PHYLOGEOGRAPHY AND POPULATION GENETICS OF GREATER ONE-HORNED RHINOCEROS *(Rhinoceros Unicornis)* IN INDIA

Thesis submitted for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
WILDLIFE SCIENCE

by

TISTA GHOSH

to

Saurashtra University
Rajkot - 360005 (Gujarat)

Under the supervision of

Dr. Samrat Mondol, Scientist - E

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

August, 2022

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DECLARATION

I hereby declare that the work conducted under this thesis titled "**Phylogeography and population genetics of greater one-horned rhinoceros (*Rhinoceros Unicornis*) in India**" is a record of original and independent research work done by me and subsequently submitted for the award of the degree of **Doctor of Philosophy in Wildlife Science** to the **Saurashtra University, Rajkot (Gujarat)**. This research work has been carried out under the guidance and supervision of Dr. Samrat Mondol, Scientist-E of Wildlife Institute of India, Dehradun. The work has not formed the basis for the award of any other degree, diploma or any other qualification. I also declare that the thesis embodies my own work, analysis, observation, understanding and the particulars given in it are true to the best of my knowledge.

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CERTIFICATE

This is to certify that the thesis by **Miss Tista Ghosh** titled "**Phylogeography and population genetics of greater one-horned rhinoceros (*Rhinoceros Unicornis*) in India**" is an original and independent research work submitted to the **Saurashtra University, Rajkot (Gujarat),** for the award of the degree of **Doctor of Philosophy in Wildlife Science.**

Miss Tista Ghosh has put more than six semesters of research work embodied in this thesis under my guidance and supervision. The work presented in this prethesis has not been submitted to any other University or Institute for the award of any degree, diploma or distinction.

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I certify that the research work was appreciated by all who were present, and the comments made by the faculty and researchers have been appropriately included in the thesis.

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List of publications:

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Ghosh T, Sharma A, Mondol S. (2021) Optimisation and application of a forensic microsatellite panel to combat Greater-one horned rhinoceros (*Rhinoceros unicornis*) poaching in India. Forensic Science International: Genetics. 52:102472. https://doi.org/10.1016/j.fsigen.2021.102472.

Ghosh T, Kumar S, Sharma K, Kakati P, Sharma A, Mondol S. (2022) Consideration of genetic variation and evolutionary history in future conservation of Indian one-horned rhinoceros (*Rhinoceros unicornis*). BMC Ecology and Evolution. 92. https://doi.org/10.1186/s12862-022-02045-2.

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List of conferences:

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Ghosh T, Kumar S, Sharma K, Kakati P, Sharma A, Mondol S. (2021) A mixture of genetic uniqueness and diversity: Recipe for genetic rescue of Greater one-horned rhinoceros in India. Oral presentation (virtual) at International Congress of Conservation Biology (ICCB), Rawanda, 16th December, 2021.

Ghosh T, Kumar S, Kakati P, Sharma A, Mondol S. (2022) Insight into RhODIS-population assignment of seized rhino horns to identify their origin. Oral presentation at Student Conference on Conservation Science (SCCS), Cambridge, 29-31st March, 2022.

Institutional conferences

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Ghosh T, Sharma A, Mondol S. (2018) Insight into RhoDIS: A molecular approach for rhino forensics and population management. e-poster presentation at XXXIInd Annual Research Seminar, September10-September 20, Wildlife Institute of India, Dehradun, India.

Ghosh T, Kakati P, Sharma A, Mondol S. (2019) DNA detective: population assignment of seized rhino horn to identify its origin. Oral presentation at XXXIIIrd Annual Research Seminar, August 19-August 27, Wildlife Institute of India, Dehradun, India.

