhinoceros sondaicus*). Conservation Genetics, 7: 439-448.

Fitch, W. M. (1971). Toward defining the course of evolution. Minimum change for a specific tree topology. Systematic Zoology, 20: 406-416.

Foose, T.J. and van Strien, N. (1997). Asian rhinos: Status survey and conservation action plan. IUCN, Gland & Cambridge.

Frankham, R. (1995a). Conservation genetics. Annual Review of Genetics, 29:305-327.

Frankham, R. (1995b). Effective population size/adult population size ratios in wildlife: a review. Genetical Research, 66:95-107.

Frankham, R., Lees, K., Montgomery, M.E., England, P.E., Lowe, E.H. and Briscoe, D.A. (1999). Do population size bottlenecks reduce evolutionary potential? Animal Conservation, 2: 255–260.

Freeman, S. and Herron, J. C. (1998). Evolutionary analysis. Upper Saddle River, New Jersey: Prentice-Hall.

Ganai, N. A., and Yaday, B. R. (2001). Genetic variation within and among three Indian breeds of goat using heterologous microsatellite markers. Anim Biotechnol., (2):121-36.

Garnier, J.N., Bruford, M.W., Goossens, B. (2001). Mating system and reproductive skew in the black rhinoceros. Mol Ecol., 10 (8):2031-2041.

Garza J.C. and Williamson, E.G. (2001). Detection of reduction in population size using data from microsatellite loci. Mol Ecol., 10:305–318.

Gee, E.P. (1953a). The life history of the Greater Indian One-horned Rhinoceros (*Rhinoceros unicornis*). J.Bombay, Nat. Hist. Soc, 34 (2):341–348.

Gee, E.P., (1953b). Further observation on the great Indian One-horned Rhinoceros (*Rhinoceros unicornis*). J. Bombay, Nat. Hist. Soc., 51 (4): 765 –772.

Gee, E.P. (1959). Report on a survey of the rhinoceros area of Nepal. Oryx, 5:59–85.

Gee, E.P. (1963). Report on brief survey of the Wildlife Resources of India including the Rhinoceros. Oryx, 7: 67-76.

Ghosh, D. (1991). Studies on the Eco-Status of the Indian Rhinoceros with special reference to altered habitat due to human interference in Jaldapara Sanctuary, West Bengal. Ph.D thesis, University of Ranchi, India, pp 1 – 305.

Goddard, J. (1967). Home range, behavior, and recruitment rates of two black

rhinoceros populations. East African Wildlife Journal, 5: 133-150.

Goldstein, D. B., Ruiz Linares, A., Cavalli-Sforza, L. L., and Feldman, M. W. (1995). An evaluation of genetic distances for use with microsatellite loci. Genetics, 139(1):463-71.

Goswami, U. C. (1993). Analysis of certain strategies of conservation and propagation of *Rhinoceros unicornis* (L). Tiger Paper XX:16-22.

Goudet, J. (1995). FSTAT (version 12): A computer program to calculate F- statistics. Journal of Heredity, 86:485–486.

Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available at http://www.unil.ch/izea/software/fstat.htm.

Grivell, L.A. (1983). Mitochondrial DNA. Sci Am., 248:78–89.

Groves, C. (1983). Phylogeny of the living species of rhinoceros. Journal of Zoological Systematics and Evolutionary Research, 31: 293–313.

Guerier, A.S., Bishop, J.M., Crawford, S. J., Schmidt-Kuntzel, A. and Stratford, K.J. (2012). Parentage analysis in a managed free ranging population of southern white rhinoceros: genetic diversity, pedigrees and management. Conserv Genet., (online version) DOI 10.1007/s10592-012-0331-4.

van Gyseghem, R. (1984). Observations on the ecology and behaviour of the Northern White Rhinoceros (*Ceratotherium simum cottoni*). Zeitschrift fur Saugetierkunde, 49(6): 348-458.

Haberfeld, A., Cahaner, A., Yoffe, O., Plotsky, Y., and Hillel, J. (1991). DNA fingerprints of farm animals generated by microsatellite and minisatellite DNA probes. Anim Genet., 22(3):299-305.

Halliburton, R. (2004). Introduction to population genetics. Pearson Education, Inc., USA.

Hansen, M.M., Ruzzante, D.E., Nielsen, E.E. and Mensberg, K.L.D. (2001). Brown trout (*Salmo trutta*) stocking impact assessment using microsatellite DNA markers. Ecological Applications, 11: 148–160.

