Evaluation of genetic status of *Rhinoceros unicornis* in India employing noninvasive molecular methods

Thesis

Submitted to
Gauhati University for the award of the
Degree of Doctor of Philosophy in Biotechnology

Faculty of Technology

Submitted
by
Pranjal Kr Das
G.U. Registration No. 002218 of 2007-2008
Ph. D. Enrolment No. B.T. - 07/11

Department of Biotechnology
Gauhati University
Guwahati-781014, Assam

2017

DEPARTMENT OF BIOTECHNOLOGY
GAUHATI UNIVERSITY
GUWAHATI-781014

Hridip Kumar SarmaMSc., PhD
Assistant Professor
Biochemistry & Environmental Biotechnology

Phone: 03612700231
Cell: 9613861702
Email: hridip123@yahoo.com

Certificate

This is to certify that the Ph.D. thesis entitled "**Evaluation of genetic status of *Rhinoceros unicornis* in India employing noninvasive molecular methods**" submitted by **Pranjal Kumar Das** (Ph.D. Enrolment No. B.T.-07/11), carried under my supervision and is submitted to the Gauhati University for the award of the Degree of Doctor of Philosophy in Biotechnology under the Faculty of Technology. Mr Das has fulfilled all the requirements of the regulations relating to the research work, the period of research and submission of thesis.

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Declaration

I, Pranjal Kumar Das, hereby declare that the thesis entitled, **"Evaluation of genetic status of *Rhinoceros unicornis* in India employing noninvasive molecular methods"** is original work of research submitted in fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Biotechnology under Gauhati University, Guwahati, supervised by Dr. Hridip Kumar Sarma, Assistant Professor, Department of Biotechnology, Gauhati University. I further declare that the whole or any part of this thesis has not been submitted to any other University/ Institute for the award of any degree or diploma.

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Place: Guwahati

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ABSTRACT

The greater one-horned rhinoceros is a large free ranging animal, historically distributed throughout the northern part of Indian subcontinent. Due to habitat destruction and indiscriminate hunting, the population size of the species gradually declined and was on the verge of extinction in the beginning of the 20th century. Presently, about 3550 individuals of greater one-horned rhinoceros are known to thrive in isolated patches in India and Nepal, of which, more than 80% live in the protected areas of India. Reduction in population size and habitat fragmentation may have serious implications on genetic variability of a species which is crucial for maintaining its evolutionary potential. Although, the greater one-horned rhinoceros revived from the brink of extinction, the potential effect of population bottlenecking and habitat fragmentation on its genetic status is not yet known. In the present study, genetic diversity, signatures of past bottlenecking, extent of population genetic structure and migration patterns among five rhino populations of India were assessed using nine microsatellite markers. A moderate to high level of genetic diversity was observed with allelic richness ranging from 2.589 to 3.635 and expected heterozygosity ranging from 0.352 to 0.59. Although, signature of past bottlenecking was observed in two rhino populations of Assam, its effects on their genetic diversity was not very apparent, unlike that previously thought. A significant level of genetic differentiation was also observed among the greater one-horned rhinoceros populations of India, especially, the Gorumara population, showing a unique genetic signature (pair wise $F_{ST} \geq 0.25$; $p < 0.001$, with all other populations). An asymmetric pattern of migration among the

rhinoceros populations of Assam was also observed with mean migration rate ranging from 0.056 to 0.139. The results obtained in the present study signify the need for long term genetic monitoring of greater one-horned rhinoceros, which will assist in designing effective conservation and management strategies that will ensure long term survival of the species in its natural habitat.

ACKNOWLEDGEMENT

I express a deep sense of gratitude to my supervisor Dr. Hridip Kumar Sarma, Assistant Professor, Department of Biotechnology, Gauhati University for his able guidance, valuable suggestions and constructive criticisms during the course of research work and successful documentation of this thesis. I take this solemn opportunity to extent my heartiest gratitude to the Head, Department of Biotechnology, Gauhati University for providing me necessary facilities during the course of this work. I am also thankful to all the faculty members of the Department of Biotechnology for their inspiration, encouragement and support.

I am extremely grateful to the Mr. Udayan Borthakur, Head, Wildlife Genetics Division (WGD), Aaranyak, a *"Scientific and Industrial Research Organization (SIRO)"*, Guwahati for his constant guidance in experimental works, constructive criticism, incessant motivation, and moral support during the entire course of this work. I am also thankful to Aaranyak, for providing financial support and allowing me to use the laboratory facility of WGD all along my research tenure.

I would also like to extend my gratitude to Dr. Lounes Chikhi, Principal Investigator, Population and Conservation Genetics Unit, Instituto Gulbenkian de Ciência (IGC), Portugal, for providing basic understanding on the subject and clarifying conceptual incertitude during five day training from 23rd April to 27th April 2012 on *"Theory and Practical Training on Population and Conservation Genetics"*.

Further, I would also like to extend my sincere appreciation to Mrs. Rumi Deb Barman, Mr. Chatrapati Das, Dr. Anjan Kr. Talukdar, Mr. Biraj Saikia, Mr. Dipul Saikia, Mr. Anil Das, Ms. Rumana Maheen and all other colleagues in Aaranyak

who extended their help and support during the work. Thanks are also due to Mr. Arup Kr Das, Manager, Geo-spatial Technology and Application Division, Aaranyak and Mrs. Madhumita Borthakur for their help in preparing maps and in developing GIS based model during the study.

I would also like to express my gratitude to Mr. Mrinmoy Rajbongshi, Mrs. Jupitara Deka, Mr. Debaraj Baishya and Mrs. Monsumee Alam, who have always been a source of inspiration for me.

I am indebted to my parents, my brother, sister-in-law and my wife Ripshikha, for their immense support, love and inspiration throughout my research work. Lastly, but not the least, I would like to thank all who have directly or indirectly facilitated in the smooth completion of this work.

Pranjal Kr Das

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ABBREVIATIONS

ADO	Allelic dropout
AMOVA	Analysis of molecular variance
Ar	Allelic richness
CMR	Capture-Mark-Recapture
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dudhuwa National Park
EDTA	Ethylenediaminetetraacetic acid
F_0	First generation
FA	False allele
F_{ST}	Fixation index
GNP	Gorumara National Park
GPS	Global Positioning System
H_e	Expected heterozygosity
H_o	Observed heterozygosity
HWE	Hardy-Weinberg equilibrium
JNP	Jaldapara National Park
KNP	Kaziranga National Park
LD	Linkage Disequilibrium
MCMC	Markov Chain Monte Carlo

MCT	Microcentrifuge tube
MNP	Manas National Park
NA	Number of allele per locus
NaCl	Sodium Chloride
N_e	Effective population size
NGS	Noninvasive genetic sampling
ONP	Orang National Park
PA	Protected Area
PCR	Polymerase Chain Reaction
P_{ID}	Probability of identity
P_{ID}-sibs	Probability of identity among siblings
PP	Posterior Probability
PVP	Polyvinyl Polypyrrolidone
PWLS	Pabitora Wildlife Sanctuary
q	Percentage of membership value

CHAPTER 1
INTRODUCTION

The greater one-horned rhinoceros (*Rhinoceros unicornis)* is one of the largest free ranging mammals living on earth. It is characterized by a single large nasal horn and heavily built body, along with odd number of toes in fore and hind limbs, grey brown hide with skin folds and tubercles. Individuals of greater one-horned rhinoceros may live upto 70 years (Mukherjee, 1982). The adult male individuals of greater one-horn rhinoceros may weigh upto 2132 kgs while the weight of adult females may reach upto 1608 kg (Laurie et al., 1983). At maturity, the males attain height upto 6.3 ft while in case of the females upto 5.6 ft. The length of the horn of greater one-horned rhinoceros varies between 20 to 61 cm and may weigh upto 3 kg (Srivastav and Nigam, 2010). It is composed of keratin fibers, which remains epidermally connected to a bony knob on the head (Jha et al., 2015). However, the horn lacks bony structure at the center, unlike the true horns seen in other ungulate species (Sinha and Sinha, 2007).

The greater one-horned rhinoceros in general is solitary in nature. Individual rhinos are seldom seen in groups except for mother- calf pairs. Although, range exclusivity is observed in breeding males to a certain degree, no true territory is observed to be maintained by the individuals (Laurie et al., 1983). The greater one-horned rhinoceros mostly prefers riverine grassland habitats and swampy areas (Mukherjee, 1966; Laurie et al., 1983). They are most active during night,

particularly in the morning and evening hours, while they spend the remainder of the day resting in shades or wallowing. The greater one-horned rhinoceros typically feeds on grasses which may constitute upto 89% of the diet (Laurie et al., 1983) but are also known to browse on herbs and shrubs (Sinha and Sinha, 2007).

Since time immemorial, the greater one-horned rhinoceros has found its place in mythological stories, ancient literature, parietal art works as well as many popular beliefs (Briggs, 1931; Dutta, 1991; Nandagopal, 2007). In various folk tales that are prevalent in India, the greater one-horned rhinoceros has been linked to *Hindu Gods* such as lord *Vishnu,* lord *Krishna and* lord *Rama* (Dutta, 1991). The species was declared as a sacred animal in the *"5th pillar edict" of* Ashoka, the famous emperor of Maurya Dynasty. The "*Kalika Purana"* mentions about sacrificing rhinoceros to worship the *Godess Kamakhya or Kamakski*. There are also references of sacrifice of rhinoceros being offered by *Pandavas* in the great epic *Mahabharata.* Similar references of the species are also found in Chinese legends (Briggs, 1931). Artifacts' with inscribed rhinoceros motifs, which were recovered from archeological sites of Mohenjo- Daro and Harrapa, show the close association of the species with the human society of Calcolithic period in the Indian subcontinent (Rookmaaker, 2000). Similarly, depiction of greater one-horned rhinoceros in rock paintings dating back to Mesolithic and protohistoric period in India reflect the admiration of the species by human beings and its association with the human society in various forms (Manuel, 2007; Manuel, 2008).

1.1. The taxonomy, evolution and phylogeny of rhinoceros

The greater one-horned rhinoceros is one of the five extant species of the family Rhinocerotidae which include the black rhinoceros (*Diceros bicornis*), white rhinoceros (*Ceratotherium simum*), Javan rhinoceros (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*). The black rhinoceros and white rhinoceros are native to Africa while the Sumatran and Javan rhino along with greater one-horned rhinoceros are native to Asia.

The taxonomic status of greater one-horned rhinoceros is given below,

Kingdom : Animalia

Phylum : Chordata

Class : Mammalia

Order : Perissodactyla

Family : Rhinocerotidae

Genus : *Rhinoceros*

Species : *R. unicornis* L. 1758

The fossil and paleozoological records indicate that ancestors of present day rhinoceros species (super- family Rhinocerotoidae comprising of sister families Amynodontidae, Hyracodontidae and Rhinocerotidae) were abundant on earth since the middle of Eocene epoch (Prothero et al., 1986). Although, nasal or frontal horns are one of the distinguishing characters of the modern day rhinoceroces, most of the ancestors of the family Rhinocerotidae were, however,

hornless during the early stages of evolution as evident from primitive morphs such as *Hyracodon* and *Hyrachyus* (Woodward, 1898; Prothero et al., 1986). The members of the super- family Rhinocerotoidae gradually attained higher degree of speciation with more than 170 reported species representing 42 genera which inhabited the earth during different eras of geographical time scale. Although the fossil records of rhinocerotoids are well documented, there is still ambiguity over the phylogenetic relationship of the members of the super- family Rhinocerotoidae (Prothero et al., 1986). However, cladistic analysis of the living rhinoceros species and their nearest fossil relatives show a clear division between African rhino under the Tribe Dicerotini and Asian rhinos under Tribe Rhinocerotini comprising of sub tribes Rhinocerotina and Dicerorhinina respectively (Groves, 1983).

1.2. Greater one-horned rhinoceros conservation crisis

Historically, the greater one-horned rhinoceros inhabited the northern ranges of Indian subcontinent, starting from the Punjab foothills, Peshawar, Sind and lower Indus in the west to Northeastern India in the east (Rao 1947; Rookmaaker, 1980). Reports also suggested possible existence of the species in parts of Bangladesh, China and Burma (Tun Yin, 1956; Tun Yin, 1967; Rookmaaker, 1980). Even though, the species was once widely distributed all through the northern ranges of the Indian subcontinent, the population size of greater one-horned rhinoceros gradually started to diminish in number due to habitat destruction and indiscriminate hunting in various forms (Talukdar et al., 2008). In the beginning of the 20^{th} century, the species was at the verge of extinction, as the global

population size of the greater one-horned rhinoceros was reduced to only a few hundred individuals. It is also noteworthy, that the greater one-horned rhinoceros population size in Kaziranga was reduced to only 12 individuals during the period (Laurie et al., 1983). Although, the species was given protection under various wildlife protection acts since the beginning of the 20th century, continuation of illegal hunting posed a serious threat to the existence of the species. Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) in the year 1975 enlisted the greater one-horned rhinoceros under *Appendix- I* with the aim to restrict global trade on any sort of body parts of the species. In 1986, the species was included in the *Red List of Threatened Species* as 'Endangered' by International Union for Conservation of Nature (IUCN) considering the reduction in population size of the species worldwide. In the year 2008, IUCN reviewed the global status of the greater one-horned rhinoceros and included it to the vulnerable category of IUCN in the *Red List.* In India, the species is protected under Wildlife (Protection) Act, 1972 and is listed as a 'Schedule I' species. The population size of greater one-horned rhinoceros has increased in numbers in the last few decades as a result of effective conservation and management plans put in place. Nonetheless, habitat loss, illegal hunting and biotic pressures such as cattle grazing, extraction of forest resources and encroachment still remains a threat to the existence of the species.

1.3. Present status of greater one-horned rhinoceros in India

Presently, the greater one-horned rhinoceros is found only in isolated patches of protected areas in India and Nepal (Laurie et al., 1983). In India the species is

mainly distributed in seven protected areas situated in the states of Assam, West Bengal and Uttar Pradesh.

In Assam, the greater one-horned rhinoceros is found in four protected areas viz., the Kaziranga National Park, Orang National Park, Pabitora Wildlife Sanctuary and Manas National Park of Assam with estimated population sizes of 2401, 100, 92 and 32 individuals respectively (Rookmaaker et al., 2016). Apart from the Manas National Park, rest of the protected areas of Assam lie in the flood plains of the river Brahmaputra providing a favourable habitat for the species that are characterized by dry and marshy grasslands. Moreover, these protected areas are connected through a series of river islands of the river Brahmaputra which are often used by greater one-horned rhinoceros for movement (Talukdar et al., 2007). It is noteworthy that the natural population of Manas National Park was completely wiped out during 1990's and in an effort to reintroduce the species in Manas National Park under the initiative of "Rhino Vision 2020", a total of 25 individuals from Kaziranga National Park and Pabitora Wildlife Sanctuary were reintroduced in to Manas in a phase wise manner during 2008-2012.

In West Bengal, the greater one-horned rhinoceros are found in two protected areas viz., Gorumara National Park and Jaldapara National Park with estimated population size of 50 and 200 individuals respectively (Rookmaaker et al., 2016). Both the protected areas are located in the foothills of the Eastern Himalayas. The rhinoceros populations of West Bengal were once connected to Assam through Sankosh- Rydak region where rhinos existed till 1960's (Bist, 1994). However, most of the natural connectivity was lost due to clearing of forests in last six

decades restricting their movement. Notably, although the two rhinoceros bearing areas i.e., Gorumara and Jaldapara are located in close geographical proximity, yet, in recent years, there has not been any report on the movement of individual rhinos between these two protected areas which may have been influenced by human settlements and agricultural practices that separate the two areas.

To a greater satisfaction, the greater one-horned rhinoceros population of Dudhwa National Park has been raised from 7 individual rhinos (2 male and 5 females) that were translocated from Pabitora Wildlife Sanctuary, Assam and Royal Chitwan National Park, Nepal during 1984- 1985 (Sale and Singh, 1987) to 32 individuals at present (Rookmaaker et al., 2016)

1.4. Genetic status of greater one-horned rhinoceros

The conservation efforts over the years, to protect the fate of the greater one-horned rhinoceros mainly concentrated on protecting the habitats suitable for its persistence. Although, the greater one-horned rhinoceros population in India was revived from the brink of extinction, the potential effects of population size contraction (i.e., population bottlenecking) and habitat fragmentation on the genetic status of the species were not clearly understood. The remnants of the rhinoceros population in India have mostly remained in isolation for years and considering the present scenario of habitat fragmentation, it is possible that the existing rhinoceros populations will continue to remain in isolated patches. Prolonged separation may have its effects on the genetic status of the species in terms of genetic diversity and population differentiation. As a result of bottlenecking natural populations tend to lose their genetic diversity which

eventually reduces the adaptive capabilities of the population under the influence of various population processes (Lacy, 1987; Frankham, 1995; Hoelzel, 1999). Concurrently, the correlation of genetic diversity with population fitness has been demonstrated earlier by various authors (Koehn et al., 1988; Vandewoestijne et al., 2008; Markert et al., 2010). Besides, alterations in habitat configuration can also lead to the reduction in population size resulting in loss of genetic variability (Soule and Wilcox, 1980).