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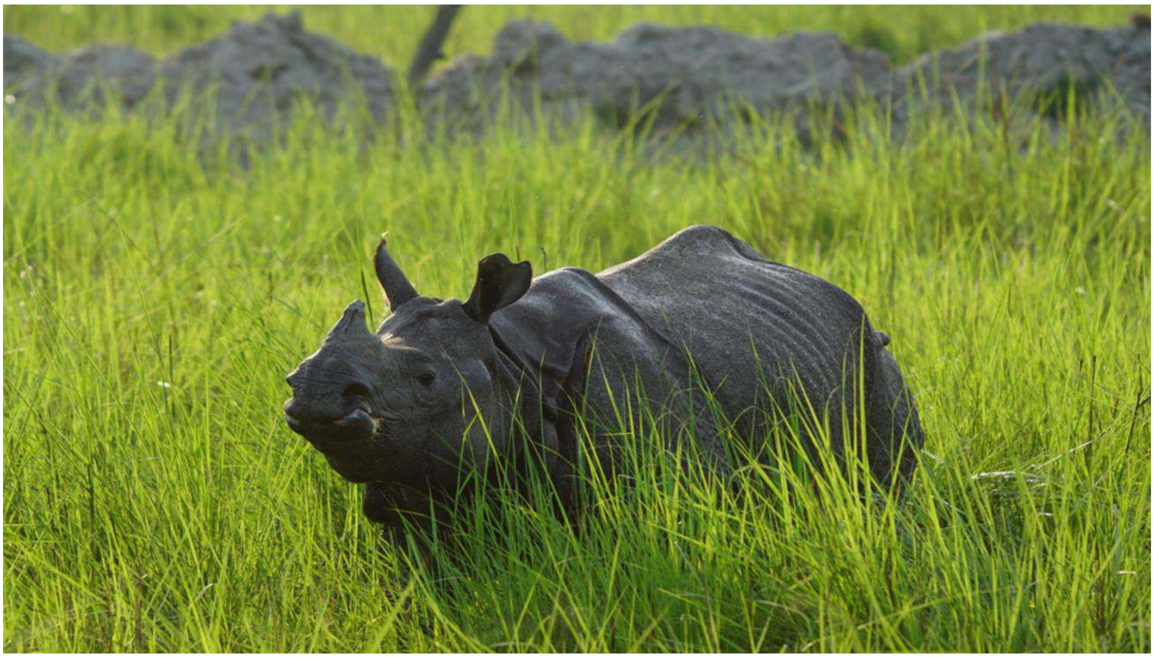

Introduction

1.1 Background and review of literature:

Globally 60% of the large herbivores are threatened with extinction due to continuous anthropogenic pressures in the form of defaunation, habitat encroachment, poaching and human-wildlife conflicts (Braje et al., 2013; Ripple et al., 2015; Lindsey et al., 2017; Mahmood et al., 2021). Such megafaunal biodiversity loss has major top-down effect on vegetation structure and composition, thus altering the ecological processes such as nutrient cycling, hydrology, fire regime etc. (Malhi et al., 2015; Ripple et al., 2015; Le Roux et al., 2018). These have cascading effects on other animals ranging from small herbivores to large carnivores, thus resulting in loss of major ecological services (Ripple et al., 2015; Le Roux et al., 2018). Significant efforts have been made for recovery of the large herbivores in terms of their population size, historical ranges or focusing on the external threats faced by them (Emslie and Brooks 1999; Madhusudan 2004; Brodie et al., 2011; Williams, 2011; Sylven et al., 2012; Rookmaker et al., 2016; Lindsey et al., 2017). Although these endeavours are made to reduce the risk of extinction, the success is mostly quantified only by population growth measurements, which may not be a direct indicator of increased absolute fitness (Coleman et al., 2013; Whiteley et al., 2015). Instead there is a plethora of studies both empirical and experimental that show direct relation of genetic diversity with fitness (Mills et al., 1996; Tallmon et al., 2004; Hogg et al., 2006; Keller et al., 2010; Heber et al., 2013; Lacy et al., 2013; Whiteley et al., 2015; DeWoody et al., 2021). Further recent studies on species recovered from bottleneck event indicate that they are prone to genetic erosion as a result of fragmentation, lower effective population size and amplified population structuring (de Flaming et al., 2015; Goosens et al., 2016; Moodley et al., 2017 and 2018; Modi et al., 2021; Biswas et al., 2022). In fact, these negative consequences are found to be acute for herbivores with large body size (Lino et al. 2019; Jhala et al., 2021) and specialised habitat requirements (Prugh et al., 2008; Karanth et al., 2010). Thus assessing the genetic diversity of the extant populations is important for monitoring purpose as well as for establishing new habitats (Mace et al., 2010; Coleman et al, 2013; Ralls et al., 2018; O'Brien et al., 2017; Weeks et al., 2017; Garner et al., 2020). This will ensure resilience in the recovered population by maintaining biodiversity and evolutionary potential of the threatened species (Fredrickson et al., 2007; Benson et al., 2011; Johnson et al., 2014; Whiteley et al., 2015; Scott et al., 2020; Willi et al., 2022).