Harley, E.H., Baumgarten, I., Cunningham, J. and O'Ryan, C. (2005). Genetic variation and population structure in remnant populations of black rhinoceros, *Diceros bicornis* in Africa. Mol Ecol., 14: 2981–2990.

Harpending, H.C. (1994). Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Human Biology, 66: 591–600.

Hartl, D. L. and Clark, A. G. (1989). Darwinian evolution in mendelian populations. In: Principles of population genetics; Sinauer Associates Inc, Saunderland, Massachusetts.

Hartl, D. L. and Clark, A. G. (1997). Principles of population genetics, 3rd ed. Sinauer Associates. Saunderland, Massachusetts. pp 542.

Hazarika, B.C. (2007). "Studies on the Eco-behaviour aspect of Greater Indian One-horned Rhinoceros (*Rhinoceros unicornis*) in the Orang National Park, Assam, India." Ph.D thesis, Gauhati University.

Hazarika, B. C. and Saikia P. K. (2009). Habitat Clafissification and Habitat Utilization of Great Indian One-Horned Rhino *(Rhinoceros unicornis* Lin.) in Rajib Gandhi Orang National Park. Proceeding of National Seminar on use of Remote sensing and GIS for wild life studies; pp53-60.

Hazarika, B. C. and Saikia P. K. (2010). A study on the behaviour of Great Indian One-horned Rhino (*Rhinoceros unicornis* Linn.) in the Rajiv Gandhi Orang National Park, Assam, India. NeBIO, 1(2): 62- 74.

Hazarika, B. C. and Saikia P. K. (2011). A Study on the Habitat Utilization Pattern of Great Indian One-hornrd Rhino *(Rhinoceros unicornis* Lin.) in the Brahmaputra flood plain-Orang National Park. NeBIO, 2(2): 37-49.

Hedrick, P. W., Brussard, P. F., Allendorf, F. W., Beardmore, J. and Orzack, S. H. (1986). Protein variation, fitness, and captive propagation. Zoo Biology, 5: 91-99.

Hedrick, P.W. (1999). Perspective: Highly variable loci and their interpretation in evolution and conservation. Evolution, 53: 313-318.

Hedrick, P.W. (2005). A standardized genetic differentiation measure. Evolution, 59: 1633–1638.

Hirayama, M., Mukai, T., Miya, M. et al. (2010). Intraspecific variation in the mitochondrial genome among local populations of Medaka *Oryzias latipes*. Gene, 457, 13–24.

Hooijer, D.A. (1968). A rhinoceros from the late Miocene of Fort Teman. Kenya. 2001. Zoo. Med.Leiden, 43: 77 -92.

Hsieh, H.M., Huang, L.H., Tsai, L.C., Kuo Y.C., Meng, H.H., Linacre, A. and Lee, J. C.I. (2003). Species identification of rhinoceros horns using the cytochrome b gene. Forensic Science International, 136: 1-11.

Hu, J., Fang, S. G. and Wan, Q. H. (2006). Genetic diversity of Chinese water deer (*Hydropotes inermis inermis*): implications for conservation. Biochemical Genetics, 44: 161–172.

Hughes, A.R., Inouye, B.D., Johnson, M. T. J., Underwood, N.and Vellend, M. (2008). Ecological consequences of genetic diversity. Ecology Letters, 11: 609–623.

Hunter, M. L. J. (1996). Fundamentals of conservation biology. Blackwell science, Inc., Cambridge, Massachusetts, U.S.A. pp 482.

Hutchins, M. and Kreger, M.D. (2006). Rhinoceros behaviour: implications for captive management and conservation. Int Zoo Yearb., 40:150–173.

Idaghdour, Y., Broderick, D., Korrida, A. and Chbel, F. (2004). Mitochondrial control region diversity of the houbara bustard *Chlamydotis undulata* complex and genetic structure along the Atlantic seaboard of North Africa. Molecular Ecology, 13: 43–54.

IRF, (2010). International Rhino Foundation. Available at: www.rhinos-irf.org.

IUCN (2004). IUCN Red List of Threatened Species. Available at:www.iucnredlist.org.

Jarne, P. and Lagoda, P.J.L. (1996). Microsatellites, from molecules to populations and back. Trends Ecol. Evol., 11, 424–429.

Jeffreys, A. J., Wilson, V., and Thein, S. L. (1985). Hypervariable 'minisatellite' regions in human DNA. Nature, 314(6006): 67-73.