The process of genetic monitoring presents the scope of evaluating population genetic parameters using molecular markers (Schwartz et al., 2007). With the advancement of molecular techniques and statistical approaches in recent years, molecular markers systems have been extensively used to study the effect of inbreeding on populations, genetic drift, past bottlenecks, changes in effective population size, sex biased dispersal, founder effects, migration pattern, genetic differentiation and population structure (Hedrick, 2001; Frankham, 2010). The nuclear microsatellite markers are considered as one of the best marker systems used extensively in genetic monitoring of natural populations of wild animals (Brufford and Wayne 1993). The microsatellites are short tandem repeats of 1-6 nucleotides that are flanked by unique non-repetitive DNA sequences that are present throughout the genome of most organisms (Tautz, 1989; Selkoe and Toonen, 2006). Due to the highly polymorphic nature of microsatellite markers, they have been widely used to evaluate genetic diversity, past bottlenecks, genetic differentiation and population structure as well as tracing patterns of migration (Paetkau et al., 1995; Waits et al., 2000; Harley et al., 2005; Serrano et al., 2009; Vonholdt et al., 2010).

Above all, collection of samples of biological origin for monitoring natural populations, especially, free ranging mammals is a challenging task. Initially, when the concept of genetic monitoring came in to force, the destructive sampling methods were commonly used which resulted in the frequent killing of animals to obtain samples for scientific studies (Taberlet and Luikart, 1999). Gradually, with the advent of PCR technology and advancement in laboratory tools and techniques, the focus shifted from destructive sampling procedures to non destructive methods of sampling, although the involvement of animal capture was still prevalent (Taberlet and Luikart, 1999). In recent years, the noninvasive genetic sampling approach has provided an alternative to such unfavourable techniques which allowed biologists to obtain DNA from biological sources like feces, hair, shed skin, urine etc without capturing, handling or even observing the individuals (Taberlet et al., 1999). In 1992, several authors successfully demonstrated that noninvasive sampling technique can be used to obtain DNA from a variety of biological sources (Hoss et al., 1992; Taberlet and Bouvet, 1992; Morin and Woodruff, 1992). Since then, the method has been used extensively in genetic monitoring of a wide variety of wild animals including rhinos, royal bengal tiger, Asian elephants, brown bear, coyote, chimpanzee and snow leopard (Taberlet et al., 1997; Kohn et al., 1999; Vidya et al., 2005; Bhagavatula and Singh, 2006; Arandjelovic et al., 2011; Borthakur et al., 2011; Karmacharya et al., 2011; Borthakur et al., 2016).

In the above context, it is important to know the genetic status of greater one-horned rhinoceros as isolated populations of rhinoceros in India may become vulnerable to stochastic factors under the influence of which, the rhinoceros

population in India may decline in size or even bear the risk of extinction. Additionally, it is also important to define the population boundaries at spatial level for species like greater one-horned rhinoceros as it will help in determining the conservation/management units that need immediate attention. Although, genetic status of the greater one-horned rhinoceros was studied earlier (Zschokke et al., 2011; Das, 2014), yet, a comprehensive analysis of the genetic parameters at a landscape level, was not previously done. Henceforth, it was felt that a comparative study involving all rhino populations of India was pertinent and therefore, the present study focused on evaluating the contemporary level of genetic diversity, population structure, gene flow and effect of habitat fragmentation on gene flow of the greater one-horned rhinoceros populations of India, based on genetic data obtained from microsatellite markers following a noninvasive genetic monitoring approach.

1.5. Objectives

The objectives of the present work were to:

1. Evaluate the contemporary extent of genetic diversity of greater one-horned rhinoceros *Rhinoceros unicornis* in India.

2. Evaluate the population structure among the proposed study sites, using multilocus microsatellite genotyping of DNA obtained from dung samples.

3. Evaluate the effect of habitat fragmentation as a barrier to gene flow among the proposed study sites in India.

CHAPTER 2
REVIEW OF LITERATURE

2.1. Historical distribution and evidences of greater one-horned rhinoceros

The greater one-horned rhinoceros was a quite common animal in India until recent historical times (Bhaduri et al., 1972). The evidences available from archeological surveys, commentaries of travelers at different periods, autobiographies, hunting stories etc suggest that the historical distributional range of the species broadly extended from Indus valley in Pakistan in the west through the Ganges Valley and Terai region into the northeastern part of India in the east covering the entire northern region of the Indian sub-continent (Figure 1; Sclater, 1891; Blanford, 1891; Laurie et al., 1983; Rookmaaker 2002). The historical distribution of greater one-horned rhinoceros has been discussed by Rookmaaker in great detail (1980, 1884, 1999, 2000, 2002, and 2004).

It is apparent from the evidences from archeological surveys and ancient literatures, that the greater one-horned rhinoceros was a common species across Indus Valley and the mountainous terrains of East Afghanistan (Rookmaaker, 2000). During the excavations of ancient cities like Mohenjo-Daro and Harappa of the Indus Valley Civilization, objects such as seals carved with rhinoceros motifs and inscriptions, rhinoceros figurines and clay pottery models were recovered (Marshall, 1931; Lang, 1961; Bhaduri et al., 1972; Rookmaaker, 1998). Rhinoceros depicted in such items had a single nasal horn and well defined skin

folds that are characteristics of greater one-horned rhinoceros. This indicates that the species was well known to the people of Harappan culture which flourished during 2600-1900BC (Rookmaaker, 2000). This view is also strengthened by the discovery of right shoulder girdle fragment of greater one-horned rhinoceros at Harappan site (Prashad, 1931).

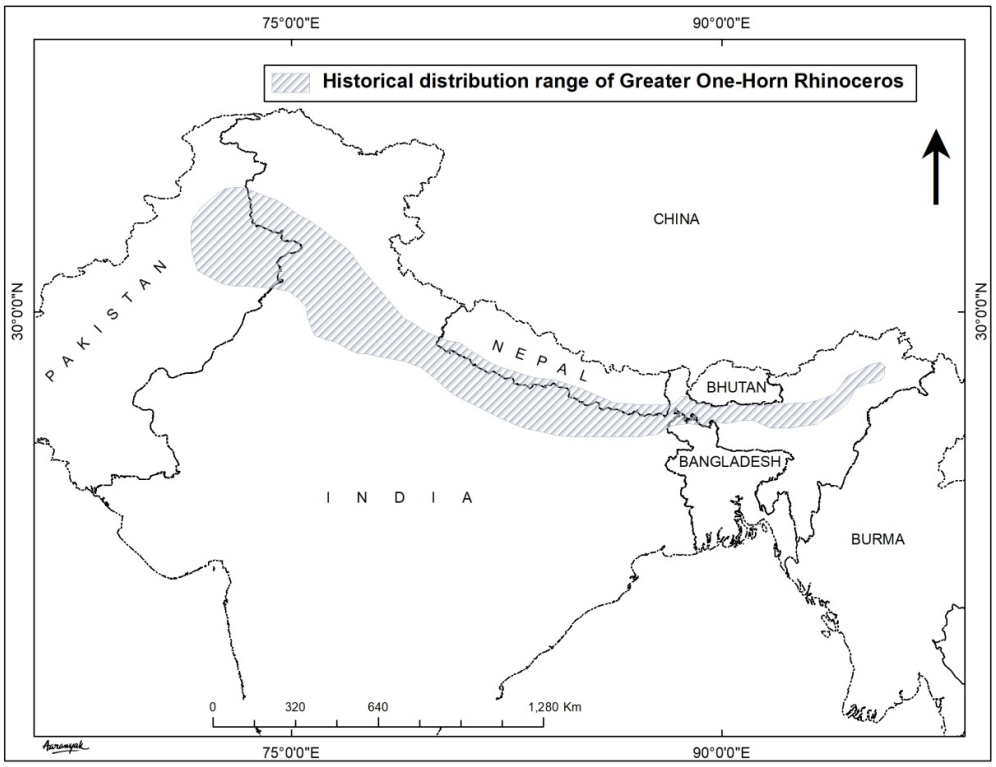

Figure 1: Historical distributional range of greater one-horned rhinoceros

Ibn Battuta, a Medieval traveler and scholar, who travelled to Sind from Kabul through Ghazni (a historical city in the central east part of Afghanistan) and then through the Sulaiman mountains (southern extension of the Hindu Kush mountain system) during 1325 to 1354 mentioned about encountering greater one-horned rhinoceros in the forests of lower Indus (Gibb, 1971). Also, Moghul emperor Zahiruddin Mohamed Babur (1483-1530) while illustrating his hunting

expeditions in his autobiographical work, the "*Baburnama*", mentioned about greater one-horned rhinoceros on the eastern bank of the river Indus and in the vicinity of Peshawar (Suleiman, 1970). He also provided a detailed description on physical characteristics, behavior and habitat of the species (Beveridge, 1922). Sidi Ali Reis, son of Husein Reis, Steward of the Imperial Arsenal in Turkey who travelled through Pakistan, Afghanistan, Iran and Azerbaijan, reaching Istanbul via Baghdad in 1556 mentioned about sighting of greater one-horned rhinoceros in his book "*Miratul Memalik"* (Vambery, 1899). The location described by Reis may be located in the present-day Afghanistan (Rookmaaker 2000). The great Mongol conqueror Timur (1336- 1405), the founder of the Timurid Empire hunted greater one-horned rhinoceros along the Kashmir frontier in 1389 (Yule and Burnell, 1903; Guggisberg, 1966; Dutta 1991; Rookmaaker, 2002)

During archeological excavations carried out between the years 1942 to 1963 at Langhnaj, Mehsana district in the state of Gujarat, two scapulae of greater one-horned rhinoceros were recovered (Clutton-Brock, 1965). Remains of greater one-horned rhinoceros were also recovered from Lothal, a notable Harappan civilization site in the state of Gujarat, indicating its historical distribution in the region (Nath, 1968; Nath and Rao, 1985). Momin recovered semi fossilized fragments of cervical vertebrae of greater one-horned rhinoceros from Khaksar in Anand district and Valotri village of Kheda district in Gujarat along with piece of tibia from Kaneval Lake situated along north-west of Cambay taluka in Gujarat during archeological exploration carried out between 1972-73 by the Department of Archaeology and Ancient History of Maharaja Sayajirao University of Baroda (Momin et al., 1973). Similarly, Banerjee and Chakraborty (1973) reported

skeletal fragments of greater one-horned rhinoceros from excavation site of Kalibangan of Ganganagar district in the state of Rajasthan. Fossilized and semi-fossilized remains of greater one-horned rhinoceros were also recovered from Narbada valley and Madras (Guggiesberg, 1966).

The greater one-horned rhinoceros inhabited the flood plains of the river Ganga till early part of the 19th century (Rookmaaker, 2002). Jean-Baptiste Tavernier, a French gem merchant and traveler mentioned about greater one-horned rhinoceros from Gianabaad situated about 50km south of present day Kanpur (Ball, 1889). British artists Thomas Daniell and William Daniell who travelled from Calcutta to Delhi through the Ganges between 1788 and 1791 saw footprints of greater one-horned rhinoceros in the year 1788 near Moti Jharna, about 21 miles from Rajmahal (Archer, 1980). Cockburn (1883) in the year 1881 discovered fossil remains of greater one-horned rhinoceros during a hunting expedition in the bank of the river Ken near Banda. He also described petroglyphs from Ghormangur Cave near Bidjeygurh fortress in Mirzapur district of Uttar Pradesh (Cockburn, 1883). A hunting excursion report published in the *Asiatic Journal and Monthly Register for British India and its Dependencies* mentioned about the killing of a greater one-horned rhinoceros by Capt. Brook of Commissariat Department in 1820, near Seerkunda, Rajmahal Hills (Annonymous, 1821). The *Catalogue of Mammalia* in the Indian Museum, Calcutta listed a sample of male greater one-horned rhinoceros from Purneah District of Bihar which was collected in the year 1871 by G. W. Shiilingford (Sclater, 1891). According to Baker (1887), greater one-horned rhinoceros roamed along the "churs" of Koasee river in Purneah and Sikrigully, near Rajmahal Hills till early 19th century. Sightings of the animal

were reported from Champaran and Saharsa districts of Bihar during mid 19th century (Mukherjee, 1963). Manners-Smith (1909) reported the sighting of greater one-horned rhino along the banks of river Bagmati in north of Muzaffarpur in Bihar.

The greater one-horned rhinoceros was a common species in Nepal. Although evidence for the historical distribution of greater one-horned rhinoceros in Nepal is limited, the available resources indicate that the species was abundant in today's Chitwan National Park and its adjoining areas (Rookmaaker, 2004). Hodgson (1834) in his paper published in the Proceedings of Zoological Society of London listed greater one-horned rhinoceros as an abundant species in the Terai regions of Nepal. Oldfield (1881) mentioned the abundance of rhinoceros in the high, dense grasslands along the Rapti river valley and Chitwan, Nepal. Flower (1884) listed three specimens of greater one-horned rhinoceros from Nepal Terai, that were preserved in the museum of the Royal College of Surgeons of England, London, which was presented to *His Royal Highness* the Prince of Wales in the year 1876 by the then Nepalese Ambassador, Sir Jung Bahadur. Blanford (1891) mentioned rhinoceros inhabiting the Terai of Nepal. Manners-Smith (1909) mentioned about greater one-horned rhinoceros in the Terai regions of Nepal, Morang, banks of river Koshi, Bagmati, and in the Chitwan and Naolpur valleys along the banks of the river Gandak and the Rapti in Nepal.

One of the earliest evidences of greater one-horned rhinoceros inhabiting in the state of West Bengal comes from the map of Bengal developed by Colonel Jean-Baptiste-Joseph Gentil, the official French agent in the court of Awadh, during

1767 to 1777 (Rookmaaker, 2014). The map contains an image of greater one-horned rhinoceros drawn on the north-eastern border of the state. Rookmaaker (2014) argued that, although the images of rhinoceros in the map might be merely decorative, it is interesting to note that the image appear only on the map where rhinoceros possibly existed. Hunter (1875-1876) mentioned greater one-horned rhinoceros occurring commonly in the districts of 24 Parganah, Sunderbans, Terai sub-division of Darjeeling district, Jalpaiguri and Cooch Behar. Although, not common, rhinoceros were occasionally seen in the Maldah District of West Bengal (Hunter, 1875-1876). Jentink (1887, 1892) in his catalogue of mammals in the Museum Naturalis, Leiden, Netherland, listed a skull of female greater one-horned rhinoceros which was collected by Frank in the year 1852 from Bengal. Maharaja of Cooch Behar, Nripendra Narayana Bhupa Bahadur, organized several hunting expeditions in the forest areas of Cooch Behar and hunted around 207 greater one-horned rhinoceros during 1871 to 1905 (Nripendra, 1908). Bladwin (1877) reported sighting of greater one-horned rhinoceros in the year 1865 between Bala and Buxa in West Bengal.

Although there are less literary resources enumerating presence of greater one-horned rhinoceros in Bhutan, the available evidences indicate that the species was also found in the foothills of Bhutan (White, 1909; Rookmaaker, 1980). Beavan (1865) reported rhinoceros being found in the forests of Bhutan. Baldwin in 1877 mentioned greater one-horned rhinoceros inhabiting the forests of Bhutan. Hobley (1932) mentioned about greater one-horned rhinoceros being found in Bhutan.

Greater one-horned rhinoceros was known to the people of Assam from ages. The species finds its place in "*Kalika Purana"* an ancient religious book (ca. 10th century) written probably in Kamrupa (modern Assam). It is considered as one of the 18 Upapuranas dedicated to worship of the *Goddess Kali*, especially *Goddess Kamakhya or Kamakski* one of the manifold incarnations of *Kali*. The book describes sacrifice of rhinoceros as a form of worshiping *Goddess Kamakhya* (Wilkins, 1882). The description of the ritual also finds place in the notes of Jean-Baptiste Chevalier, the French Governor of Chandemagore (1767-78) who travelled to the forbidden kingdom of Assam long back in 1755, which was then ruled by the King Rajeshvara Singha, the 33rd King of the Ahom Dynasty. The translations of his memoirs mentions sacrifice of rhinoceros along with other wild animals such as elephants, tiger, and buffalos at the Kamakhya temple on the Nilachal Hills (Deloche, 2008). Between 1746 and 1758, a greater one-horned rhinoceros which was popularly known as *"The Dutch Rhinoceros"* was displayed in many cities of Europe. It was captured in 1738 in Assam and was brought to Netherland in the year 1741 by its owner Douwe Mout van der Meer (Rookmaaker et al., 1998). According to Major James Rennell, the First Surveyor-General of India, the greater one-horned rhinoceros was abundant in the vicinity of the Goalpara district of Assam (La Touche, 1910). Horsfield (1851) in his catalogue of the Mammals in the Museum of the Hon. East India Company, London, listed a greater one-horned rhinoceros horn which was hunted in Goalpara District in the year 1777 by Mr. Thomas Craigie. M'Cosh (1836a, 1836b) in his illustration on fauna of Assam mentioned the species inhabiting dense and inaccessible forests of Assam. Butler (1847), a Major in Bengal Native

Infantry, in his account mentioned about greater one-horned rhinoceros being distributed in high grass forests and swampy areas of Assam. Baldwin (1877) reported hunting of the greater one-horned rhinoceros in the vicinity of Tezpur where the species was abundantly found during that period. Hunter (1879) in his compilation *"Statistical Account of Assam"* mentioned the species as one of the common wild animal inhabiting the forests of Assam. Barker in 1884 in his book "A Tea Planter's Life in Assam" mentioned greater one-horned rhinoceros being abundant in the *"out-of-the-way districts"* of erstwhile Assam.

Rookmaaker (1980) has discussed distribution of greater one-horned rhinoceros from Bangladesh, China and Indo-China regions. He suggested that the species might have existed in the low lands of Nasirabad and Sylhet in Bangladesh. However, to him, records of greater one-horned rhinoceros from China and Indo-China region (Tun, 1956; Tun, 1967) are questionable (Rookmaaker, 1980).