The greater one horned rhinoceros (*Rhinoceros unicornis*) (GoH rhinoceros henceforth) typifies such endangered group of megaherbivores endemic to grasslands of India and Nepal. It is the second largest of the five extant genus of rhinos present in the world and the fourth largest mega herbivore (Johnsingh and Manjrekar 2015). Historically the GoH rhinoceros was distributed throughout the Indian subcontinent, ranging from the Punjab foothills, Peshawar, Sind and lower Indus from far west to northeastern India, with discrete records of its existence from Bangladesh, China and Burma (Rookmaker 1980, Rookmaker et al., 2016) (Figure 1.1). At present they are found in 11 protected areas across the northern part of the Indian sub-continent: four in Assam i.e. Kaziranga National Park (NP), Pobitora Wildlife Sanctuary (WLS), Orang NP and Manas NP; two in West Bengal i.e Gorumara NP and Jaldapara NP, one in the state of Uttar Pradesh i.e. Dudhwa NP and four in Nepal, Chitwan NP, Bardia NP, Sukhlaphanta Wildlife Reserve and Parsa NP. With a present population size of ~3000 individuals (~83% of global species population, Ellis and Talukdar 2019) Indian rhinos have faced severe range contraction and population decline owing to rapid habitat loss and poaching pressure (Menon 1996). The decline started with habitat loss and sport hunting from mid-1800 to 1900 (Menon 1996; Rookmaker et al., 2016). It took a severe turn from 1960s when about 46% of the entire population were lost in about 20 years due to consistent poaching pressure (Menon 1996). Since the beginning of 20th century significant conservation efforts including anti-poaching programmes, translocation, re-introduction etc. have helped in its recovery. Now it is considered as vulnerable by IUCN Red List and the species is listed in Appendix I in CITES and Schedule I of Wildlife Protection Act of India (1972 i.e. highest level of protection).