Jnawali, S.R. (1995). Population Ecology of Greater One-horned Rhinoceros with particular emphasis on habitat preference, food, ecology and ranging behaviour of a reintroduction population in Royal Bardia National Park in lowland Nepal. Ph.D thesis, Agricultural University, Norway.

Jobling, M. A., Hurles, M. E. and Tyler-Smith, C. (2004). Human Evolutionary Genetics: origins, peoples and disease. London/New York: Garland Science Publishing, 523 pp.

Jorde, L.B., Watkins, W.S. and Bamshad, M. J. (2001).Population genomics: a bridge from evolutionary history to genetic medicine, Hum. Mol. Genet., 10 (20): 2199-2207.

Kalinowski, S. T. (2002). Evolutionary and statistical properties of three genetic distances. Mol Ecol., 11(8):1263-73.

Karsten, M., van Vuuren, B.J., Goodman, P. and Barnaud, A. (2011). The history and management of black rhino in KwaZulu-Natal: a population genetic approach to assess the past and guide the future. Anim Conserv., 14:363-370.

Kashi, Y., Tikochinsky, Y., Genislav, E., Iraqi, F., Nave, A., Beckmann, J. S., Gruenbaum, Y. and Soller, M. (1990). Large restriction fragments containing poly-TG are highly polymorphic in a variety of vertebrates. Nucleic Acids Res., 18(5):1129-32.

Keller, L.F. and Waller, D.M. (2002). Inbreeding effects in wild populations. Trends Ecol Evol., 17 (5):230-241

Kemf, E. and Strien, N.J. van (2002). Asian rhinos in the wild - a WWF species status report. Gland, WWF, pp. 1-24.

Kimura, M. and Crow, J. F. (1964). The Number of Alleles That Can Be Maintained in a Finite Population. Genetics, 49:725-38.

Kiwia, H. Y. D. (1989). Ranging patterns of the black rhinoceros *Diceros bicornis* (L.) in Ngorongoro Crater, Tanzania. African Journal of Ecology, 27: 305-312.

Kushwaha, S.P.S. and Madhavan Unni, N.V. (1986). Application of remote sensing techniques in forest cover monitoring and habitat evaluation: A case study in Kaziranga National Park, Assam. Proc. Seminar-cum-Workshop on Wildlife Habitat Evaluation using Remote Sensing Techniques, 22-23 October, Dehradun, pp. 238-247.

Kushwaha, S.P.S., Roy, P.S., Azeem, A., Boruah, P. and Lahan, P. (2000). "Land area change and rhino habitat suitability analysis in Kaziranga National Park, Assam." Tigerpaper, 27 (2): 9 – 16.

Kushwaha, S.P.S.(2008).Mapping of Kaziranga Conservation Area, Assam, Final Technical Report, Mapping of National Parks and Wildlife Sanctuaries, Volume-V, pp 1-61.

Lacombat, F. (2005). The evolution of the rhinoceros. In Fulconis, R. (ed). Save the Rhinos: EAZA Rhino Campaign 2005/6. London: European Association of Zoos

and Aquaria. Pp 46-49.

Lacy, R. C. (1987). Loss of genetic variation from managed populations: Interacting effects of drift, mutation, immigration, selection and population subdivision. Conservation Biology, 1: 143-158.

Lande, R. (1988). Genetics and demography in biological conservation. Science, 241(4872):1455-60.

Lande, R. and Barrowclaugh, G. F. (1987). Effective population size, genetic variation, and their use in population management. In: Viable populations for conservation (Edited by M.E. Soule). University press. pp 87-123.

Larizza, A., Pesole, G., Reyes, A., Sbisà, E. and Saccone, C. (2002). Lineage specificity of the evolutionary dynamics of the mtDNA D-loop region in rodents. Journal of Molecular Evolution, 54:145-155.

Laurie, W. A. (1978). The ecology and the behaviour of the Great one horned rhinoceros (*Rhinoceros unicornis*). Ph D. dissertation, University of Cambridge, Cambridge, pp 450.

Laurie, W. A. (1982). Behavioral Ecology of Great Indian one-horned Rhinoceros . J. Zool., 196: 307-341.

Laurie, W.A., Lang, E.M. and Groves, C.P. (1983). *Rhinoceros unicornis*. Mammalian Species, 211: 1–6.

Leberg, P. L. (1992). Effects of a population bottleneck on genetic variation as measured by alloyzme electrophoresis. Evolution, 46: 477-494.