2.2. Present distribution of greater one-horned rhinoceros

With the increase of human population since late 15th century the alluvial plain grasslands of northern Indian subcontinent were gradually encroached for cultivation (Laurie, 1978). Incidentally, change in habitats of the greater one-horned rhinoceros due to clearing of forests for agricultural practices along with rampant hunting in various forms has led to rapid decline in population size of the species from most of its historical distributional range in the last six hundred years (Guggiesbern, 1966; Laurie and Olivier, 1977; Laurie, 1978; Laurie, 1984; Menon, 1996; Rookmaaker et al., 2016). Besides, during the pre independence era

of India the British Government had placed reward to kill crop raiding rhinos, which further deteriorated the fate of the species (Ellis, 2012).

Presently, the population of greater one-horned rhinoceros is restricted to isolated patches of protected areas (PAs) of India and Nepal. These include the Kaziranga National Park (429.93 km^2), the Orang National Park (78.8 km^2) and the Pabitora Wildlife Sanctuary (38.81 km^2), the Manas National Park (500 km^2) in the state of Assam, the Jaldapara National Park (216.6 km^2) and the Gorumara National Park (79.99 km^2) in the state of West Bengal and the Dudhwa National Park (490.29 km^2) in the state of Uttar Pradesh in India while such protected areas of Nepal include Chitwan National Park (932 km^2), Bardia National Park (968 km^2) and Shukla Phanta Wildlife Reserve (305 km^2).

2.3. Population trends and greater one-horned rhinoceros conservation and management efforts

Although, population of greater one-horned rhinoceros was fast diminishing and was restricted to only a few isolated patches, yet, hunting was still rampant till early part of the 20th Century. The Maharaja of Cooch Behar, Nripendra Narayana Bhupa Bahadur hunted more than 200 individual rhinos in the vicinity of Cooch Behar during 1871 to 1905 (Nripendra, 1908). Col. Pollock, a Military Engineer shot a rhino or a wild buffalo before breakfast every day (Dey, 2000). Such incidents resulted in dwindling of the greater one-horned rhino population which was at the verge of extinction during early part of the 20th century with the global population size reduced to only few hundred individuals (IUCN; Zschokke, 2016).

It is to be mentioned that various laws have been enacted since late 19th century in India with the aim to save the species from extinction. In 1891, Assam Forest Regulation was introduced as an extension of the Indian Forest Act, 1878 which offered legal protection to the greater one-horned rhinoceros. However, the act was later repealed as it was only related to the Assam province. Hunting of rhinoceros was subsequently banned making it an illegal practice in India in the year 1910 (Laurie, 1978; Menon, 1996; Ellis, 2012). The population size of greater one-horned rhinoceros in Kaziranga at that point of time was to only 12 individuals (Laurie, 1978). In order to conserve rhinos inhabiting in Kaziranga, it was designated as a Reserve Forest in the year 1908. In the year 1916, Kaziranga Reserve forest was converted to a Game Reserve and then to a Wildlife Sanctuary in 1950. In the year 1974 it was recognized as a National Park. In the year 1915, the "Assam Rhinoceros Prevention Act" was enacted, which was later, upgraded to "Assam Rhinoceros Act", 1954 that prohibited killing, injuring and capturing of the animal. In 1927, the "Indian Forest Act" was re-enacted to regulated laws related to forests and forest-produce which provided some protection to the greater one-horned rhinoceros in India along with other wildlife. Presently, the species is listed as Schedule I species under the "Wildlife Protection Act" of India, 1972.

Although, the population size of greater one-horned rhinoceros of Kaziranga during early 20th century has been a subject of debate (Zschokke et al., 2011), but, the implementation of effective conservation action plan has resulted in a steady growth of its population in Kaziranga National Park in the past century and has successfully made possible for Kaziranga to harbor the largest population of

greater one-horned rhinoceros in the world with estimated size of 2401 individuals (Rookmaaker et al., 2016).

In 1971, the Government of Assam converted Pabitora Grazing Reserve to a Wildlife Reserve and subsequently to a Wildlife Sanctuary in 1987 in an effort to protect the species inhabiting the area (Talukdar, 1999). Since 1987, the rhino population in Pabitora Wildlife Sanctuary has increased from an estimated number of 54 individuals to 92 individuals as per 2015 census report (Talukdar and Sarma, 2007; Rookmaaker et al., 2016).

According to Forest Department estimates, Orang Game Reserve was inhabited by 35 rhinoceros individuals in 1972. In 1985 Orang's status was upgraded to a Wildlife Sanctuary and then to a National Park in 1999. Until 1991 the trend of rhinoceros population in Orang was on rise. However, 1999 estimates show a sharp decline (>50%) in number (Hussain, 2001; Talukdar and Sarma, 2007; Momin, 2008). However, the population has gradually revived with a present population estimate of 100 individuals (Rookmaaker et al., 2016).

Although, Kaziranga, Pabitora and Orang represent a rhino conservation success story, sadly, the Manas National Park and Laokhowa Wildlife Sanctuary, the two other major rhinoceros bearing areas of Assam, lost the whole population during civil unrest between early 80's to 90's of the 20th century (Menon, 1996; IUCN).

The greater one-horned rhinoceros populations of West Bengal have been showing a continuous fluctuating trend during past 100 years (Bist, 1994). It is believed that, in the beginning of the 20th century the number of greater one-

horned rhinoceros individuals in West Bengal varied between 40 to 50 individuals (Dey, 2000). Similar to the state of Assam, the "Bengal Rhinoceros Preservation Act" was introduced in the state of West Bengal in the year 1932 barring killing, injuring and capturing of rhinoceros. However, as a result of continued habitat loss and poaching the species became locally extinct in many areas within the state (Malik, 2015; Bist, 1994). During 1960's to 1980's the Jaldapara and Garumara population of the species recorded a sharp decline in number due to increased poaching activities (Bist, 1994). Fortunately, the number has increased in both the protected areas since 1986 and has remained steady for last one decade (Bist, 1994; Rookmaaker et al., 2016). Bist (1994) has documented the population trend of the greater one-horned rhinoceros in northern parts of West Bengal in detail.

The Indian Board of Wildlife in 1979 constituted a committee to evaluate the status of rhinoceros conservation and possible reintroduction within historical distributional range in India (Sale and Singh, 1987). Based on the committee recommendations, nine individuals (five from Assam and Four from Nepal) were reintroduced in Dudhuwa National Park in the state of Uttar Pradesh (Sale and Singh, 1987). Although, two of the individuals translocated to Dudhuwa died, nonetheless, the population in last three decades has shown an increasing trend with an estimated size of 32 individuals (Rookmaaker et al., 2016). In a similar effort, rhinos were phase wise reintroduced in the Manas National Park of Assam in a phase wise manner since 2008 under Indian Rhino Vision 2020, a joint initiative of Assam Forest Department, the Bodoland Territorial Council, World Wide Fund for Nature (WWF), International Rhino Foundation (IRF) and the US

Fish and Wildlife Service which was initiated in 2005 (Rookmaaker et al., 2016). Although, the numbers of individual rhinos have increased in Manas National Park to 32 individuals, during 2011 to 2013, six individuals have been killed by poachers (Rookmaaker et al., 2016). On March 29th 2016, two female rhinoceros (a mother and a calf) were translocated to Burachapori Wildlife Sanctuary from Kaziranga National Park. Unfortunately, the mother died on 22nd May 2016 due to unknown reasons.

In 1989, three rhino individuals from Bardia National Park in Nepal got settled in Karteniaghat Wildlife Sanctuary, Uttar Pradesh (Sinha and Singh, 1999). The population size of Karteniaghat in 1997 was estimated to be four (Foose and van Strien, 1997) but according to 2015 estimate the number has reduced to two (Rookmaaker et al., 2016). Talukdar and Sinha (2013) reported movement of four rhino individuals from Chitwan National Park, Nepal to Valmiki Tiger Reserve, Bihar, India. However, the number of rhino individual in Valmiki Tiger Reserve as of 2013 stand at two (Talukdar and Sinha, 2013).

Until 1950, the greater one-horned rhinoceros was well protected in Nepal due to restrictions on hunting under the then Rana regime (Thapa et al., 2013). The population size of greater one-horned rhinoceros in Nepal was believed to be between 300-400 individuals (Smythies, 1942). According to the Nepal forest department estimate, the population size of the species was 1000 individuals in 1953 which got reduced to 600 individuals in 1957 (Gee, 1959). However, Stracey's (1957) records suggest that the numbers were not more than 300 individuals. Interestingly, Gee estimated 300 individual rhinoceros in the year

1959. In the same year the Nepal Government constituted "*Gainda Gasti*", a task force for protection of the species. At the same time, due to continued poaching activities the population dwindled to less than 100 individuals in 1960's and was restricted to only the Chitwan valley (Adhikari et al., 1999). In an effort to conserve the greater one-horned rhinoceros and other wildlife species "*National Park and Wildlife Conservation Section*" was created within the Forest Department of Nepal in the year 1971 and Chitwan was formally declared as the first National Park in Nepal in the year 1973. The Nepal Government in 1975 handed over the responsibility to protect rhinos in Chitwan National Park to the Nepal Army. Since 1960's the rhinoceros population in Chitwan gradually revived with current population size of 605 individuals (Rookmaaker et al., 2016). In an effort to establish new founder population of the species, the Government of Nepal initiated Rhino Action plan in Nepal and eventually, a total of 87 individual rhinoceros were translocated to Bardia National Park and Suklaphanta Wildlife Reserve between 1986 to 2003 (Thapa et al., 2013; Rookmaaker et al., 2016). At present the population sizes in Bardia and Suklaphanta stands at 29 and 8 individuals respectively (Rookmaaker et al., 2016).

2.4. Genetic monitoring of wild populations

Extinction is a natural process, but, the rate at which species are disappearing from the earth as a result of the direct or indirect consequences of anthropogenic activities is of grave concern and calls for effective conservation and management strategies to protect the worlds' biodiversity from extinction. The efforts for conservation of biodiversity, in general, emphasizes conserving ecological

diversity at large which facilitate conservation of species in their natural habitats and thus genetic diversity (Lundqvist et al., 2008). Protecting natural habitat, however, may not be sufficient to protect a species and require genetic monitoring to strategize effective conservation action plan (Aravanopoulos, 2011). Thus, genetic monitoring can be defined as the '*quantification of temporal changes of population genetic parameters using molecular markers'* (Schwartz et al., 2007).

Recent advances in laboratory techniques, molecular approaches (Luikart et al., 2003; Miquel et al., 2006) and statistical tools (Piry et al., 2004; Beaumont and Ranala, 2004; Excoffier et al., 2005; Evanno et al., 2005) have enabled conservation biologists to use genetic tools to answer questions pertaining to the conservation and management of wild animals (Haig, 1998; Hedrick, 2001; Deyoung and Honeycutt, 2005; Schwartz et al., 2007; Stetz et al., 2011). Schwartz and his colleagues classified genetic monitoring into two categories; firstly, identifying individuals, populations and species using molecular markers and secondly, monitoring of genetic parameters of a population such as genetic variation, admixture, migration, effective population size etc. (Schwartz et al., 2007).

2.4.1. Molecular markers for genetic monitoring

Allozymes were the first molecular markers to be used in population studies (see Schlotterer, 2004). These have been widely used in monitoring natural populations of several species (Hubby and Lewontin, 1966; Lewontin and Hubby, 1966; O'Brien et al., 1985; Slip et al., 1985; Reusing et al., 2011). Although, widely used, the allozyme markers are often criticized for being an indirect

method to assess polymorphism because of its lack of ability to detect mutations at the level of DNA (Schlotterer, 2004). This led to a shift, towards the use of DNA based markers such as mitochondrial DNA (mtDNA) based markers, Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD), Microsatellites, Single Nucleotide Polymorphism (SNP) etc., in place of enzyme based markers in population genetic studies (Schlotterer, 2004).

The microsatellite marker (or simple sequence repeats) in recent years has emerged as one of the most reliable DNA based molecular marker systems. Microsatellites represent short tandem repeats of 1-6 nucleotides flanked by unique non-repetitive DNA sequences that are present throughout the genome of most organisms (Tautz, 1989; Selkoe and Toonen, 2006). Typically, microsatellites vary between 5 to 40 repeats in length with di-, tri- and tetra-nucleotide repeats (Selkoe and Toonen, 2006). The microsatellite markers are co-dominant, highly polymorphic, easily detectable, and follow Mendelian inheritance, making them highly suitable for genetic studies (Queller et al., 1993; O'Connell and Wright, 1997). These have been extensively used in genetic monitoring of natural populations of wild animals (Paetkau et al., 1995; Waits et al., 2000; Harley et al., 2005; Vidya et al., 2005; Borthakur et al., 2011; Tende et al., 2014; Borthakur et al., 2016). The references cited above give an idea of the wide use of microsatellite markers in population genetic studies in recent times.

2.4.2. Traditional population monitoring and study of demographic parameters

Molecular markers have been successfully used in identification of individuals, species and other taxonomic groups, to estimate population sizes, monitor population turnover, study relationships within the individuals of a population and even identify hybrids between closely related species (Schwartz et al., 2007). Alacs et al., (2003) used mtDNA based markers in successful identification of quokka (*Setonix brachyurus*) from other sympatric macropod species. Piggott et al., (2006) estimated the population size of brush-tailed rock-wallaby (*Petrogale penicillata*) following a capture-mark-recapture (CMR) analysis using data on individuals identified through microsatellite markers. Similarly, Borthakur et al. (2011) estimated population size of tiger (*Panthera tigris*) using single session CMR analysis in Orang National Park, Assam. Neaves et al., (2010) using mtDNA as well as microsatellite markers, detected a rare event of introgressive hybridization between sympatric species of grey kangaroos, viz., western grey kangaroo (*Macropus fuliginosus*) and eastern grey kangaroo (*Macropus giganteus*).

2.4.3. Study of population genetic parameters

Application of molecular marker systems often gives a better understanding of the evolutionary process acting upon natural populations of wild animals in comparison to the traditional methods used for population studies (Hedrick and Miller, 1992; Hedrick, 2001; Schwartz et al., 2007). The selection of appropriate marker system along with proper statistical approach and suitable parameter for

evaluating evolutionary processes, however, plays a key role in drawing inferences that are relevant to conservation (Wan et al., 2004).

2.4.3.1. Genetic diversity in natural populations

Genetic diversity is one of three key components of biological diversity along with ecological diversity and species diversity (McNeely et al., 1990). It refers to the total genetic information present in the gene pool of a species (McNeely et al., 1990). Loss of genetic diversity reduces adaptive capabilities of a species, thereby, increasing the risk of extinction from events of stochastic population changes (Soule and Wilcox, 1980; Frankham, 1995; Saccheri et al., 1998; Ebert et al., 2002). The extent of genetic diversity present in natural populations of a species is, typically, measured in terms of polymorphism, average heterozygosity and allelic richness (Frankham et al. 2004, Dyke, 2008). Polymorphism represents the occurrence of more than one variant (or allele) at a locus in the population. Heterozygosity corresponds to the proportion of individuals that are heterozygous at a locus. In population studies, heterozygosity in general, is represented in terms of expected heterozygosity which corresponds to the expected proportion of heterozygotes present at a locus (Frankham et al. 2004). Allelic richness, which measures the number of alleles that are present per locus, is also a suitable estimator of genetic diversity present in a population (Petit et al, 1998).

2.4.3.2. Small populations, inbreeding and genetic drift

Populations having a small size or those that are declining in number are often vulnerable to extinction in comparison to large and stable populations (Lacy,

2000; Frankham et al. 2004). The viability of small populations is affected by two factors viz., inbreeding and genetic drift (Lacy, 1997).

Inbreeding occurs when closely related individuals mate with each other. Although, it has been argued by many authors that the effect of inbreeding is only transient in natural populations, yet, studies have shown that genetic variability is reduced as a consequence of inbreeding in natural population and can affect population viability in an irreversible manner (Lacy, 2000; Frankham et al., 2004; Dyke, 2008). Inbreeding is typically measured in terms of *inbreeding coefficient*. Inbreeding coefficient can be estimated in terms of increase in homozygosity as a result of mating between closely related individuals, also known as *pedigree inbreeding coefficient (F)*. Ellegren (1999) estimated, *F* in a captive Scandinavian grey wolf population with known pedigree using 29 microsatellite markers. Presence of inbreeding can also be determined in terms of nonrandom mating among individuals of a local population (Keller and Waller, 2002). In such instances inbreeding is typically measured through fixation index F_{IS}, which represents the deviation of heterozygosity observed in an individual relative to expected heterozygosity under random mating (i.e., Hardy–Weinberg equilibrium). Keller and Waller (2002) have reviewed the effects of inbreeding and the various approaches used to measure inbreeding in wild population.

Genetic drift is a random shift in allele frequencies occurring in a population over successive generations (Allendorf and Luikart, 2007). Genetic drift may have deleterious effects such as loss of genetic variability, fixation of allele and population differentiation (Frankham et al., 2004; Dyke, 2008). As a consequence

of genetic drift, alleles, especially the rare alleles, are lost from small, isolated population resulting in reduction of overall genetic variability of the population (Dyke, 2008).