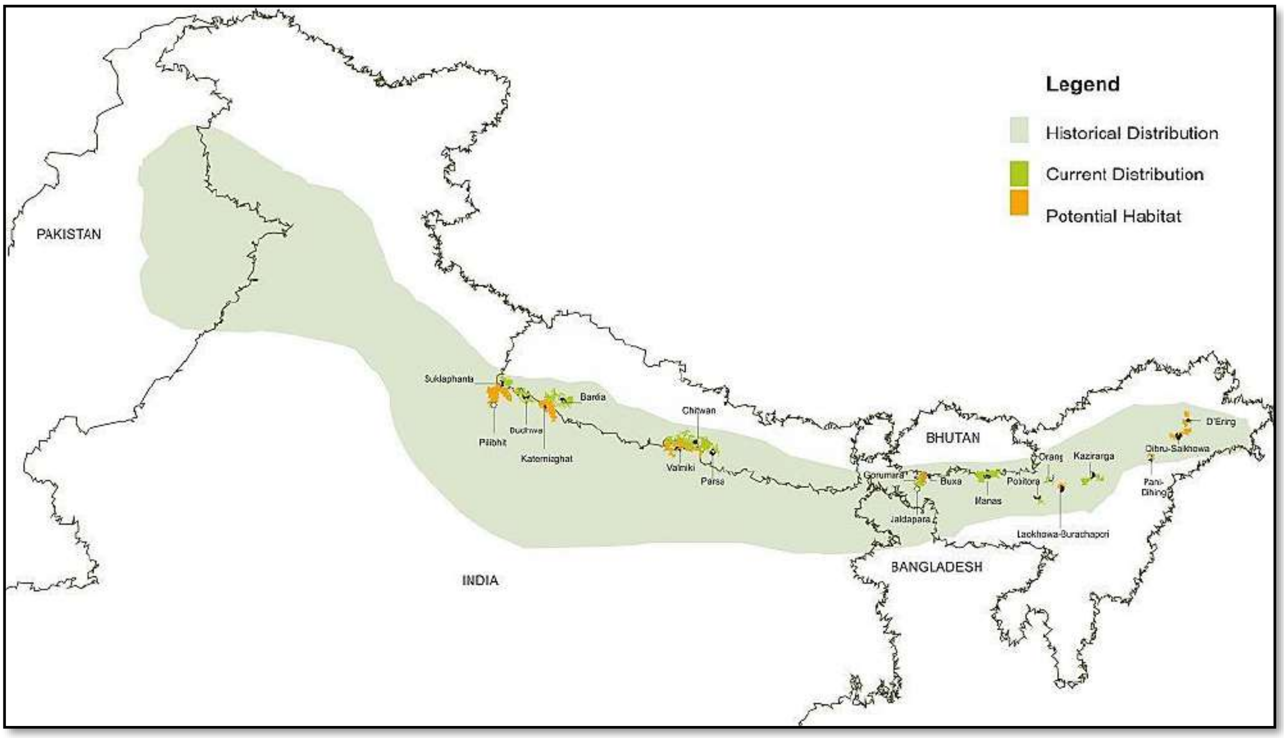

Figure 1.1: The figure (Rookmaker et al., 2016) represents the past distribution (grey shaded area) of one-horned rhinoceros across the floodplains of Indus, Ganga and Brahmaputra of Indian subcontinent. Green areas indicate current distribution whereas the orange depicts the potential habitats for rhino translocation within India.

The revival of GoH rhinoceros is one of the successful conservation stories in Indian wildlife history. However, with increasing population trend and continuous decline of grasslands especially due to biological invasions within protected areas of India (Sinha et al., 2022), most of the rhino bearing parks are reaching their carrying capacities (Jhala et al., 2021). As a result future management plans for the species are directed towards grassland restoration, identification of potential habitats and reintroduction programmes (Rookmaker et al., 2016; Jhala et al., 2021; Talukdar et al., 2021; Sinha et al., 2022). All these efforts are made to achieve the goal of large population size with the expectation of maintaining genetic diversity. But most of the time these revived populations are fragmented and suffers from genetic erosion due to bottleneck effects (Coleman et al., 2013; Mondol et al., 2013; Moodley et al., 2017). Thus in addition to habitat rehabilitation, the management plans like translocations/reintroduction should aim towards genetic rescue to minimise the effects of genetic erosion (Fredrickson et al., 2007; Johnson et al., 2010; Ralls et al., 2018; Weeks et al., 2017; Willi et al., 2022). It has also been suggested in Convention on Biological Diversity for maintenance of viable populations in wild (Garner et al., 2010; Mace et al., 2010). Compared to other rhino genus where detailed genetic assessments of demography, behaviour and evolutionary aspect of species (Steiner et al., 2017; Moodley et al., 2017 & 2018; Mays et al., 2018; Margaryan et al., 2020) are available, there is a dearth of knowledge in case of Indian rhinos (Pant et al., 2020). The literature present in hand like Zschokke et al., (2011 & 2016), Das et al., (2015) and Borthakur et al., (2016) give preliminary baseline information on Indian rhino genetic diversity (see Table 1.1 for details). However, due to limited geographical focus and analytical approaches, none of the studies can connect the genetic pattern with the life history traits of the species. Given these facts, studying Indian rhino genetics will help in gaining a better understanding of genetic variation and how it is influenced by population decline and habitat fragmentation.

The second cause of concern for current population of Indian rhinos is the continuously growing poaching pressures (Rookmaker et al., 2016; Ewart et al., 2018; Eikelbloom et al., 2020). The steep decline in rhino population was not only the outcome of habitat shrinkage but was also driven by their rampant killing in late 1900s due to political unrest in north east India (Menon 1996). Similar to its other sister groups Indian rhino is poached for their horns as status symbol and/or consumed as Chinese medicines in South-east Asian countries (Ewart et al., 2018; Eikelboom et al., 2020). The situation is severe because of the geographical proximity of India to two of the major consumer countries, Vietnam and China besides Myanmar, the primary route for cross border illegal trading of rhino horns (Emslie et al., 2016). Thus, to ensure the species' survival, it is important to improvise our ways of tackling wildlife poaching.. The illegal wildlife body trafficking (IWT) is a result of complex network that involves multiple middlemen ensuring the safe transfer of body parts from source to consumers (Wasser et al., 2007; Mondol et al., 2014). Finding out these trading spots is critical to break the network which can be done by identifying the origin/source population of seized sample (Mondol et al., 2014; Wasser et al., 2015). However, detecting the origin of seized wildlife body parts is challenging, especially for large free ranging mammals (Linacre et al., 2011; Ogden et al., 2015). In such scenarios, the wildlife forensic genetics has been successful by identifying trading routes and poaching hotspots (Wasser et al., 2007 & 2015; Mondol et al., 2014; Harper et al. 2018), thus strengthening on-ground law enforcement. The best example of one such effort is the Rhino DNA Indexing System or RhoDIS developed by Veterinary genetics laboratory, University of Pretoria in partnership with the South African National Parks supported by WWF for African rhinos. Under this project a panel of 23 microsatellite markers and one sex marker were standardised for African rhinos (Harper et al., 2013) to generate a DNA fingerprint library which were successful in tracing the seizures (Vietnam, Yemen etc.) back to their origin (Harper et al., 2018). For Indian rhino, identifying species from seizure (Jha et al. 2017; Ewart et al., 2018) and differentiating it from the fake ones is well established (Jha et al., 2017). But no measures has been taken regarding the DNA profiling or population origin which can help in pointing out poaching hotspots or trade routes for rhino horns (Table 1.1). Thus generating such database will be important to collect systematic information on illegal rhino horn trade, subsequently linking potential perpetrators to specific crime scenes and thus helping in prosecutions.