Lewontin, R. C. and Kojima, K. (1960). The evolutionary dynamics of complex polymorphisms, Evolution, Vol. 14: 458-472.

Li, M. H., Zhao, S. H., Bian, C., Wang, H. S., Wei, H., Liu, B., Yu, M., Fan, B., Chen, S. L., Zhu, M. J., Li, S. J., Xiong, T. A., and Li, K. (2002). Genetic relationships among twelve Chinese indigenous goat populations based on microsatellite analysis. Genet Sel Evol., 34(6): 729-44.

Luikart, G. and England, P.R. (1999). Statistical analysis of microsatellite DNA data. Trends Ecol. Evol., 14, 253–256.

Luikart, G. and Cornuet, J. M. (1998). Emperical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conservation biology, 12(1)**,** 228-237.

Luikart, G., Allendorf, F. W., Cornuet, J. M. and Sherwin, W. B. (1998). Distortion of allele frequency distributions provides a test for recent population bottlenecks. J Hered**.,** 89(3)**,** 238-47.

Lwin, T. U. (1998). Htamanthi Wildlife sanctuary: Last foothold of Rhinos in Myanmyar. Tiger Paper, 25(2): 1-3.

Lydekker, R., (1908). The game animals of Africa. London, Rowland Ward.

Mate, M. L., Rocco, F., Zambelli, A. and Vidalrioja, L. (2004). Mitochondrial DNA structure and organization of the control region of South American camelids. Molecular Ecology Notes, 4:765-767.

Matson C.W., and Baker, R.J. (2001). DNA sequence variation in the mitochondrial

control region of redbacked voles (*Clethrionomys*). Molecular Biology and Evolution, 18:1494-1501

McArdle, B. H. (1996). Levels of evidence in studies of competition, predation, and disease. New Zealand Journal of Ecology, 20:7-15.

McCracken, K.G., Johnson, W.P. and Sheldon, F.H. (2001).Molecular population genetics, phylogeography, and conservation biology of the mottled duck (*Anas fulvigula*), Conservation Genetics, 2: 87–102.

Meghen, C., MacHugh, D. E. and Bradley, D. G. (1994). Genetic characterization and West Africa cattle. World Animal Review, 78(1): 59-66.

Merenlender, A., Woodruff, D., Ryder, O., Kock, R. and Vahala, J. (1989). Allozyme variation and differentiation in African and Indian rhinoceroses. Journal of Heredity, 80: 377–382.

Mills, S. L., and Smouse, P. E. (1994). Demographic consequences of inbreeding in remnant populations. American Naturalist, 144, 412-431.

Moore, S. S., Sargeant, L. L., King, T. J., Mattick, J. S., Georges, M. and Hetzel, D. J. (1991). The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. Genomics, 10(3):654-60.

Morales, J. C. and Melnick D.J. (1994). Molecular systematics of the living rhinoceros. Molecular Phylogenetics and Evolution, 2: 129-134.

Morales, J. C., Andau, P.M., Supriatna, J., Zainuddin, Z. Z. and Melnick, D.J. (1997).

Mitochondrial DNA variability and conservation genetics of the Sumatran rhinoceros. Conservation Biology, 11: 539-543.

Morin, P. A., Moore, J. J., Chakraborty, R., Jin, L., Goodall, J. and Woodruff, D. S. (1994). Kin selection, social structure, gene flow, and the evolution of chimpanzees. Science, 265 (5176): 1193-201.

Moritz, C., Dowling, T. E. and Brown, W. M. (1987). Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. Ann. Rev. Ecol. Syst. 18:269–292.

Moritz, C. (1994a). Defining "evolutionary significant units" for conservation. *Trends in* Ecology and Evolution, 9, 373-375.

Moritz, C. (1994b). Applications of mitochondrial DNA analysis in conservation: critical review. Molecular Ecology, 3, 401- 411.

Moritz, C. and Cicero, C. (2004). DNA barcoding: Promise and pitfalls. PloS Biol. 2:1529–1531.

Mukherjee, A.K., (1982). Endangered animals of India. Calcutta, Zoological Survey of India, pp 1-122.

Munwes, I., Geffen, E., Friedmann,A., Tikochinski, Y. and Gafny, S. (2011) Variation in repeat length and heteroplasmy of the mitochondrial DNA control region along a core–edge gradient in the eastern spadefoot toad (Pelobates syriacus), Molecular Ecology 20: 2878–2887.