2.4.3.3. Population differentiation, migration and gene flow

At a metapopulation level, it is often observed that natural populations of a majority of species are subdivided into local, randomly mating subpopulations or demes (Allendorf and Luikart, 2007). For designing effective conservation action plans, it is important to understand the pattern and the extent of structuring among subpopulations. It has been observed that delineating population boundaries in many species is often difficult (Manel et al, 2005). Natural populations of wild animals are often defined based on prior assumptions on sampling locations. However, such definitions do not necessarily correspond to the natural population boundary in genetic terms (Pritchard et al., 2000). In the last two decades, a number of statistical tools based on Bayesian analysis and maximum likelihood approach have been developed to evaluate population structure using molecular marker data (Paetkau et al., 1995; Rannala and Mountain, 1997; Cornuet et al., 1999; Pritchard et al., 2000; Dawson and Belkhir, 2001; Corander et al., 2003; Guillot et al., 2005). Moreover, the degree of differentiation among subpopulations depends on a number of factors that include genetic drift, mutation, migration and effective gene flow between subpopulations and interactions between such factors over time (Marko and Hart, 2011). Over the years, effort has been given by various authors to develop statistical tools which can measure population differentiation using data obtained from molecular

markers. Population differentiation is often assessed in terms of *fixation index* (F_{ST}), which represents heterozygosity deficit relative to the heterozygosity expected under Hardy-Weinberg proportion in a panmictic population (Allendorf and Luikart, 2007). The concept of *fixation index* was first developed in the year 1921 by Wright to explain effect of inbreeding, but later, was extended to population subdivision (Wright, 1951). Subsequently, *fixation index* has been redefined by various authors (Nei, 1977; Cockerham and Weir 1987; Hartl and Clark, 2006). Simultaneously, similar to F_{ST}, a number of other estimators such as G_{ST}, Φ_{ST}, AMOVA and R_{ST} have also been derived to assess degree of genetic differentiation among subpopulations of a species (Nei, 1973; Excoffier et al., 1992; Slatkin, 1995). In recent years, assignment based methods have also been used by various authors to evaluate genetic differentiation along with traditional estimators (Waits et al., 2000; Hamill et al., 2007; Giraldo et al., 2008).

Gene flow brings about genetic homogeneity among populations of a species, thus, reducing the degree of differentiation. Moreover, the genetic variation within subpopulations of a species increases as a result of gene flow, thereby, counter the effect of genetic drift (Allendorf and Luikart, 2007). The rate of gene flow among populations of a species may be estimated in terms of average migration rate (*Nm*) by calculating difference in allele frequency distribution among populations assuming conditions of symmetrical migration among populations and identical population size. However, such conditions rarely hold in natural populations (Allendorf and Luikart, 2007). Rannala and Mountain (1997) presented a likelihood based method for detecting migrants and migrant ancestry using genotypic data on multiple loci. Subsequently, a number of estimators have been

developed by various authors based on maximum likelihood or Bayesian analysis to estimate migration rate and detect migrants between populations (Beerli and Felsenstein, 2001; Pritchard et al., 2000; Wilson and Rannala, 2003).

2.4.4. Study of population fragmentation

In recent times, habitat fragmentation due to anthropogenic activities is one of the major threats to the world's biodiversity. As a consequence of habitat fragmentation, large areas of suitable habitat of an organism are transformed into smaller patches that are isolated by matrix of unsuitable habitats (Wilcove et al., 1986). It has been argued my many authors that, various population processes are often more complex than thought and are affected by the structure of landscape mosaic where the population occur (Dunning et al., 1992; Wiens, 1997). Habitat fragmentation may have serious implications as small, isolated populations tend to lose genetic variability under the influence of factors such as genetic drift and inbreeding. Furthermore, fragmentation may act as a dispersal barrier, thereby restricting gene flow between isolated patches, which could result in genetic differentiation (Gerlach and Musolf, 2000). In recent years landscape genetics has emerged as a promising tool in the field of population genetics which allows the study of the interactions between evolutionary processes like gene flow, genetic drift and landscape features including landscape composition and matrix quality (Manel et al., 2003; Holderegger and Wagner, 2006). The general approach is to determine the spatial genetic pattern present in a species and find its correlation with landscape features (Manel et al., 2003). Most studies related to effect of landscape features on gene flow involves modeling hypothesis of

landscape permeability over a cost surface to estimate landscape distances among subdivided populations and evaluate the relationship between landscape distance with observed genetic distance using test statistics such as Mantel's test or partial Mantel's test (Manel and Holderegger, 2013).

2.5. Noninvasive genetic tools in conservation biology

Noninvasive genetic sampling (NGS) has been a method of choice for biologist for collection of samples in studies related to genetic monitoring of population in the last 25 years (Waits and Paetkau, 2005). The method was first developed in 1992 for obtaining genetic material from rare and elusive animals such as brown bear (*Ursus arcto;* Hoss et al., 1992; Taberlet and Bouvet, 1992) and chimpanzees (*Pan troglodytes*; Morin and Woodruff, 1992). NGS allows biologists to obtain DNA from a variety of biological sources which include feces, hair, feathers, skin, saliva etc (Waits and Paetkau, 2005).

Feces are a good source of genetic material for genetic studies of wild population. They contain mucosal cells shed from intestinal lining allowing isolation and analysis of DNA of the host organism (Waits and Paetkau, 2005). DNA obtained from feces has been successfully used in various studies which include identification of species (Palomares et al., 2002; Alacs et al., 2003), individuals, sex and estimation of population size (Hansen and Jacobsen, 1999; Kohn et al., 1999; Murakami, 2002; Kurose et al., 2005; Borthakur et al., 2011; Liu et al., 2014), determination of home range and territory size (Bischof et al., 2016), evaluation of genetic diversity (de Barbara et al., 2010; Sugimoto et al., 2014; Wang et al., 2015; Goossens et al., 2016), effect of inbreeding (Liberg et al.,

2005), effects of bottlenecking (Russello et al., 2004; Nyström, 2006), estimation of effective population size (Diefenbach et al. 2015), gene flow (Bergl and Vigilant, 2007; Schregel et al., 2012; Baden et al., 2014), genetic differentiation and population structure (Vidya et al., 2005; Dalén et al., 2006, Munshi-South, 2011; Nater et al., 2013).

Various studies have shown that the quantity of DNA obtained from noninvasively collected samples largely depend on the preservation and storage methods (Wasser et al., 1997; Piggott and Taylor, 2003). Although, different methods of sample storage have been proposed, yet, no method has been observed to be superior to the others (Waits and Paetkau, 2005). However, optimal storage can be obtained through silica desiccation, emersion in ethanol or other storage buffer such as DMSO-EDTA-Tris-Salt (DETs) buffer (Wassere et al., 1997; Frantz et al., 2003; Frantzen et al., 1998). Moreover, quality of extracted DNA may also depend on environmental factors, age of sample, interactions between storage and DNA extraction procedures and so forth (Piggot, 2004; Frantz et al. 2003). Additionally, noninvasively collected samples are often prone to contamination (Taberlet et al., 1999). Therefore, it is pertinent that while working with such samples one has to be cautious and take special precautions in order to avoid contamination during field sampling and also performing laboratory analysis to obtain reliable data (Waits and Paetkau, 2005).

2.6. Greater one-horned rhinoceros conservation genetics

Genetic studies on greater one-horned rhinoceros are rather limited with very few comparative reports available that involve wild populations. Moreover sample

sizes in many of these studies were either low (Merenlender et al., 1989; Morales and Melnick, 1994; Ali et al., 1999) or restricted to a single population (Dinerstein and McCracken, 1990) which may often lead to wrong interpretation of the underlying population processes.

Merenlender et al., (1989) studied allozyme variation in rhinoceroces including the greater one-horned rhinoceros, the African northern white rhinoceros (*Ceratotherium simum simum*), southern white rhinoceros (*Ceratotherium simum cottoni*) and the black rhinoceros (*Diceros bicornis*). They observed low level of polymorphism along with high degree of differentiation between greater one-horned rhinoceros and African rhinos. Interestingly, Dinerstein and McCracken (1990) observed high level of heterozygosity in the Chitwan population of greater one-horned rhinoceros. Fernando et al., (2006) reported 2.4– 2.7% of interspecies divergence between Javan (*Rhinoceros sondaicus)* and greater one-horned rhinoceros based on study involving 12S gene sequence.

Morales and Melnick (1994) studied molecular systematics of living rhinoceros species using restriction mapping of mitochondrial ribosomal gene fragment. Interestingly, no intraspecific variation was observed in the two sampled populations of greater one-horned rhinoceros from Assam and Nepal (Morales and Melnick, 1994).

Xu et al., (1996) sequenced complete mitochondrial genome of greater one-horned rhinoceros and reported heteroplasmy associated with the mitochondrial control region. They observed a total of 36 identical control region motifs within the 16,829 bp mitochondrial genome.

Ali et al., (1999) reported low level of allelic heterozygosity in greater one-horned rhinoceros using Microsatellite Associated Sequence Amplification (MASA) and Southern blot analysis of genomic DNA with pSS(R)2 and other synthetic oligo probes.

Zschokke et al., (2003) developed eleven greater one-horned rhinoceros specific polymorphic microsatellite markers. Zschokke et al., (2011) further reported moderate to high level of genetic diversity along with significant differentiation among greater one-horned rhinoceros populations from Assam (captive individuals with known origin) and Nepal (wild) based on microsatellite as well as mitochondrial D-loop markers.

Ghosh et al., (2013) developed DNA barcodes of greater one-horned rhinoceros which may be applicable in forensic investigations. Das (2014) evaluated the genetic status of greater one-horned rhinoceros in three protected areas of Assam viz. Kaziranga National Park, Orang National Park and Pabitora Wildlife Sanctuary. Das (2014) used mitochondrial D-loop markers and six microsatellite marker and reported higher levels of genetic diversity with some degree of genetic differentiation between the individuals of greater one-horned rhinoceros inhabiting in these protected areas. Borthakur et al., (2016) estimated population size of greater one-horned rhinoceros using fecal DNA. This demonstrates feasibility of the use of noninvasive genetic sampling methods in population genetic monitoring of greater one-horned rhinoceros.

CHAPTER 3
MATERIALS AND METHODS

The present study evaluated the genetic status of greater one-horned rhinoceros (*Rhinoceros unicornis)* in India, which was once widely distributed throughout the northern region of the Indian sub-continent. The contemporary level of genetic diversity, population structure and extent of gene flow persistent among greater one-horned rhinoceros populations of India living in isolated patches were assessed employing a noninvasive sampling strategy.

3.1. Study area

Faecal samples from five wild greater one-horned rhinoceros bearing protected areas (PAs) from India were included for the present study which comprised three PAs of Assam viz., Kaziranga National Park (KNP), Orang National Park (ONP) and Pabitora Wildlife Sanctuary (PWLS) situated along the flood plains of Brahmaputra river basin and two PAs from West Bengal viz., Jaldapara National Park (JNP) and Gorumara National Park (GNP) located at the Terai belt along Eastern Himalayan foothills (Figure 2).

3.1.1. Kaziranga National Park

The Kaziranga National Park (KNP) is situated between the southern bank of the river Brahmaputra and foothills of the Mikir - Karbi Anglong hillock, within the administrative boundary of Golaghat and Nagaon districts of Assam. KNP is spread over an area of 429.93 km^2 and lies between 26°34' N to 26°46' N latitudes

and 93°08' E to 93°36' E longitudes along the flood plains of the river Brahmaputra. The park represents a mosaic patchwork of inundated alluvial grasslands and reed beds, alluvial savanna woodland, tropical moist mixed deciduous forests and tropical semi-evergreen forests (Talukdar, 1995) harbouring the world's largest population of greater one-horned rhino with an estimated population size of 2401 individuals (Rookmaaker et al., 2016). Along with greater one-horned rhinoceros, KNP is also known to harbour a total of 52 mammalian species, which include major herbivores such as Asian elephants (*Elephas maximus*), water buffalo (*Bubalus arnee*), swamp deer (*Rucervus duvaucelii*), sambar deer (*Rusa unicolor*), hog deer (*Axis porcinus*), barking deer (*Muntiacus vaginalis)* and wild boar (*Sus scrofa*); large carnivores such as tiger (*Panthera tigris*) and leopard (*Panthera pardus*); 43 species of reptiles and more than 490 species of birds (Choudhury, 2013; Rahmani et al., 2016) .

3.1.2. Orang National Park

Orang National Park (ONP) is situated along the northern bank of river Brahmaputra within the administrative boundary of Darrang and Sonitpur districts of Assam. It is spread over an area of 78.8 km^2. It lies between 26º29′ N to 26º40′N latitudes and 92º16′E to 93º27′E longitudes along the flood plains of the river Brahmaputra. The vegetation type of the park include Eastern Himalayan moist mixed deciduous forest, dry savannah grassland, wet alluvial grassland, seasonal swamp forest and degraded grassland (Sharma et al., 2011). Presently, the park inhabits 100 rhino individuals (Rookmaaker et al., 2016). ONP is also known to be inhabited by species such as Asian elephant (*Elephas maximus*),

water buffalo (*Bubalus arnee*), hog deer (*Axis porcinus*), wild boar (*Sus scrofa*), tiger (*Panthera tigris*), fishing cat (*Felis viverrina*), jungle cat (*Felis chaus*) and leopard cat (*Prionailurus bengalensis*) (Ahmed et al., 2009).

3.1.3. Pabitora Wildlife Sanctuary

Pabitora Wildlife Sanctuary (PWLS) is situated at the southern bank of the river Brahmaputra within the administrative boundary of Morigaon district bordering the Kamrup district of Assam. PWLS is spread over an area of 38.81 km^2. It is located between 26°12′N to 26°15′N latitudes and 91°57′ E to 92°50′E longitudes. The sanctuary represents a mosaic patchwork of heterogeneous alluvial tropical plain which is comprised of moist deciduous forests and woodland, grassland and savannah, swampy vegetation and tropical semi-evergreen forests (Bora and Kumar, 2003). PWLS has the highest density of greater one-horned rhinoceros with estimated population size of 92 individuals (Rookmaaker et al., 2016). The other major sympatric mammalian species known to inhabit PWLS include wild boar (*Sus scrofa*), leopard (*Panthera pardus*), fishing cat (*Felis viverrina*), jungle cat (*Felis chaus*) and Golden Jackal (*Canis aureus*) (Barua, 1998).

3.1.4. Jaldapara National Park

Jaldapara National Park (JNP) is situated in the Alipurduar district of West Bengal. The park covers an area of 216.6 km^2 and is located between 25° 58′ N and 27° 45′ N latitude and 89° 05′ E and 89° 55′ E longitude along the flood plains of the river Torsa. JNP lies within the Eastern Himalayan submontane Terai

belt and is characterized by dry alluvial savanna woodland, eastern alluvial grassland and dry-wet-mixed sal and evergreen forests (Biswas et al., 2014). The current population size of greater one-horned rhinoceros in JNP is about 200 (Rookmaaker et al., 2016). Apart from rhinos JNP is known to be inhabited by mammalian species which include Asian elephants (*Elephas maximus*), bison (*Bos gaurus*), sambar deer (*Rusa unicolor*), hog deer (*Axis porcinus*), barking deer (*Muntiacus vaginalis)*, wild boar (*Sus scrofa*), pigmy hog (*Porcula salvania*), sloth bears (*Melursus ursinus*) and leopard (*Panthera pardus*) (Dubey et al., 2015).

3.1.5. Gorumara National Park

Gorumara National Park (GNP) is situated in the Jalpaiguri district of West Bengal. The park covers an area of 79.99 km^2. GNP is located between 26°44′N to 26°75′N latitudes and 88°50′E to 88°60′E longitudes along the flood plains of the Murti and Raidak rivers within the Eastern Himalayan submontane Terai belt. The vegetation type of the park includes mosaic ecology of tall riverside grasslands, savannas and evergreen sub-Himalayan secondary wet-mixed forests and deciduous forests (Das et al., 2012). Gorumara has a known small population size of about 50 individual rhinos (Rookmaaker et al., 2016). Other important sympatric species found in GNP are Asian elephants (*Elephas maximus*), bison (*Bos gaurus*), spotted deer (*Axis axis*), hispid hare (*Caprolagus hispidus*) and leopard (*Panthera pardus*) (Dubey et al., 2015).

It is noteworthy that, the greater one-horned rhinoceros population of Manas National Park (MNP) and Dudhuwa National Park (DNP) were not sampled for

the present study as both the PAs host reintroduced rhinoceros population from Assam.