Table 1.1: Details of the paper available on genetic studies of Greater one-horned rhinoceros

Subject	Citations	Objective	Key findings
Population genetics	Zschokke et al., 2011	Genetic differentiation between rhino populations of Chitwan NP, Nepal and Kaziranga NP, India.	Two populations are significantly distinct from one another and were suggested to conserve as separate units
	Das et al., 2015	Population genetic assessment of extant populations (except two reintroduced ones) of rhinoceros in India.	They found Gorumara NP, Jaldapara NP and Assam populations as three distinct genetic subpopulations (K=3)
	Borthakur et al., 2016	Conducting a pilot study for carrying non-invasive population census in Gorumara NP, West Bengal	They identified 43 unique individuals out which 34 are males and nine female
	Zschokke et al., 2016	Review on available genetic studies. It also present the result of outbreeding and inbreeding between the rhino individuals of Nepal and Assam based on the studbook data	This paper suggests that outbreeding is not an issue in One-horned rhino populations
Phylogenetics	Xu et al., 1997	Whole mitogenome data of One-horned rhinoceros using a zoo individual	This is the first paper providing information on one-horned rhino mitogenome
	Steiner et al., 2011	Molecular phylogeny and evolution of perissodactyls	Confirm the topology among the extant rhino genus and supported the sister cladding of Javan and one-horned rhino
Forensic genetics	Jha et al., 2017	Species identification of One-horned rhino horn	Standardisation of mitochondrial marker for court case prosecutions in alleged Indian rhino crime cases

This doctoral work aimed to generate an exhaustive genetic database at landscape level for the Indian GoH rhinoceros which can be used for population management plans (like translocation programmes) and scientific evidence in court prosecutions of alleged rhino poaching crimes. The study focuses on assessing the evolutionary history and genetic health of each rhino bearing areas in India to suggest conservation mitigation plans at the park level. Further, it also attempts to generate an allele frequency map of forensic standards for a large trade-targeted mammal. This thesis re-emphasizes the application of genetic data for making long term conservation plans and promotes its inclusion in future efforts as proven in many other cases (Fredrickson et al., 2007; Hostetler et al., 2010; Johnson et al., 2010; Benson et al., 2011; Knuas et al., 2011; O'Brien et al., 2017; Weeks et al., 2017). In conclusion, the doctoral study focuses on securing the genetic diversity of extant populations of Indian rhino by providing information for the targeted conservation effort.

1.2 Study area:

This doctoral study was conducted across all the seven rhino bearing area of India distributed in three states of Assam (Kaziranga National Park (NP), Orang NP, Pobitora Wildlife Sanctuary (WLS) and Mans NP), West Bengal (Gorumara NP and Jaldapara NP) and Uttar Pradesh (Dudhwa NP) (Figure 1.2). Currently they harbour ~3000 rhino individuals ranging from 40-2413 population size with varying density (0.2-6/km^2). Details of each park with respect to its recovery history, density, grassland area and connectivity status (isolated or connected) are discussed in Table 1.2. These parks are present along the floodplains of Ganga and Brahmaputra which comes under the category of Terai and riverine alluvial grasslands (Rawat and Adhikary 2015).Main feature of this region is the mosaic of forest-grassland-wetland complex resulting alluvial rich soil with high silt deposition owing to tall grasslands. The dominant grass species is kans grass (*Saccharum spontaneum*) which colonise the silt plains after the monsoon, whereas in the hilly areas the dominant tree is sal (*Shorea robusta*), which can grow to a height of 45 m (148 ft). The vegetation is alluvial inundated grasslands and reedbeds, alluvial savannah woodland, tropical moist mixed deciduous forests, tropical semi-evergreen forests (Rawat and Adhikary 2015). It is the habitat of many charismatic mammals like Asian elephant, tigers, wild buffaloes, swamp deer, hog deer, fishing cats etc. along with wetland birds like Black-necked stork, sarus crane, bar-headed goose.