Muya, S. M., Bruford, M. W., Muigai, A.W.T., Osiemo Z. B., Mwachiro, E., Okita-

Ouma B. and Goossens, B.(2011). Substantial molecular variation and low genetic structure in Kenya's black rhinoceros: implications for conservation. Conserv Genet., DOI 10.1007/s10592-011-0256-3.

Neff, B.D. and Gross, M.R. (2001). Microsatellite evolution in vertebrates: inference from AC dinucleotide repeats. Evolution, 55, 1717–1733.

Nei, M. (1972). Genetic distance between populations. American Naturalist, 106:283-292.

Nei, M., Maruyama, T. and Chakraborty, R. (1975). The bottleneck effect and genetic variability in populations. Evolution, 29:1–10.

Nei, M. (1977). F-statistics and analysis of gene diversity in subdivided populations, Annals of Human Genetics, 41(2): 225–233.

Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA, 76: 5269-5273.

Nei, M., Tajima, F. and Tateno, Y. (1983). Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. J Mol Evol., 19(2):153-70.

Nei, M. (1984). Molecular Evolutionary Genetics. Colombia University press/New York.

Nei, M. (1987). Molecular Evolutionary Genetics. Columbia University press, New York. pp 506.

Nei, M. and Takezaki, N. (1994). Estimation of genetic distances and phylogenetic trees

fromDNA analysis. In: proceeding of the 5th world congress of genetic applied to livestock production. 7-12 August, 1994 Guelph, Canada. Vol 21: 405-412.

Nei, M. (1996). Phylogenetic analysis in molecular evolutionary genetics. Annu Rev Genet., 30:371-403.

Nielsen, L., Meehan-Meola, D., Kilbourn, A. and Alcivar-Warren, A. (2008). Characterization of microsatellite loci in the black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*): their use for cross-species amplification and differentiation between the two species. Conserv Genet., 9:239–242.

O'Brien, S.J. and Evermann, J.F. (1988). Interactive influence of infectious disease and genetic diversity in natural poputations. Trends in Ecology & Evolution, 3: 245–259.

Okumus, I. and Ciftci, Y. (2003). Fish Population Genetics and Molecular Markers: II-Molecular Markers and Their Applications in Fisheries and Aquaculture. Turkish Journal of Fisheries and Aquatic Sciences, 3: 51-79.

Onuma, M., Suzuki, M. and Ohtaishi, N. (2006). Possible conservation units of the sun bear (*Helarctos malayanus*) in Sarawak based on variation of mtDNA control region. Japanese Journal of Veterinary Research, 54: 135–139.

Owen-Smith, R.N. (1975). The social ethology of the white rhinoceros. Zeitschrift fur Tierpsychologie, 38:337-384.

Orlando, L., Leonard, J.A., Thenot, A., Laudet, V., Guerin, C. and Hanni, C. (2003).

Ancient DNA Analysis Reveals Wooly Rhinoceros Evolutionary Relationship. Molecular Phylogenetics and Evolution, 28: 485-499.

Pluhacek, J., Sinha, S.P., Bartos, L. and Sipek, P. (2007). Parity as a major factor affecting infant mortality of highly endangered Indian rhinoceros: Evidence from zoos and Dudhwa National park, India. Biological Conservation, 139: 457-461.

Parsons, J. (1743). A letter to Martin Folkes, esq.; President of the Royal Society, containing the natural history of the rhinoceros. Philosophical transactions of the Royal Society, 42: 523–541.

Paetkau, D. and Strobeck, C. (1995). The molecular basis and evolutionary history of a microsatellite null allele in bears. Mol Ecol., 4(4):519-20.

Peakall, R. and Smouse, P.E. (2006). GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes, 6:288–295.

Pesole, G., Gissi, C., Chirico,A. De. and Saccone. C. (1999). Nucleotide substitution rate of mammalian mitochondrial genomes. Journal of Molecular Evolution, 48:427-34.

Piry, S., Luikart, G. and Cornuet, J.M. (1999). BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity, 90:502–503.

Prater, S.H. (1980). The book of Indian animals, Bombay Natural History Society, Twelth reprint 2005, pp 324

Primack, B.R. (1993). Essentials of Conservation Biology, Sinauer Associates, Inc., Sunderland, MA, USA. pp 564.

Pritchard, J. K., Stephens, M. and Donnelly, P. (2000). Inference of population structure using multilocus genotype data. Genetics 155(2):945-59.