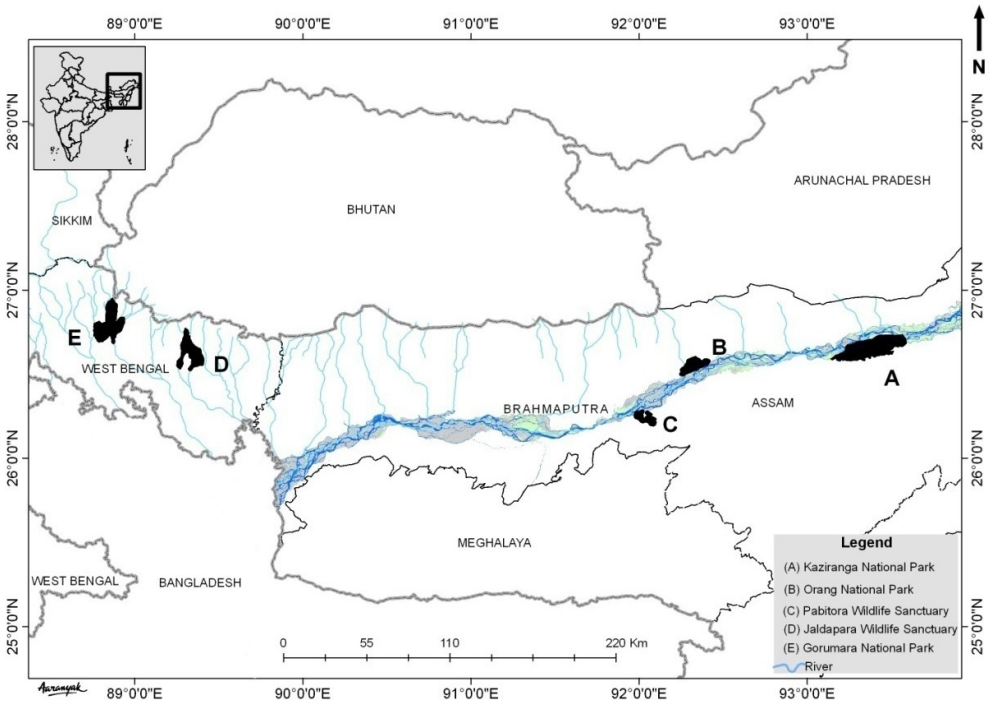

Figure 2: Map showing rhino bearing protected areas (PAs) of Assam and West Bengal sampled for the present study. (A) Kaziranga National Park (KNP), (B) Orang National Park (ONP), (C) Pabitora Wildlife Sanctuary (PWLS), (D) Jaldapara National Park (JNP) and (E) Gorumara National Park (GNP) respectively.

3.2. Collection of samples

3.2.1. Sampling Strategy

Greater one-horned rhinoceros generally defecates on common dung piles (Laurie et al., 1983). In the present study, a single session sampling strategy was followed and only fresh faecal samples, not more than 24 hours old were collected within a period of 3 to 7 days. Collection of only fresh samples within a short period of time reduces the probability of recapturing the same individuals within sampled population and thereby, increases the probability of maximum number of individuals being genotyped.

3.2.2. Sampling Technique

For collection of faecal material, multiple teams comprising of four to six persons were formed and all the PAs were surveyed to locate the active dung piles used by rhinos for defecation. About 10 to 15 grams of dung samples were collected in plastic vials containing DMSO EDTA Tris salt saturated (DETs; 20% DMSO, 0.25M EDTA, 10mM Tris at pH 7.5 and NaCl to saturation) buffer (Frantzen et al., 1998). To avoid cross contamination from multiple samples that were collected from the same dung pile, sampling of faecal material was done only from the top most bolus of two visibly different heaps of faeces. The Global Positioning System (GPS) coordinate readings were recorded for each of the sampled location for future reference. During the sampling sessions, vehicle and/ or elephants were used as a mode of transport.

A total of 292 dung samples were collected which included 58 samples from PWLS, 84 samples from KNP, 54 samples from ONP, 60 samples from GNP and 36 samples from JNP and were used for microsatellite genotyping. Additionally ten tissue samples were also collected from rhino carcasses during the period. All the tissue samples were collected in absolute ethanol. These samples were utilized for initial standardization of microsatellite markers used in the present study. The samples were stored at -20°C until DNA was extracted for further analyses.

3.3. Extraction of DNA

DNA from dung samples were extracted following two methods. First, by using standard commercial kit protocol (QIAamp DNA Stool Kit, QIAGEN Ag., Germany) and second, by adopting the guanidine isothiocyanate- silica based

protocol (Boom et al., 1990) with minor modifications. Briefly, 500 µl of DETs buffer containing faecal sample was added to 1000 µl of L6 lysis solution (5M Guanidine isothiocyanate, 100mM Tris, pH 6.4, 20mM EDTA, pH 8.0, and 1.3% Triton X-100) in a sterile 1.5 ml microcentrifuge tube (MCT) and was incubated overnight at room temperature with intermittent vortexing. This was followed by centrifugation at 8,000 rpm for 1 minute. The supernatant was then transferred to a new 1.5 ml MCT to which 100 µl of 10% polyvinyl polypyrrolidone (PVP) solution was added. The suspension was mixed by gentle inversion and was incubated again at room temperature for 30 minutes followed by centrifugation at 12,000 rpm for 2 minutes. Then the supernatant was aspirated out and was mixed with 50 µl of 6% silica solution in a new 1.5 ml MCT. The mixture was further incubated at room temperature for 30 minutes. The silica matrix was then pelleted through centrifugation at 12,000 rpm for 1 minute. This was followed by washing of the silica pellet twice with 500 µl of L2 (5M Guanidine isothiocyanate, 100mM Tris, pH 6.4, and 20mM EDTA, pH 8.0) Solution and 500 µl of Ethanol wash buffer (100mM Tris, pH 7.5, 100mM sodium chloride, 1mM EDTA, pH 8.0, and 60% ethanol) and then washing with 500 µl of ice-cold 80% Ethanol (v/v) and 500 µl with ice-cold acetone once. All centrifugation steps during washing were carried out at 12,000 rpm. The washed pellet was then incubated at 55°C for drying and DNA was finally eluted in 75 µl of TE buffer (10 mM Tris-Cl at pH 7.5, 1mM EDTA at pH 8.0). For each batch of extraction, a negative control was included to cross check contamination.

DNA extraction from all the reference tissue samples was carried out using commercially available DNeasy Blood and Tissue Kit (QIAGEN Ag., Germany)

following standard kit protocols except for the final elution volume which was reduced to 80 µl. All the DNA extractions were performed in an isolated facility dedicated for low quality DNA work.

3.4. Amplification of microsatellite markers

3.4.1. Screening of Microsatellite markers

A total of eleven polymorphic microsatellite markers developed for the greater one-horned rhinoceros (Table 1; Zschokke et al., 2003; Scott, 2008) were initially screened for their utility by Borthakur et al., (2016) and used in the present study.

3.4.2. Polymerase Chain Reaction (PCR)

All the microsatellite markers were amplified employing a multiplex PCR assay. Initially, each multiplex PCR reaction was carried out with a reaction volume of 10µl containing 3 to 4 microsatellite markers for standardization. The forward primers of each microsatellite marker were labelled with one of the either four different fluorescent tags viz., 6-FAM, VIC, NED and PET in DS-33 dye system (Applied Biosystems, USA). Based on the preliminary observations, nine microsatellite markers were selected and were grouped into three panels of microsatellite markers, each comprising of three pairs of primes for multiplex PCR reactions (Table 2). All the reactions were performed using QIAGEN Multiplex PCR Kit (QIAGEN, Germany) following standard kit protocol designed for reagent concentration with 0.25 µM of each primer and 2.5 µl of template DNA. The thermal cycling conditions for panel 1 and 2 was set with initial denaturation at 95°C for 15 min, followed by 40 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 1 min, and a final extension at 72°C for 30 minutes.

For panel 3, annealing temperature was set at 52°C for 1min, in lieu of 55°C keeping the rest of the reaction conditions the same as for panel 1 and 2.

To obtain reliable genotype data, a multiple tube approach was adopted in the present study and consensus genotypes were created from the repeats (Taberlet et al., 1996). Notably, DNA obtained from noninvasively collected samples is often very low. Genotyping of such samples are prone to contamination and may result in incorrectly detecting a heterozygote individual as homozygote at a particular locus (Taberlet et al., 1996; Taberlet et al., 1999). Taberlet and his colleagues proposed a two step multiple tube approach in order to avoid potential genotyping errors and obtain reliable data at 99% confidence level, especially for samples with low and often unknown quantity of DNA (Taberlet et al., 1996).

Following Taberlet et al., (1996) all the PCR reactions were first performed in triplicates for each sample and microsatellite locus typed. Alleles were called only if they were observed at least twice in the first set of PCR. In case of ambiguity, additional four PCR were performed to determine whether an observed allele is a true or false allele. Samples with ambiguous genotypes after seven independent PCRs as suggested by Taberlet et al., (1996) were removed from further analysis.

Table 1: Repeat motif structure, primer and Tm values for all microsatellite loci screened for use in this study

Sl. #	Microsatellite Marker	Repeat Motif	Primer (5'-3')	Tm (°C)
1	Rh1*	$(TG)_{13}$	F: GTGCCATTATTATCCCAGGTC R: CGTAAGACCTCAAGGGATGC	60
2	Rh2*	$(GT)_{36}$	F: GACTTCAAACTTCGCAGCAATC R: GCCCTAGACCTGGAAATAACC	60
3	Rh3*	$(TC)_8TG(TC)_7CCTG(TC)_4$ $TG(TC)_{16}$	F: TGTGTGGAGCACATCAGTCTTC R: CCAGGGACCCGTGAGGAT	62
4	Rh4*	$(AC)_{22}$	F: CAAAATGTGGGTTTTGTGAGC R: GACGAGCTTTGTTTGAATGC	60
5	Rh5*	$(TG)_{15}$	F: CCCATTAGAGGCTGTAGAGTAATATC R: GGACTCTAAACTCCAGGGTCAC	58
6	Rh6*	$(CA)_4GT(AT)_2(GTAT)_4$ $GCAT(GT)_2(AT)_2(GT)_{11}$	F: CCTTACTGTTGGGAAGATGTTATAGG R: CATCACCTGTGCGTAAGTGC	58
7	Rh7*	$(TG)_{17}$	F: CCGTCACATATGACAGTGTGC R: GGGCAGCTTATGCTCAAGTC	62
8	Rh9*	$(TG)_4TT(TG)_{17}TA(TG)_5$	F: TCTGGTACCACCAAATGTAGC R: ACGATTACGTCTTTCAGTTGC	60
9	Rh10*	$(GT)_{24}(GC)_7$	F: TATGCCAGGGAAGAATCTGGTC R: TCCCTCACCAACTCTCGTAAAC	60
10	Rh11*	$(CA)_{23}$	F: CTCGCATCCTCATCAATGC R: GCAGGTGTACCAGGCTGAG	64
11	IR14**	$(GT)_{15}$	F: CCTAGTAGTCAACGGCAAGG R: TGGACTCTTGCATAGGCTCC	62

* Zschokke et al., 2003; ** Scott, 2008; F= Forward Primer; R= Reverse Primer.

Table 2: Multiplex panel information and PCR conditions

Sl #	Marker	Fluorescent Tag	Panel	PCR Thermocycler Condition
1	Rh4	6-FAM	Panel I	95ºC for 15 minute 94 ºC for 1 minute 55 ºC for 30 second 72 ºC for 1 minute (40 cycle) 72 ºC for 30 minute
2	Rh10	NED		
3	Rh3	VIC		
4	Rh11	NED	Panel II	95ºC for 15 minute 94 ºC for 1 minute 55 ºC for 30 second 72 ºC for 1 minute (40 cycle) 72 ºC for 30 minute
5	Rh7	VIC		
6	Rh9	PET		
7	Rh1	6-FAM	Panel III	95ºC for 15 minute 94 ºC for 1 minute 52 ºC for 1 minute 72 ºC for 1 minute (40 cycle) 72 ºC for 30 minute
8	Rh5	NED		
9	Rh6	PET		

3.5. Analysis of genetic data

3.5.1. Allele sizing

The PCR products for each microsatellite marker were run on a ABI 3130 Genetic Analyzer (Applied Biosystems, USA) and alleles were sized relative to an internal control (500 LIZ) for marker system DS-33 using software GENEMAPPER v3.7 (Applied Biosystems, USA). Furthermore, the raw data of each marker for each sample were manually inspected using the software 'Peak Scanner v1.0' (Applied Biosystems, USA). This combined approach was used to mitigate the potential scoring errors like stochastic amplifications within the size range, mistyping of

allele due to stuttering, allelic dropout or null alleles respectively (Pompanon et al., 2005; Dewoody et al., 2006).

3.5.2. Estimation of genotyping error rate and generation of consensus genotypes

Microsatellite genotyping are often associated with two types of errors viz. allelic dropout (ADO) and false allele (FA) (Taberlet et al., 1999; Miller et al., 2002; Fernando et al., 2003).

ADO represents non-amplification of one of the two alleles at a microsatellite locus of a heterozygous individual. ADO often generates false homozygotes which lead to underestimation of observed heterozygosity while overestimating inbreeding co-efficient (Taberlet et al., 1996; Taberlet et al., 1999; Miller et al., 2002; Wang et al., 2012). False allele represents amplification of products within the allele range of a microsatellite marker that are not part of true genotypic data which may arise due to PCR artefacts. False allele also leads to misidentification of homozygote as heterozygote or introduces error in case it gets amplified in a heterozygote (Goossens et al., 1998; Taberlet et al., 1999).

Estimates of error rates and consensus genotypes were determined by comparing the alleles scored against all the samples for all the microsatellite loci from PCR repetitions using the software 'GIMLET v1.3.3' (Valiere, 2002).

3.5.3. Quality index criterion for genotype selection

As a result of variations in quality and quantity of DNA obtained from noninvasively collected samples, the quality of genotyping data often varies

across sample set (Miquel et al., 2006). Miquel and his colleagues developed a quality index criterion for assessing reliability of genotypic data obtained from noninvasively collected sample following multiple tube approach. They proposed to assign values '1' or '0' to each genotype for each locus typed for a sample relative to the consensus genotype. Furthermore, to estimate the quality index value for each locus typed for each sample as the sum of assigned value to each genotype divided by total number of repeats. Assigning quality index allows making comparisons among genotypic data at each locus and thus, helping in identifying problematic loci as well as samples (Miquel et al., 2006).

In the present study, the quality index criterion, as proposed by Miquel et al., (2006) was followed to select the final set of samples for analysis. The quality index value was calculated for each genotype at each locus typed and a value of 0.67 was considered as the cut-off for retaining genotypic data at a particular locus for a sample.

3.5.4. Determining the power of microsatellite marker

For population studies involving estimation of population size, genetic tracking and long term monitoring of populations, determination of home range as well as dispersal pattern, require accurate identification of individuals of a population which can be achieved through multilocus genotyping (Waits et al., 2001). Identification of individuals based on multilocus genotypic data, is therefore, critical and often depends on the ability of genetic markers used in such studies to differentiate between samples of two different individuals. The power of genetic markers to resolve individual identity of samples within and among population

can be determined through *probability of identity* statistic (P_{ID}) that represents the probability of two individuals in a population sharing the same genotype at multiple loci (Waits et al., 2001). Typically, P_{ID} is calculated under the assumptions that individuals are not related to each other, are randomly sampled, and the loci are at Hardy-Weinberg equilibrium**.** However, such assumptions may not always hold in natural populations and therefore, result in underestimation of true P_{ID} (Waits et al., 2001; Allendorf and Luikart, 2007). To avoid underestimation of true P_{ID}, a related statistic, the *probability of identity among siblings* (P_{ID}-sibs), therefore, should also be estimated along with P_{ID} to determine the power of markers used in a study (Waits et al., 2001; Allendorf and Luikart, 2007).

In the present study, P_{ID} and P_{ID}-sibs values were estimated for 10 tissue samples as well as for each rhino population using the software 'GIMLET v1.3.3' (Valiere, 2002).

3.5.5. Individual identification

The unique multilocus microsatellite genotypes, i.e. individual rhinos within the dataset were identified using the *'Identity Analysis'* module as implemented by the software 'CERVUS v3.0' (Marshall et al., 1998). The '*Identity Analysis'* module identifies the matching genotypes from a multilocus genotype data file. A strict criterion was followed for identification of individual rhinos allowing no mismatch at any locus to be accepted while executing the *'Identity Analysis'* module.

3.5.6. Estimating genetic diversity

In the present study the genetic diversity of the five rhino populations were estimated in terms of allele frequency, allele richness and observed and expected heterozygosity. The allele frequency and allelic richness (Ar) were estimated using software F_{STAT} v2.9.3 (Goudet, 2001). The Allelic richness was measured in terms of the number of alleles independent of sample size, to facilitate comparisons amongst different sample sizes. It had been observed that the number of alleles in a sample is highly dependant on sample size (Mousadik and Petit, 1996). To overcome this problem the software F_{STAT} adopts the rarefaction index of Hurlbert (1971) and estimates the expected number of alleles from a pool of 2n genes (where n is fixed and represents the smallest number of individuals typed for a locus in a sample) that were sampled (Goudet, 2001).

The observed heterozygosity (H_o) and expected heterozygosity (H_e) as well as inbreeding coefficient (F_{IS}) were calculated using software 'GENETIX v.4.05.2' (Belkhir et al., 1996-2004).

3.5.7. Testing Hardy-Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD)

Hardy-Weinberg principle is one of the fundamental principles in population genetics which describes an equilibrium state between allele and genotype frequencies in a large, randomly mating population that is devoid of evolutionary forces acting upon it. Any deviation from the underlying assumptions of Hardy-Weinberg equilibrium (HWE) would lead to departure from equilibrium state, thereby, essentially providing a baseline to detect evolutionary change in a

population (Frankham et al., 2004). In the present study, to detect any significant deviation from HWE, the genotypic data was tested using an 'exact test' as implemented in the software 'ARLEQUIN v3.5.1.2' (Excoffier et al., 2005). The exact test was performed using 100,000 steps in Markov-Chain and 10000 dememorization steps. The *'p'* values for HWE were adjusted with Bonferroni correction for multiple comparisons (Rice, 1989). In statistical hypothesis testing, if multiple comparisons are carried out the chance of a rare event occurring increases and thus, the probability of incorrectly rejecting the null hypothesis increases (Mittelhammer, 2000). The Bonferroni correction compensates this chance by testing individual hypothesis at a significance level of *'α/m'* where α is the desired overall alpha and *m* is the number of tests (Miller, 1966).