Prothero, D.R., Manning, E. and Hanson, C.B. (1986). The phylogeny of the Rhinocerotoidea (Manmlalia: Perissodactyla). Zoological Journal of the Linnean Sociely, 87: 341 - 366.

Prothero, D.R., and Schoch, R.M. (1989). Origin and evolution of the Perissodactyla: a summary and synthesis. New York: Oxford Univ. Press, pp. 504-529.

Prothero, D., Manning, E. and Hanson, C. (1986). The phylogeny of the Rhinocerotoidea (Mammalia, Perissodactyla). Zoological Journal of the Linnean Society 87: 341–366.

Queller, D. C., Strassmann, J. E. and Hughes, C. R. (1993). Microsatellite and Kinship. Trends in Ecology and Evolution, 8:285-288.

Randi, E., Pierpaoli, M. and Danilkin, A. (1998). Mitochondrial DNA polymorphism in population of Siberian and European roe deer (*Capreolus pygargus* and *C. capreolus*). Heredity, 4: 429-437.

Raymond, M. and Riousset, F. (1995). GENEPOP (version 1.2): Population genetics software for exact test and ecumenicsm. J.Heredity, 86:248-249.

Richards, R. I. and Sutherland, G. R. (1994). Simple repeat DNA is not replicated simply. Nat Genet., 6(2): 114-6.

Rogers, J. S. (1972). Measures of genetic similarity and genetic distances. Studies in Genetics, Univ. Texas Publ., 7213:145–153.

Rogers, A.R. and Harpending, H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol., 9:552-569.

Rookmaaker, L. C. (1978). Two collections of rhinoceros plates compiled by James Douglas and James Parsons in the eighteenth century. Journal of the Society for the Bibliography of Natural History, 9 (1): 17–38.

Rookmaaker, L.C. (1980). The Distribution of Rhinoceros in Eastern India, Bangladesh China and Indochinese region. Zool. Anz. Jena., 20: 253-268.

Rookmaaker, L.C. (1982). The former distribution of the Indian Rhinoceros (*Rhinoceros unicornis)* in India and Pakistan. Journal of Bombay Natural History Society, 80: 555-563.

Rookmaaker, L.C. (2002). Historical records of the Rhinoceros (*Rhinoceros unicornis*) in northern India and Pakistan. Zoos' Print Journal 17: 923–929.

Rose, G., Passarino, G. and Scornaienchi, V., Romeo, G., Dato, S., Bellizzi, D., Mari, V., Feraco, E., Maletta, R., Bruni, A., Franceschi C. and De Benedictis, G. (2007). The mitochondrial DNA control region shows genetically correlated levels of heteroplasmy in leukocytes of centenarians and their offspring. BMC Genomics, 8: 293.

Roy, M. S., Geffen, E., Smith, D., Ostrander, E. A. and Wayne, R. K. (1994). Patterns of differentiation and hybridization in North American wolflike canids, revealed by

analysis of microsatellite loci. Mol Biol Evol., 11(4):553-70.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X. and Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497.

Ruegg, K.C. and Smith, T.B. (2002). Not as the crow flies: a historical explanation for circuitous migration in Swainson's thrush (Catharus ustulatus). Proceedings of the Royal Society B: Biological Sciences, 269:1375–1381.

Russell, J.P. (2003). Essential Genetics. Benjamin Cummings (Pearson Education Inc.), San Francisco. ISBN: 0-8053-4697-X, pp 600.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory Manual, 2nd edition, Vol. 2&3, Cold Spring Harbour Laboratory Press, New York.

Sarma, P.K., Takudar, B.K., Sarma, K. and Baruah, M. (2010). Assessment of rhino habitat change in Pabitora Wildlife Sactuary of Assam, India, Pachyderm, 46:18 – 24.

Sarma, P. K., Mipun, B. S., Talukdar, B.K., Kumar, R. and Basumatary, A.K. (2011). Evaluation of Habitat Suitability for Rhino (*Rhinoceros unicornis*) in Orang National Park Using Geo-Spatial Tools. International Scholarly Research Network, ISRN Ecology, 1-9. doi:10.5402/2011/498258

Sarma, P.K. (2010). Habitat suitability for rhino (*Rhinoceros unicornis*) and utilization pattern in Rajiv Gandhi Orang National Park of Assam, Ph.D thesis, North Eastern Hill University, Shillong. pp 1-300.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol, 4 (4):406-25.