Further, presence of nonrandom association between alleles of the microsatellite markers used in the study i.e., linkage disequilibrium (LD) was assesed using software ARLEQUIN v3.5.1.2 (Excoffier et al., 2005). In studies related to population monitoring, LD can provide valuable information on evolutionary forces such as, mutation, genetic drift, and natural selection acting upon wild population of a species along with population history and breeding behaviour (Slatkin, 2008).

3.5.8. Evaluating past population bottleneck

The effect of population bottlenecking was evaluated through Wilcoxon's signed rank test following two-phase model (TPM) (Di Rienzo et al., 1994) using software 'BOTTLENECK v1.2.02' (Piry et al., 1999). The software test for recent decline in effective population size (N_e) occurring in a temporal range of

$2N_e$– $4N_e$ generations based on the principle that allelic diversity reduces faster as compared to the heterozygosity when a population experiences recent reduction in effective population size. Consequently, a past bottleneck can be inferred when the observed heterozygosity is higher than the heterozygosity expected at mutation drift equilibrium (Piry et al., 1999). The parameters used were 95% of single-step mutation and 5% multistep mutations with a variance among multiple steps equal to twelve (Piry et al., 1999). Simulations with different parameter values (80–99% of single- step mutation) were also performed to assess the probability of occurrence of any variation.

3.5.9. Evaluation of population differentiation and genetic structuring

In the present study, population differentiation was evaluated using two tests viz., pairwise F_{ST} and analysis of molecular variance (AMOVA). The pairwise F_{ST} between the five PAs and estimates of molecular variance (AMOVA) between and within PAs were calculated using software ARLEQUIN (Excoffier et al., 2005).

To investigate the pattern of genetic structure in the greter one-horned rhinoceros populations of India two approaches were followed. Firstly, a Bayesian clustering method was followed by using the software 'STRUCTURE v2.3.1' (Pritchard et al., 2000) which does not require any prior information on population. Secondly, a Bayesian approach following Rannala and Mountain (1997) with 'leave one out' option (Cornuet et al., 1999) using software 'GeneClass v2.0.h.' (Piry et al., 2004) which require prior information on the populations. Under admixure model with correlated allele frequency, the software 'STRUCTURE' was run for five

replicates with different K values (K= 1 to 10) and 10^6 burnin periods along with 10^6 Markov Chain Monte Carlo (MCMC) repeats after burnin (Pritchard and Wen, 2003). The number of actual clusters was determined by estimating delta K (ΔK), an adhoc value associated with the second order rate of change of the 'log probability' of data corresponding to each K (Evanno et al., 2005) employing the software 'Structure Harvester Web v0.6.92' (Earl and VonHoldt, 2012). Once the optimal K was determined, samples were assigned to their respective subpopulations based on their highest percentage of membership value (q). A threshold value of $q \geq 0.90$ was chosen in order to maintain higher stringency during the analysis.

3.5.10. Detection of migrants and migrant ancestry

Analysis for detection of migrants and migrant ancestry were performed for 'STRUCTURE'-defined clusters of greater one-horned rhinoceros and for populations of Assam viz., PWLS, KNP and ONP separately to understand the pattern of migration between the 'STRUCTURE' defined clusters as well as in the three rhinoceros populations of Assam.

For detection of migrants and migrant ancestry three approaches were employed. First, a likelihood estimation of probability that an individual is a resident or first generation (F_0) migrant was estimated using Bayesian criterion (Rannala and Mountain, 1997), employing the software 'GeneClass2' (Piry et al., 2004). The test statistic used was L_{home}/L_{max}, a ratio of the likelihood computed from the population where the individual was sampled (L_{home}) over the highest likelihood value among all population samples including the population where the individual

was sampled (L_{max}) (Paetkau et al., 2004). A total of 10 000 individuals were simulated and alpha (α) was set at 0.005 to estimate the critical value of L_{home}/L_{max} for each individual genotyped, using Monte Carlo re-sampling method of Paetkau et al., (2004) to determine whether an individual originates from the population where it was sampled. Individuals with a probability less than the significance (α) level were classified as migrants.

Second, a model-based Bayesian clustering method was used through software 'STRUCTURE v2.3.4' (Prichard et al., 2000). The USEPOPINFO Model inherent in 'STRUCTURE' uses prior population information and user-specified prior probability to infer whether an individual is an immigrant or not (Pritchard et al., 2000). The simulations in 'STRUCTURE' were run under admixture model with correlated allele frequency among populations. For detection of migrants, we used 10^6 burnin periods along with 10^6 MCMC repeats after burnin (Pritchard and Wen, 2003) while lambda (λ) was set to 1.0. Prior population information based on geographical sampling locations was used to determine the migration history of each individual up to two generations back. As no information on migration rate were available, separate simulations were run with MIGPRIOR (*v*, the probability that individual is an immigrant to population) set to 0.05 and 0.10 which corresponds to 5% and 10% probability of an individual is an immigrant or have migrant ancestry (Prichard et al., 2000). The choice of *v* is crucial while drawing conclusions (Prichard et al., 2000).

Third, a Bayesian method for estimation of recent migration rate by estimating fraction of individuals that are migrants between population samples as well as migration ancestry of individuals in a population using MCMC was used by

employing the software 'BayesAss v3.0.3' (Wilson and Rannala, 2003). The 'assignment' method implemented in the software 'BayesAss' does not incorporate genealogy or assume that populations are in Hardy-Weinberg equilibrium (Wilson and Rannala, 2003). The mixing parameter values for migration rate (ΔM), allele frequency (ΔA) and inbreeding co-efficient (ΔF) were set to 0.3 to obtain an acceptable acceptance rate (0.2-0.6) for the proposed change in parameters viz., migration rate, allele frequency and inbreeding coefficient, respectively at 95% credible interval (Wilson and Rannala, 2003; Rannala, 2007). The MCMC was performed for 10^7 iterations with a burn-in of 10^6 while the sampling interval was set to 1000.

3.6. Evaluating influence of landscape features on gene flow

Results of Bayesian statistics provided indication of restricted geneflow between some of the greater one-horned rhinoceros populations which may be influenced by landscape features. Based on the results obtained, our hypothesis was habitat fragmentation due to human settlements along with agricultural practices, especially, paddy and tea hinder dispersal. The hypothesis was explicitly tested by developing a linear raster cost-distance model to determine the biological cost incurred during dispersal through a matrix of varying landscape features using the 'Cost Distance tool' of the 'Landscape Genetics Toolbox' an extension to the software 'ARCGIS 10' (Etherington, 2011).

We first performed an unsupervised classification of nine LANDSAT5 satellite images covering our study area using software 'Erdas Imagine 9.1'. The images used in the present study (LT51350422011035, acquisition date, Feb 04, 2011;

LT51360412011026, acquisition date, Jan 26, 2011; LT51360422011010, acquisition date, Jan 10, 2011; LT51370412011033, acquisition date, Feb 02, 2011; LT51370422011033, acquisition date, Feb 02, 2011; LT51380412011008, acquisition date, Jan 08, 2011; LT51380422011024, acquisition date, Jan 24, 2011; LT51390412011079, acquisition date, Mar 20, 2011; LT51390422011063, acquisition date, Mar 04, 2011) were obtained from United States Geological Survey (USGS) website http://landsat.usgs.gov. A total of 9 habitat classes were defined viz., Grassland, River, Sand, Open forest, Dense forest, Agriculture Type 1 (paddy), Agriculture Type 2 (tea), Orchard and Settlement. Paddy and tea are the two major agricultural crops in the study area and, hence, the class Agriculture was divided into these two groups. Furthermore, ambiguous grid cells were reclassified based on the prior knowledge of the landscape using the software 'Arc GIS'. For example, polygons were drawn around known forests, grasslands, riverbeds etc, and aberrant grid cells within the polygon were reclassified; Say, agriculture within polygon of open forest were converted to grassland because agriculture land within a open forest patch is not likely to be present. All the reclassified images were then merged to obtain the final image of the study area.

Each cell in the classified grid was assigned a value that reflected a judgment of biological cost of dispersal based on two criteria, firstly, the habitat type and secondly, the elevation (Table 3). The underlying hypothesis for generating this cost-distance model was that rhinos would face higher biological cost to traverse through a patch of poor habitat (comprising of human settlement, crop fields etc) in comparison to a patch of good habitat (comprising of forest, grassland, river and river islands etc). Similarly, biological cost to traverse through high elevation

was assumed to be higher in comparison to low elevations. The two values for each grid cell were then combined to obtain a single matrix of grid cells comprising of biological cost of dispersal which is relative to one another. The protected areas included in the present study were considered as the "source" population and each grid cell within a source was assigned cost value zero. The 'Cost Distance tool' was then used to estimate pair wise dispersal cost grid among the sources.

The relationship between gene flow and geographic distance as well as landscape features were further evaluated through Mantel test. For Mantel test, first, the pair wise population F_{ST} values were linearized using formula $F_{ST}/ (1- F_{ST})$ following Rousset (1997). Then, the linearized genetic distance matrix was correlated with the matrices of geographic distance, log transformed geographic distance and cost distance. The Mantel test was carried out with 10,000 randomization using web based source 'IBDS V3.23' (Jensen et al., 2005).

Table 3: Biological cost assigned to different habitat class and elevation

Habitat Type	Biological Cost Assigned	Elevation (m)	Biological Cost Assigned
Grasslands	1	0 - 150	1
River/ Sand	2	150 – 400	2
Open Forest	3	> 400	3
Dense Forest	4		
Agriculture Type I	5		
Agriculture Type II	6		
Orchard	7		
Settlement	8		

CHAPTER 4
RESULTS

In the present study, the extent of contemporary genetic diversity in wild populations of the greater one-horned rhinoceros in India was successfully evaluated along with the existing population structure within the rhino populations in order to determine the population boundaries at a spatial scale. Furthermore, the contemporary migration rates between spatially explicit rhino population as well as rhino bearing PAs and possible effect of landscape features on the gene flow were evaluated.

4.1. Extraction of DNA

The extracted DNA were visualized in 2% (*w/v*) Agarose gel (Figure 3) which were successfully used in generating microsatellite genotypic data.

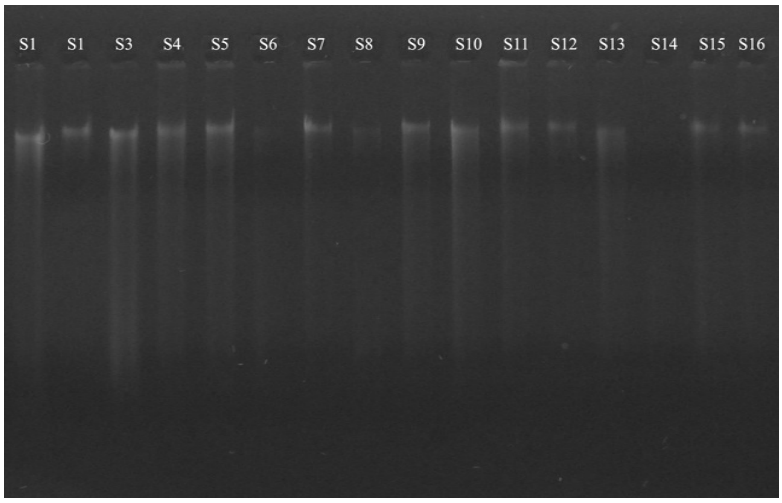

Figure 3: Photograph of DNA bands on 2% (*w/v*) Agarose Gel

4.2. Microsatellite genotyping and estimation of error rates

Out of the eleven microsatellite markers screened for this study, nine markers viz., Rh1, Rh3, Rh4, Rh5, Rh6, Rh7, Rh9, Rh10 and Rh11 produced usable data for all the sampled locations. Henceforth, genotype data for these nine microsatellite markers were considered for further analysis.

All the markers were found to be polymorphic, with a minimum of two alleles and up to a maximum of six alleles per locus (across all populations) (Table 4). However, marker Rh1 was observed to be monomorphic (i.e., only one allele was amplified) for GNP population (Table 4).

The estimates of genotyping error viz., ADO and FA were observed to be low. The ADO values ranged between 0 to 8.3% across all populations. Similarly the FA values across all populations were observed to be $\leq$ 6.9% (Table 4).

4.3. Quality index, P_{ID}, P_{ID}-sibs and individual identification

Out of the 292 samples genotyped, 249 samples conceded the quality index criteria set for selection of final set of samples. These samples were then used for further analysis.

The estimates of cumulative P_{ID} and P_{ID}-sibs were observed to be 2.66×10^{-7} and 4.17×10^{-3} in case of the 10 reference tissue samples. The estimates of cumulative P_{ID} and P_{ID}-sibs for faecal samples were observed to be 7.406×10^{-6} and 6.050×10^{-3} in PWLS, 8.145×10^{-7} and 2.310×10^{-3} in KNP, 1.793×10^{-6} and 2.990×10^{-3} in ONP, 4.961×10^{-4} and 2.907×10^{-2} in GNP and 7.148×10^{-5} and 1.439×10^{-2} in JNP respectively.

The individual identity analysis yielded 238 unique multilocus genotypes which included 45 individuals from PWLS, 72 individuals from KNP, 44 individuals from ONP, 43 individuals from GNP and 34 individuals from JNP respectively.

4.4. Genetic diversity

4.4.1. Allele frequency and allelic richness

The marker Rh4, Rh10 and Rh11 showed highest polymorphism with six observed number of alleles across all populations with allele size ranging from 89-101, 138-150 and 141-155. Marker Rh3, Rh5 and Rh9 yielded five alleles across all populations with allele size ranging from 116-150, 196-206 and 148-174 respectively. The marker Rh7 and Rh1 yielded four and three alleles across all populations with allele size ranging from 200-206 and 148-154 respectively. The number of alleles observed for marker Rh6 was two across all the populations with allele size ranging from 120-122. The alleles frequencies of microsatellite markers used in the study are represented graphically in Figure 4.

The mean observed allelic richness (Ar) was higher in the greater one horned rhinoceros populations of Assam in comparison to the populations of West Bengal. The estimates of mean observed allele richness for KNP, ONP and PWLS were 3.606±1.18, 3.635±0.93 and 3.445±1.02 respectively. In JNP and GNP the mean allele richness was found to be 3.088±1.22 and 2.589±0.88 respectively (Table 5).

Table 4: Estimates of the number of alleles per locus (NA), estimates of allelic dropout (ADO) and false alleles (FA) across microsatellite loci of greater one-horned rhinoceros populations of KNP, ONP, PWLS, JNP and GNP.

Loci	KNP			ONP			PWLS			JNP			GNP		
	NA	ADO	FA	NA	ADO	FA	NA	ADO	FA	NA	ADO	FA	NA	ADO	FA
Rh1	3	0.038	0.029	3	0.022	0.024	3	0.043	0.042	3	0.083	0.045	1	0.000	0.000
Rh3	4	0.039	0.014	3	0.042	0.031	3	0.024	0.038	3	0.000	0.000	4	0.025	0.050
Rh4	6	0.031	0.019	5	0.025	0.021	5	0.022	0.042	4	0.063	0.038	2	0.000	0.000
Rh5	4	0.022	0.034	5	0.038	0.000	3	0.028	0.034	3	0.053	0.000	3	0.045	0.000
Rh6	2	0.044	0.038	2	0.033	0.034	2	0.045	0.028	2	0.000	0.000	2	0.000	0.000
Rh7	2	0.017	0.022	4	0.042	0.031	3	0.043	0.042	2	0.000	0.019	3	0.037	0.000
Rh9	3	0.043	0.029	3	0.050	0.029	3	0.063	0.000	2	0.029	0.029	2	0.000	0.000
Rh10	5	0.043	0.019	4	0.025	0.000	5	0.045	0.020	6	0.045	0.043	3	0.036	0.069
Rh11	5	0.033	0.017	5	0.019	0.000	5	0.036	0.026	3	0.029	0.029	4	0.036	0.045

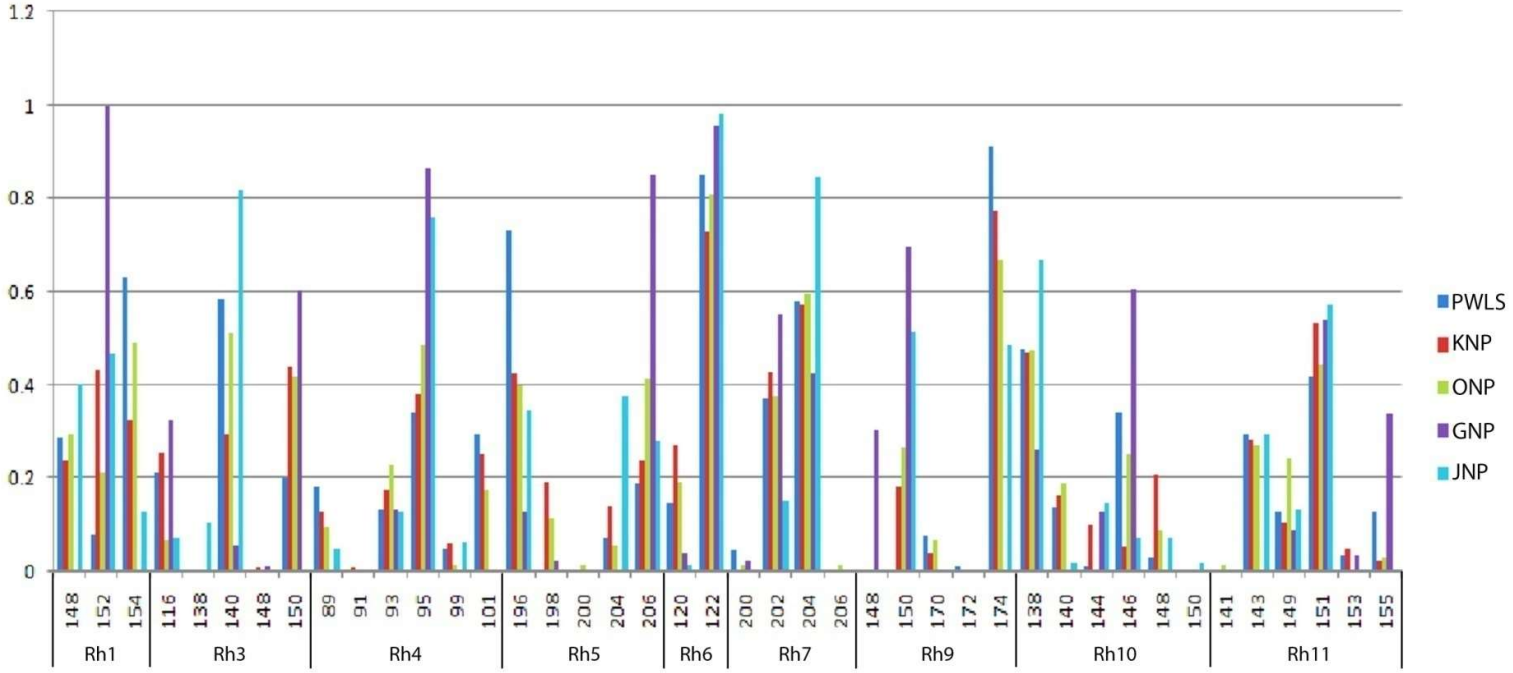

Figure 4: Bar diagram showing allele and allele frequencies of nine microsatellite markers across all the rhinoceros populations of India

4.4.2. Heterozygosity and inbreeding coefficient

The level of heterozygosity of greater one-horned rhinoceros populations was observed to be moderate to high for the microsatellite markers used in the study. The mean expected heterozygosity (He) ranged from 0.352± 0.2 to 0.59± 0.13 and was observed to be higher in the rhinoceros populations of Assam in comparison to that of West Bengal. In Assam KNP showed the highest level of expected heterozygosity (He= 0.590 ± 0.13) followed by ONP (He= 0.571 ± 0.11) and PWLS (He= 0.502 ±0.19). In West Bengal, JNP displayed the highest level of expected heterozygosity (He= 0.428 ± 0.19) while in GNP the mean expected heterozygosity was observed to be lowest (He= 0.352 ± 0.20) (Table 5).