Sbisa, E., Tanzariello, F., Reyes, A., Pesole, G. and Saccone, C. (1997). Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. Gene, 205:125-140.

Schenkel, R. and Schenkel-Hulliger, L. (1969). Ecology and Behaviour of the Black Rhinoceros (*Diceros bicornis* L.): A Field Study. In :Mammalia Depicta, Verlag Paul Parey, Hamburg and Berlin. pp 96.

Scott, C.A. (2008). Microsatellite variability in four contemporary rhinoceros species: implications for conservation. Department of Biology, Queen's University Kingston, Ontario, Canada.

Seutin, G., White B.N. and Boag P.T. (1991). Preservation of avian blood and tissue samples for DNA analysis, Canadian Journal of Zoology, 69:8290

Shoshani, J. (2006). Taxonnomy, classification, history, and evolution of elephants. In: Fowler M.E. and Mikota, S.K. (eds.), Biology, medicine, and surgery of elephants. Blackwell Publishing, Ames, IA, pp 3-14.

Slatkin, M. (1985). Gene flow in natural populations. Annu Rev Ecol Syst., 16: 393-430.

Slatkin, M. (1995). A measure of population subdivision based on microsatellite allele frequencies. Genetics, 139(1): 457-62.

Steiner, C. C. and Ryder, O. A. (2011). Phylogeny and evolution of the Perissodactyla.

Zoological Journal of the Linnean Society, 163:1289–1303.

Stracey, P.D. (1949). The Vanishing Rhinoceros and Assam Wildlife Sanctuaries. Indian Forester. 75:470-473.

Strand, T.M. (2011). Genotyping of black grouse MHC class II B using reference strand- mediated conformational analysis (RSCA). BMC Research Notes, 4:183.

Sunnucks, P. (2000). Efficient genetic markers for population biology. Trends in Ecology and Evolution, 15: 199–203.

Swart, M.K.J. and Ferguson, W.H. (1997). Conservation Implications of genetic differentiation in southern African populations of black rhinoceros (*Diceros bicornis*).

Takezaki, N. and Nei, M. (1996). Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics, 144(1):389-99.

Talukdar, B.K., Barua, M. and Sarma, P.K. (2007). Tracing straying routes of rhinoceros in Pabitora Wildlife Sanctuary, Assam, Current Science, 92(9):1303-1305.

Talukdar, B. (1995). Status of Swamp Deer in Kaziranga National Park. Department of Zoology, Ph. Thesis, Gauhati University, Assam.

Talukdar, B.K., (2000). The current state of rhino in Assam and threats in the 21st century. Pachyderm, 29:39-47.

Tauz, D. (1989). Hypervariability of simple sequences as a general source of

polymorphic DNA markers. Nucleic Acid Research, 17: 6463-6471.

Tateno, Y., Nei, M. and Tajima, F. (1982). Accuracy of estimated phylogenetic trees from molecular data. I. Distantly related species. J Mol Evol., 18(6):387-404.

Tajima, F. (1996). The amount of DNA polymorphism maintained in a finite population when the neutral mutation rate varies among sites. Genetics, 143: 1457–1465.

Toju, H. and Sota, T. (2006) Phylogeography and the geographic cline in the armament of a seed-predatory weevil: effects of historical events vs. natural selection from the host plant. Molecular Ecology, 15: 4161–4173.

Toth, G., Gaspari, Z. and Jurka, J. (2000). Microsatellites in different eukaryotic genomes: survey and analysis. Genome Res., 10:967–81.

Tougard, C., Delefosse, T., Hoenni, C. and Montgelard, C. (2001). Phylogenetic relationships of the five extant rhinoceros species (Rhinocerotidae, Perissodactyla) based on mitochondrial cytochrome b and 12s rRNA genes Molecular Phylogenetics and Evolution, 19(1): 34–44.

Tripathi, A.K. (2012). Habitat and population ecology of *Rhinoceros unicornis* in Dudhwa National Park, Uttar Pradesh. Int. J. of Pharm. & Life Sci., 3(11): 2090-2097.

Ullrich, W. (1972). Kaziranga – Tierparadies am Brahmaputra, 2nd ed. Urania Verlag, Leipzig, Jena, Berlin.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. and Shipley, P. (2004). MICROCHECKER: software for identifying and correcting genotyping errors in

microsatellite data. Molecular Ecology Notes, 4: 535–538.

Van Strien, N. J. (1986). The Sumatran Rhinoceros *Dicerorhinus sumatrensis* (Fischer, 1814) in the Gunung Leuser National Park, Sumatra, Indonesia. Hamburg.

Van Strien, N.J. (1975). *Dicerorhinus sumatrensis* (Fischer), the Sumatran or two-horned Asiatic rhinoceros: a study of literature. Netherlands Committee for International. Nature Protection Meded, 22: 1-82.

Van Strien, N. J. (2005). Asian Rhino Specialist Group report. Pachyderm 39: 13-17.

Van Coeverden de Groot, P.J., Putnam, A.S., Erb. P., Scott, C., Melnick, D., O'Ryan, C. and Boag, P.T. (2011) Conservation genetics of the black rhinoceros, *Diceros bicornis bicornis*, in Namibia. Con Genet., 12:783-792.

Vasu, N.K. (2003). Management Plan: Kaziranga National Park, World Heritage Site. Forest Department, Government of Assam, India.

von Houwald, F., Pagan, O. and Rieches, R., (2010). International studbook 2009– Greater One-Horned or Indian Rhinoceros *Rhinoceros unicornis* Linne, 1758. Zoo Basel, Basel.

Waser, P.M. and Strobeck, C. (1998). Genetic signatures of interpopulation dispersal. Trends Ecol Evol., 13:43–44.

Weber, J. L. and May, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet., 44(3): 388-96.

Weber, J. L. (1990). Informativeness of human (dC-dA)n.(dG-dT)n polymorphisms. Genomics, 7(4):524-30.

Weir, B. S. and Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. Evolution, 38:1358-1370.

Weir, B.S. (1996). Genetic data analysis II. Sunderland, MA: Sinauer Associates, Inc.

Willerslev, E., Gilbert, M., Binladen, J., Ho, S., Campos, P., Ratan, A., Tomsho, L., da Fonseca, R., Sher, A., Kuznetsova, T., Nowak-Kemp, M., Roth, T., Miller, W. and Schuster, S. (2009). Analysis of complete mitochondrial genomes from extinct and extant rhinoceroses reveals lack of phylogenetic resolution. BMC Evolutionary Biology, 9: 95.

Williamson, J.H. (2001). Broodstock management for imperilled and other fishes. In G. A. Wedemeyer, (Ed.). Fish hatchery management, second edition. American Fisheries Society, Bethesda, Maryland: 397-482.

Wright, S. (1965). The Interpretation of Population Structure by F-Statistics with Special Regard to Systems of Mating. Evolution, 19(3):395-420.

Wright, S. (1969). Evolution and the genetics of populations. University of Chicago press, Chicago.

Wu, H. L., Wan, Q. H. and Fang, S. G. (2006). Population structure and gene flow among wild populations of the black muntjac (*Muntiacus crinifrons*) based on mitochondrial DNA control region sequences. Zoological Science, 23: 333–340.

Wurster, D.H. and Benirschke, K. (1968). The chromosomes of the Great Indian Rhinoceros (*Rhinoceros unicornis* L.). Experientia, 24: 5–11.

Xu, X. and Arnason, U. (1997). The complete mitochondrial DNA sequence of the white rhinoceros, *Ceratotherium simum*, and comparison with the mtDNA sequence of the Indian rhinoceros, *Rhinoceros unicornis*. Molecular Phylogenetics and Evolution, 7: 189-194.

Xu, X., Janke, A. and Arnason, U. (1996). The complete mitochondrial DNA sequence of the greater Indian rhinoceros, *Rhinoceros unicornis*, and the Phylogenetic relationship among Carnivora, Perissodactyla and Artiodactyla (+Cetacea). Molecular Biology and Evolution, 13: 1167-1173.

Zschokke, S. and Baur, B. (2002). Inbreeding, outbreeding, infant growth, and size dimorphism in captive Indian rhinoceros (*Rhinoceros unicornis*). Canadian Journal of Zoology, 80: 2014-2023.

Zschokke, S., Gautschi, B. and Baur, B. (2003). Polymorphic microsatellite loci in the endangered Indian rhinoceros, *Rhinoceros unicornis*. Molecular Ecology Notes, 3: 233- 233.

Zschokke, S., Armbruster, F. J., Sylvain U. and Bruno, B. (2011). Genetic differences between the two remaining wild populations of the endangered Indian rhinoceros (*Rhinoceros unicornis*) Biological Conservation, 144: 2702–2709.