The observed heterozygosity (Ho) was found to be slightly higher than the expected heterozygosity (He) ranging from 0.409 (±0.27) to 0.67 (±0.15) in all the populations, generating an overall excess of heterozygosity across all the PAs with mean F_{IS} ranging from -0.027 to -0.158 (Table 5).

4.5. Hardy- Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LD)

The test for HWE showed that the locus Rh11 in KNP, ONP, PWLS and GNP, locus Rh10 in KNP and Rh5 in ONP were observed to deviate significantly from HWE after Bonferroni corrections (Table 5). Although, these three loci deviated from HWE, they were considered for further analysis owing to the fact that these loci are polymorphic in nature and HWE may not always stand true in natural populations (Dorak MT, 2014).

No significant LD was observed between any pair of loci for the microsatellite markers used in the present study.

4.6. Population Differentiation and Genetic Structuring

The test for population genetic differentiation in the present study showed considerable genetic differentiation among the greater one-horned rhinoceros population of India.

The estimates of pair wise F_{ST} were observed to be significantly high (p<0.001) between rhinoceros populations of West Bengal to that of Assam (Table 6). The pair wise F_{ST} between GNP and PWLS was observed to be highest (F_{ST} = 0.382; p< 0.001) among all pairs of populations. It was interesting to observe that, although GNP and JNP are at the closest geographical proximity, pair wise F_{ST} between the two populations was considerably high (F_{ST}= 0.312; p< 0.001). Within Assam, the pair wise F_{ST} were observed to be relatively low. The observed pair wise F_{ST} estimates between PWLS-KNP, PWLS-ONP and KNP-ONP at p<0.001 appeared as 0.06265, 0.04018 and 0.0222 respectively.

Table 5: Comparison of allelic richness (Ar), observed (Ho) and expected (He) heterozygosity, heterozygosity deficit (F_{IS}) and deviation from HWE (* significant after flat Bonferroni correction at p=0.005) across nine microsatellite loci in PWLS, KNP, ONP, GNP and JNP.

		Microsatellite Markers									Mean ± SD
		Rh1	Rh3	Rh4	Rh5	Rh6	Rh7	Rh9	Rh10	Rh11	
PWLS	Ar	3.00	3.00	4.98	2.99	2.00	2.98	2.58	4.50	4.95	3.445 ± 1.02
	Ho	0.684	0.531	0.780	0.532	0.295	0.651	0.174	0.532	0.767	0.550 ± 0.19
	He	0.511	0.572	0.744	0.419	0.252	0.521	0.160	0.634	0.706	0.502 ±0.19
	F_{IS}	-0.327	0.080	-0.037	-0.260	-0.162	-0.238	-0.073	0.172	-0.075	-0.083
	HWE	0.0421	0.7843	0.1486	0.1302	0.5679	0.0086	0.9999	0.1525	0.0001*	-
KNP	Ar	3.00	3.39	5.39	4.00	2.00	2.00	2.95	4.97	4.74	3.606 ± 1.18
	Ho	0.716	0.638	0.833	0.835	0.513	0.536	0.391	0.761	0.800	0.670 ± 0.15
	He	0.648	0.652	0.743	0.704	0.395	0.489	0.364	0.695	0.616	0.590 ± 0.13
	F_{IS}	-0.098	0.029	-0.114	-0.180	-0.295	-0.088	-0.067	-0.088	-0.290	-0.128
	HWE	0.3562	0.1591	0.0062	0.0960	0.0152	0.6205	0.7149	0.0018*	0.0001*	-
ONP	Ar	3.00	2.99	4.73	4.76	2.00	3.50	2.99	4.00	4.72	3.635 ± 0.93
	Ho	0.714	0.419	0.676	0.943	0.386	0.444	0.333	0.550	0.885	0.594 ± 0.21
	He	0.627	0.558	0.670	0.651	0.312	0.502	0.481	0.669	0.670	0.571 ± 0.11
	F_{IS}	-0.127	0.261	0.006	-0.435	-0.229	0.129	0.320	0.190	-0.309	-0.027
	HWE	0.3366	0.0942	0.0377	0.0012*	0.3189	0.4695	0.0193	0.0979	0.00001*	-
GNP	Ar	1.00	3.62	2.00	2.86	1.96	2.88	2.00	3.00	3.96	2.589 ± 0.88
	Ho	0.00	0.605	0.265	0.225	0.042	0.659	0.605	0.452	0.825	0.409 ± 0.27
	He	0.00	0.524	0.230	0.262	0.080	0.516	0.422	0.546	0.588	0.352 ± 0.20
	F_{IS}	NA	-0.140	-0.143	0.038	0.486	-0.265	-0.424	0.183	-0.392	-0.158
	HWE	NA	0.0460	0.5784	0.0172	0.0638	0.0877	0.0081	0.0485	0.0005*	-
JNP	Ar	3.00	3.00	3.99	3.00	1.79	2.00	2.00	6.00	3.00	3.088 ± 1.22
	Ho	0.581	0.357	0.354	0.844	0.029	0.303	0.666	0.482	0.618	0.470 ± 0.23
	He	0.602	0.309	0.402	0.662	0.029	0.257	0.500	0.522	0.567	0.428 ± 0.19
	F_{IS}	0.052	-0.139	0.134	-0.260	0.000	-0.164	-0.321	0.096	-0.074	-0.084
	HWE	0.8437	0.9998	0.0185	0.1131	0.9998	0.9998	0.0838	0.1992	0.0768	-

Table 6: Estimates of pair wise F_{ST} between greater one-horned rhinoceros populations of India (* p<0.001)**

	PWLS	KNP	ONP	GNP	JNP
PWLS		***	***	***	***
KNP	0.06265		***	***	***
ONP	0.04018	0.02220		***	***
GNP	0.38221	0.25040	0.26971		***
JNP	0.16330	0.11575	0.09543	0.31290	

The AMOVA results showed 16.9% variation amongst the populations in comparison to 83.1% variations within populations (Figure 5).

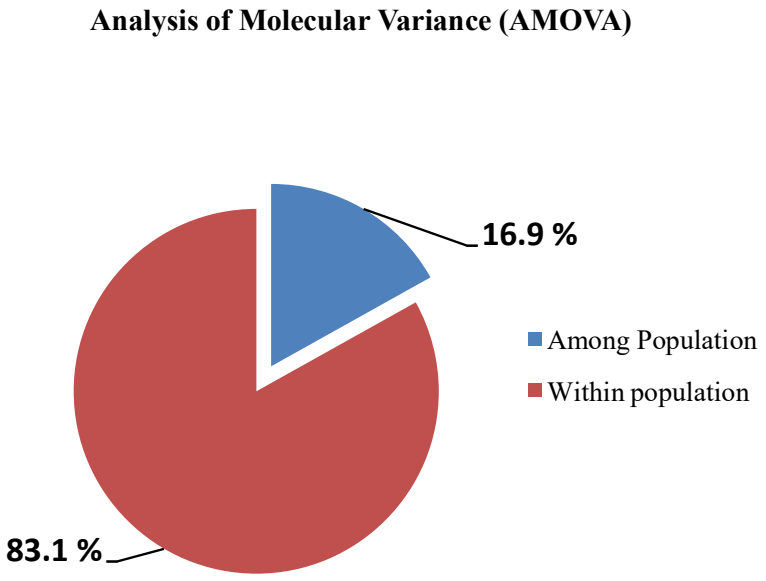

Figure 5: Estimates of percentage of variation among and within population of greater one-horned rhinoceros

The simulation run on the software 'STUCTURE' identified two modes of clustering (Figure 6), one at K=2 (ΔK= 1232.32) and the other at K= 5 (ΔK= 58.28). At K= 2, samples from PWLS, KNP, ONP and JNP were assigned to a single cluster while the samples from GNP were assigned to a separate cluster (Figure 7a). The average proportions of memberships (q) for each of the clusters were observed to be 0.9706 and 0.9707 respectively.

At mode K= 5 a greater level of admixture was observed among the individuals of different clusters. The individuals sampled from GNP were grouped to a single unique cluster with average membership proportion (q) of 0.911. Although, individuals sampled in JNP clustered together with individuals sampled from PWLS, KNP and ONP at K= 2, they formed a separate cluster at K= 5 with observed average membership proportions of q= 0.673, q= 0.668 and q= 0.675 respectively (Figure 7b).

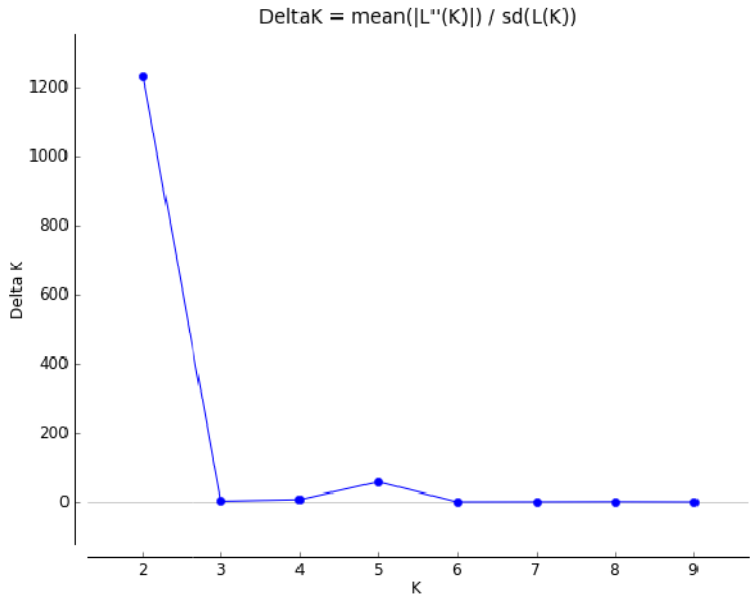

Figure 6: ΔK (adhoc) values corresponding to K from STRUCTURE simulation under admixture model with correlated allele frequency for rhinoceros samples from all populations. Two modes of clustering, one, at K= 2 and other at K= 5 were observed.

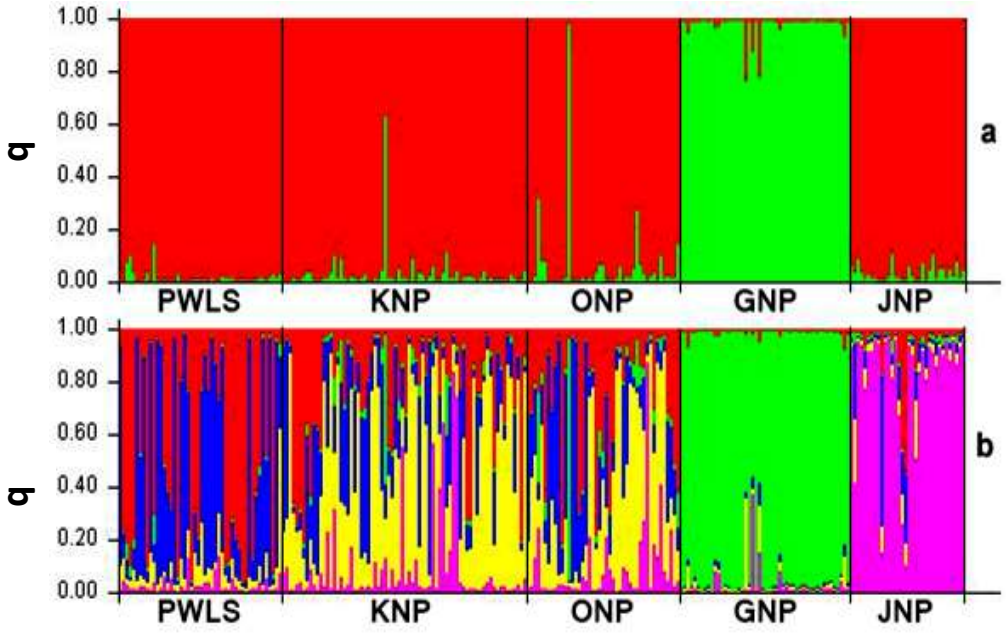

Figure 7: Bar diagram showing inferred cluster by STRUCTURE simulation under admixture model with correlated allele frequency at (a) K= 2 and (b) K= 5 for rhinoceros samples from all populations.

Keeping the aforementioned results in view, another set of 'STRUCTURE' simulations were run among the samples assigned to the cluster 1, at K=2, i.e., cluster encompassing rhinoceros populations of PWLS, KNP, ONP and JNP keeping the parameters used for simulation same as before. This was done to find out possible existence of fine scale population sub-structuring among PWLS, KNP, ONP and JNP, which was evident from the pair wise F_{ST} estimates. The ΔK value was observed to be highest at K=4. Here, the individuals from JNP were found to group into a unique cluster together with 7 samples from KNP and 3 samples from ONP (Figure 8). The average membership proportion was observed to be 0.786. Rest of the three clusters

showed admixure with observed average membership proportion of 0.621, 0.661 and 0.640 respectively.

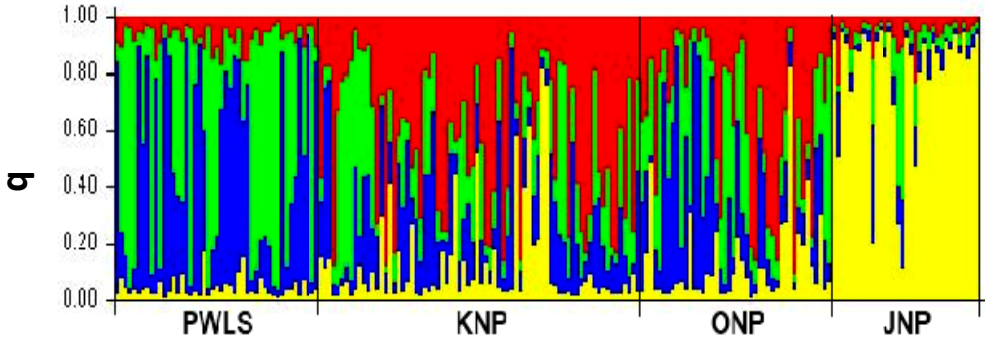

Figure 8: Bar diagram showing inferred cluster by STRUCTURE simulation under admixture model with correlated allele frequency at K= 4 for rhinoceros samples from PWLS, KNP, ONP and JNP.

The assignment test implemented in 'GeneClass2' also produced results that appeared similar to the 'STRUCTURE' simulations. A considerable amount of admixture was observed among the individuals sampled from the PAs of Assam. Only 25.5%, 20% and 9% of the individuals from these PAs were assigned correctly to their respective sampling locations i.e. PWLS, KNP and ONP. The remaining individuals were assigned to more than one population. However, 97% of the individuals from GNP and 70% of individuals from JNP were correctly assigned to their respective sampling locations.

The degree of genetic differentiation between STRUCTURE defined clusters were further evaluated in terms of pairwise F_{ST} values between JNP, GNP and populations of Assam combined as one population (STRUCTURE defined).

The pair wise F_{ST} estimates showed that, JNP differ significantly from Assam and GNP with corresponding pairwise F_{ST} values of 0.104 and 0.312 (p<0.001) respectively (Table 7). The pairwise F_{ST} between GNP and Assam was also observed to be high (F_{ST}= 0.255, p< 0.001).

Table 7: Estimates of pair wise F_{ST} between Assam population, GNP and JNP (* p<0.001)**

	Assam	**GNP**	**JNP**
Assam	-	***	***
GNP	0.25568	-	***
JNP	0.10471	0.31290	-

4.7. Past Bottleneck

Simulations conducted with software 'BOTTLENECK' showed significant deviation of expected heterozygosity (He) from heterozygosity expected at *Mutation Drift equilibrium* (Heq) in two rhinoceros populations of Assam, *viz.,* KNP (probability for heterozygosity excess p = 0.004) and ONP (probability for heterozygosity excess p = 0.018) indicating signatures of past bottlenecking in those two populations. However, it is interesting to note that the other rhinoceros populations did not show any signature of population bottleneck. Notably, even after changing the simulation parameters no appreciable change in the results was observed.

4.8. Detection of Migrants and Migrant Ancestry

The tests for migrant detection in software 'GeneClass2', 'STRUCTURE' and 'BayesAss' did not detect any migrants between the STRUCTURE defined populations of rhinoceros in India. However, a single rhinoceros individual sampled in ONP was detected as F_0 migrant ($p<0.005$) from PWLS by GeneClass2. The same individual was shown to have signature of migrant ancestry in STRUCTURE analysis at both v=0.05 (PP= 0.121) and v=0.10 (PP= 0.196). Analysis in BayesAss also identified the same individual sampled in ONP as migrant from PWLS (PP= 0.813) along with six individual rhinos sampled from KNP as first generation migrants (PP≥ 80%) from ONP (Table 8).

Table 8: List of individuals along with geographical origin, source population and posterior probability (PP) of migrant ancestry detected as migrants by software BayesAss v3.0.3

Sl. No.	Individual	Geographical Origin	Source Population	PP of Migrant Ancestry
1	Individual I	KNP	ONP	0.972
2	Individual II	KNP	ONP	0.890
3	Individual IIII	KNP	ONP	0.949
4	Individual IV	KNP	ONP	0.802
5	Individual V	KNP	ONP	0.858
6	Individual VI	KNP	ONP	0.834
7	Individual VII	ONP	PWLS	0.813

Simulations in BayesAss did not identify any considerable migration between the STRUCTURE defined rhino populations of India (Table 9). However, the mean migration rates (*m*) among the rhino populations of Assam were observed to be ≥ 0.056 (Table 10).

Table 9: Contemporary migration rates between STRUCTURE defined populations of greater one-horned rhinoceros. Values in parentheses indicate 95% confidence interval (or credible interval)

	Assam	GNP	JNP
Assam	-	0.006 (-0.002 to 0.014)	0.011 (-0.006 to 0.028)
GNP	0.008 (-0.007 to 0.022)	-	0.009 (-0.008 to 0.025)
JNP	0.015 (-0.012 to 0.043)	0.012 (-0.010 to 0.033)	-

Table 10: Contemporary migration rates between greater one-horned rhinoceros populations of Assam. Values in parentheses indicate 95% confidence interval (or credible interval)

	PWLS	KNP	ONP
PWLS	-	0.017 (-0.014 to 0.048)	0.014 (-0.012 to 0.039)
KNP	0.056 (-0.026 to 0.138)	-	0.139 (-0.047 to 0.324)
ONP	0.107 (0.047 to 0.166)	0.015 (-0.014 to 0.045)	-

4.9. Influence of Landscape Features on Gene Flow

Mantel's *r* correlation did not show any significant correlation between observed linearized genetic distance and geographic distance ($r = 0.3189$, $p = 0.1174$), log transformed geographic distance ($r = 0.2603$, $p = 0.1335$) as well

as cost distance (r = 0.3474, p = 0.0758). The scatter plot generated using allelic data set are represented in Figure 9.

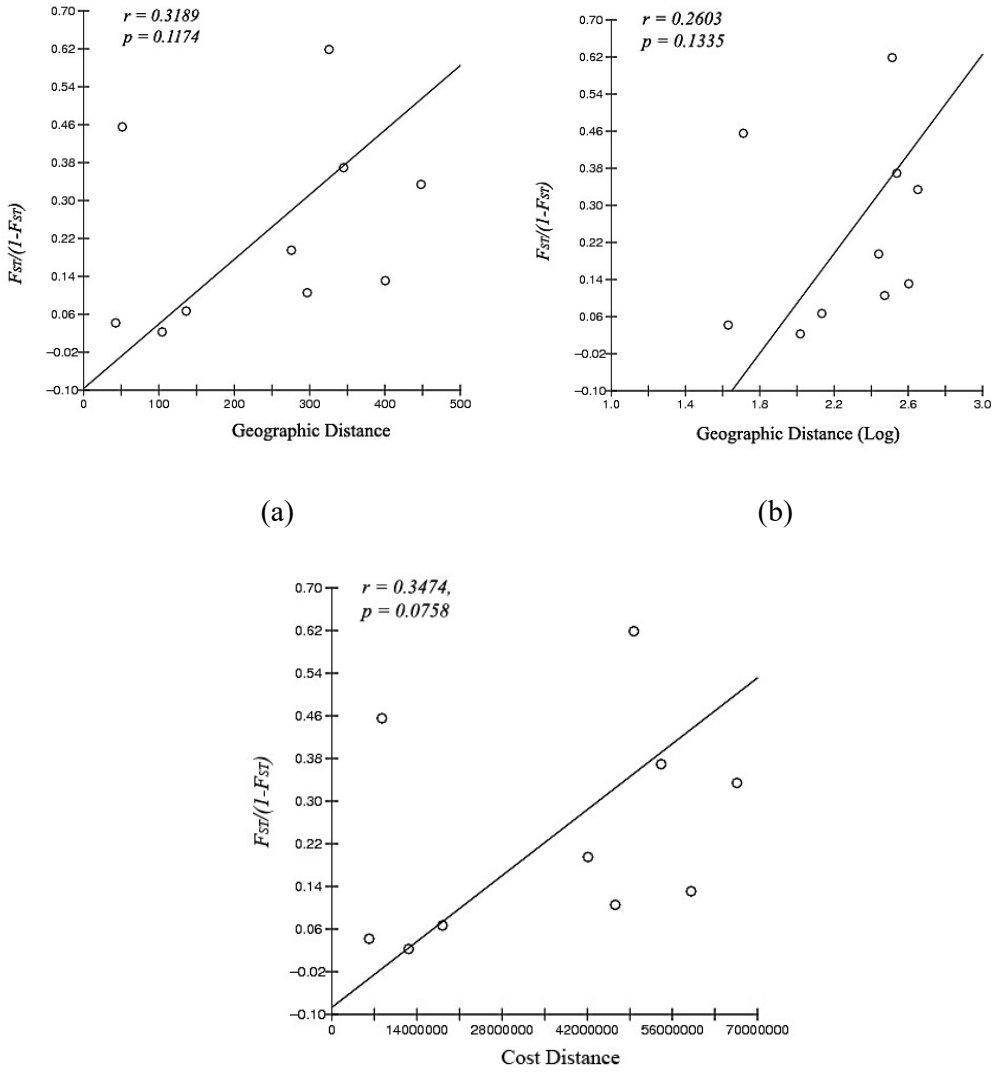

Figure 9: Scatter plot showing the relationship between linearized F_{ST} ($F_{ST}/(1 - F_{ST})$) with (a) geographic distance (r = 0.3189, p = 0.1174), (b) log transformed geographic distance (r = 0.2603, p = 0.1335) and (c) cost distance (r = 0.3474, p = 0.0758). The relationships were not found to be significant.

CHAPTER 5
DISCUSSION

In the present study the genetic status of greater one-horned rhinoceros populations of India was evaluated based on genetic information generated, following a noninvasive population monitoring approach. Although previous studies (Zschokke et al., 2011; Das, 2014) had evaluated the genetic status of greater one-horned rhinoceros, a comparative study involving all rhinoceros source populations from India was lacking till date. The results obtained in the present study provide an insight to the population processes acting upon the rhinoceros population of India at a landscape level and will be crucial in prioritizing the conservation needs of greater one-horned rhinoceros and designing an effective conservation action plan for the species.

5.1. Genetic Diversity and Bottleneck

The present study revealed that the level of genetic variability in greater one-horned rhinoceros population of India is moderate to high with estimated level of heterozygosity (He) ranging from 0.352 to 0.59. Zschokke et al., (2011) also reported similar results in greater one-horned rhinoceros population of India (He= 0.60) in a comparative study involving rhinos of India and Nepal (He= 0.45). However, Zschokke et al. (2011) in their study included only zoo samples with their origin from Assam as a representative population of India. Das (2014) also reported high level of haplotypic diversity as well as microsatellite diversity in

rhinos of Assam. Both, Zschokke et al. (2011) and Das (2014), however, did not include other source populations of India in their respective studies. Interestingly, Dinerstein and McCracken (1990) also reported higher level of heterozygosity in greater one-horned rhinoceros population of the Royal Chitwan National Park, Nepal based on allozyme allele diversity. But, it is noteworthy that, the level of heterozygosity obtained in the present study is not directly comparable to that of Dinerstein and McCracken (1990) as different marker systems were used in both the studies.

The observed level of heterozygosity in rhinoceros populations of Assam may be attributed to two factors. Firstly, the present rhino populations of Assam are connected through a chain of river islands of the river Brahmaputra. Migration of rhinos through such islands from one PA to another has been previously reported (Talukdar et al. 2007). Our results show that there is considerable amount of migration among these populations indicating that migration of individuals between these populations and their subsequent contribution to the gene pool could have helped in retaining higher levels of heterozygosity in these populations. Secondly, the long generation time (average generation time being 15 years; Wirz-Hlavacek et al. 1998) and lifespan of the rhinos might also have contributed in retaining the observed levels of diversity. The threat to the reduction of heterozygosity in a population with overlapping generation is often low in comparison to populations without overlapping generations (Harlt and Clark, 2006; Kekkonen et al., 2012). These factors together may have contributed together in retaining higher levels of heterozygosity in rhino populations of Assam. Furthermore, there are possibilities that the population size of greater one-

horned rhinoceros in Assam during the early part of the 20th century was larger than what was previously reported. Further, individuals from adjacent areas might also have immigrated into the region and contributed to the gene pool during succeeding period, thereby helping in retaining the observed level of genetic diversity in rhinos of Assam (Zschokke et al., 2011).

Although, evidence of past bottleneck was detected in KNP and ONP population, the level of genetic diversity in both the populations were observed to be high (He ≥ 0.571 and Ar≥ 3.606) which suggests that the effect of bottlenecking on genetic diversity of rhino populations of Assam is less prominent than previously thought. Reduction of genetic diversity, post bottleneck, depends on the duration of bottleneck, population size, gene flow, generation time of species concerned and severity of bottleneck (Chapman et al., 2011). Harley et al., (2005) observed high level of genetic diversity in African black rhinoceros (*Diceros bicornis*) suggesting known population bottleneck having limited effect on the extent of genetic diversity in black rhinos. African buffalo (Syncerus caffer) recorded a sharp decline in population size a result of Rinderpest epidemic that broke out in 1893 with mortality rate ranging from 90-95%. However, the species was observed to retain high levels of genetic diversity even after population crash of such intensity (van Hoof et al., 2000). Similar observations are also made in other species such as fur seals (*Arctocephalus gazella and Arctocephalus tropicalis*) in Antarctic and sub-Antarctic region Wynen et al., (2000). Zschokke et al. (2011) also reported bottlenecking in the greater one-horned rhinoceros populations of Assam, which according to them, occurred some 800 to 4200 years before, thus,

suggesting that the rhino population of Assam have suffered multiple events of bottlenecking.

The results obtained in the present study also showed that the level of heterozygosity in the rhinoceros population of West Bengal, viz., JNP and GNP were low (He= 0.428 and 0.352 respectively) compared to Assam i.e., KNP, ONP and PWLS (H_e= 0.590, 0.571 and 0.502 respectively). Although, the number of individual rhinos in GNP and JNP has gradually increased in last few decades, they had a rather fluctuating trend of population size in the past century and have been under constant biotic as well as anthropogenic stress (Bist 1994), which along with insignificant migration may have contributed to the observed estimates of low expected heterozygosity (Vucetich and Waite, 1999).

5.2. Population Differentiation and Genetic Structure

In the present context, the pair wise F_{ST} and the AMOVA results suggest that there is considerable amount of genetic differentiation among the greater one-horned rhinoceros populations of India, especially, the GNP population. The observed F_{ST} estimates between GNP and all other PAs appeared to be significantly higher (F_{ST}> 0.25; p<0.001).

It was interesting to note that, although GNP and JNP are located in close geographical proximity, the pair wise F_{ST} between the two rhino population was significantly high (F_{ST}= 0.31; p<0.001) between the two. STRUCTURE and other Bayesian based simulations assigned samples from GNP to a separate cluster which is also suggestive of a strong genetic differentiation between GNP

to the rest of the rhinoceros populations. 'STRUCTURE' simulation excluding GNP samples and the pairwise F_{ST} values observed between JNP and combined Assam population ($F_{ST=}$ 0.10; p<0.001) suggest that JNP also hold a unique genetic signature. These results clearly indicate that both JNP and GNP populations are genetically differentiated from rhino population of Assam as a whole. The observed results are important to testify the effect of genetic drift persistent in rhino populations of India that have been restricted to isolated patches only in recent times primarily due to anthropogenic factors. Similar magnitude of population structure within a short time scale was also observed in case of other species in India (Mondol et al. 2013).

Earlier, Zschokke et al. (2011) also observed a strong genetic differentiation between rhinos of Assam and Nepal. They reported a high pairwise F_{ST} estimate (F_{ST}= 0.202; p< 0.001) between the sampled populations based on microsatellite markers. Therefore, the positioning of rhinoceros populations of West Bengal with respect to the metapopulation genetic structure of rhinos in the region is important and this necessitates further study to examine the genetic relationship between the rhinos of Nepal and West Bengal.

5.3 Pattern of Migration in Greater One-horned Rhinoceros Populations of India

In the present study, a pattern of asymmetric migration was observed among the greater one-horned rhinoceros populations of Assam. The mean migration rates from PWLS to KNP (m= 0.056), PWLS to ONP (m=0.107) and ONP to KNP (m= 0.139) observed to be high. However, the expected proportion of migrants into

PWLS from ONP (m= 0.014) and KNP (m= 0.017), and similarly, to ONP from KNP (m= 0.015) were observed to be much smaller suggesting unidirectional migration among the populations. The possibility of existence of a source- sink structure within the rhinoceros populations of Assam cannot be denied. However, to ascertain the role of underlying factors driving such an asymmetric pattern of gene flow needs further study with data on habitat quality as well as social interactions, influence of environmental agents on dispersal and demographic parameters for multiple years as single point measurement of population parameters could be apparently misleading in many instances (Dias, 1996).

5.4. Effect of Landscape Features on Gene Flow

The results of Mantel test clearly indicate that geographic distance does not have a significant effect on observed level of genetic distance. The estimated landscape distance in the present study based on the information obtained from habitat heterogeneity and landscape features on a resistance surface (cost distance) did not show any correlations with the observed pair wise genetic distances among greater one-horned rhinoceros populations of India. Although, Mantel r correlation failed to establish any such association, yet, possible effects of landscape heterogeneity induced by factors such as human settlement, road network, agricultural land etc., on gene flow among the populations of India cannot be nullified. Possibly, the model used to estimate the biological cost involved in dispersal may have failed to resolve the true resistance imparted by landscape features, especially, human settlements on dispersal owing to the fact that the cost-distance model developed in the present study was based on

unsupervised classification of the satellite imageries. This could be overcome through supervised classification of the imageries used, along with using night light imageries to identify settlement and distribution of human population. However, this could not be achieved in the present study and needs to be explored in future.

It is also noteworthy that human disturbances can adversely influence behaviour of wild animals, which may have serious implications (Ciuti et al., 2012). Human activities and changes in habitat configuration may have their bearing on the behaviour of greater one-horned rhinoceros in terms of dispersal, especially, in case of individuals of GNP and JNP which are located in a human dominated landscape. However, no such study has been carried out till date to measure the extent of behavioural changes in rhinoceros in response to human disturbance and changes in the habitat configuration induced by fragmentation which needs further exploration.

5.5. Conservation Implications

From the present study, it is evident that there is a considerable amount of genetic differentiation among the greater one-horned rhinoceros populations of India, especially, the population of Gorumara National Park being significantly different from rest of the populations under investigation. In the present scenario of habitat fragmentation and persistent human disturbance, it is possible that these populations will continue to remain in isolation. However, prolonged genetic and demographic separation of rhinoceros populations is highly unwanted as it would lead to loss of genetic diversity through inbreeding and genetic drift. Immigration

and gene flow among these rhinoceros populations will, therefore, be crucial in maintaining the genetic variability and thus, long-term viability of the species. Although, there is genetic differentiation, to our view, the greater one-horned rhinoceros populations of India should be regarded as a single management unit unlike Zschokke et al. (2011) who recommended treating rhino populations of Nepal and Assam as separate management units based on similar results. The West Bengal populations lost their natural connectivity with the rest of the rhinoceros source population only in recent time and therefore, priority should be given for genetic restocking of these two populations by translocating individuals between JNP and GNP as well as Assam in order to avoid loss in genetic variability. Furthermore, steps should be taken to maintain the existing connectivity among the rhino populations of Assam which will ensure maintenance of genetic variability, thus, ensuring long term survival of the species in its natural habitat.