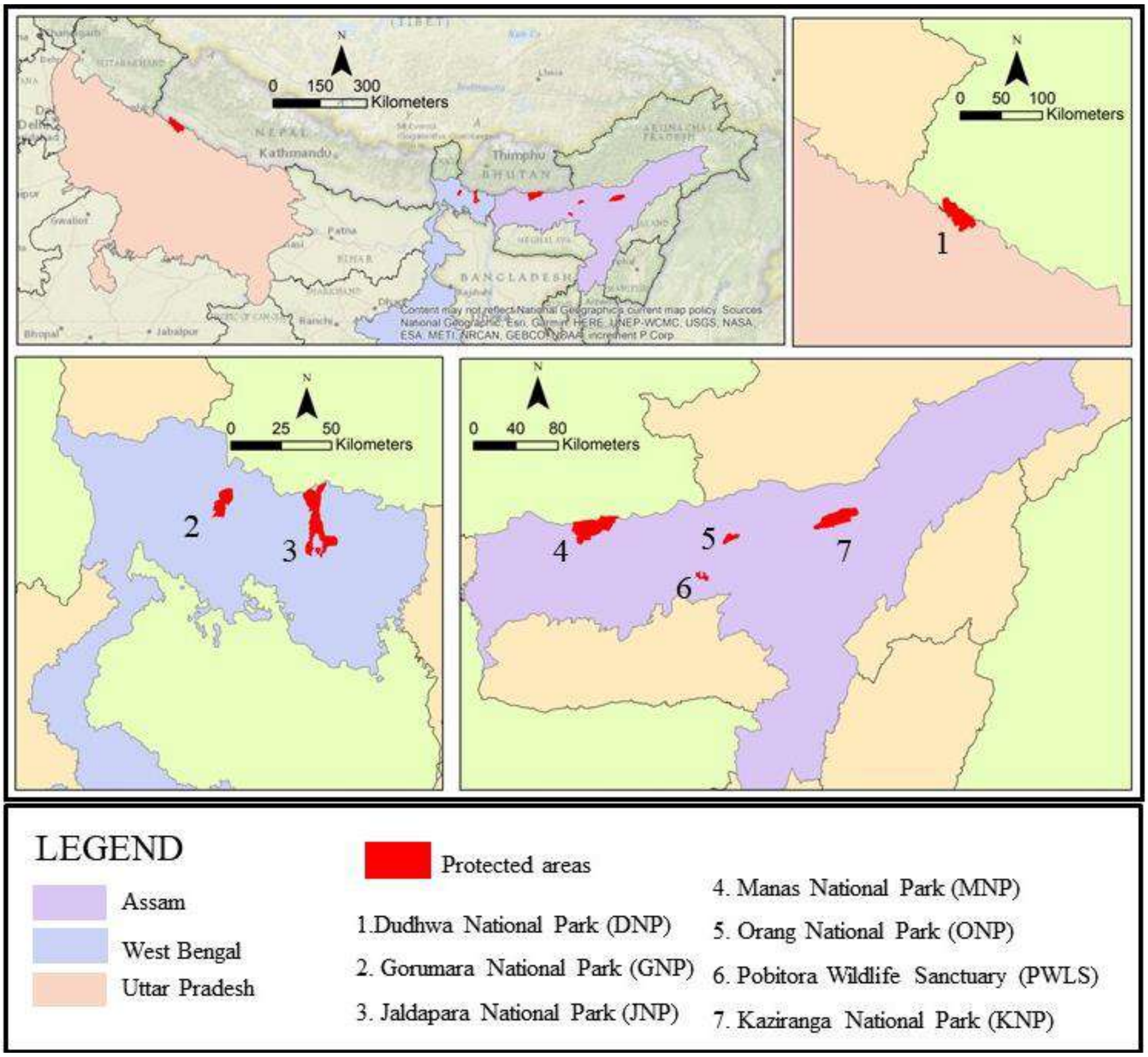

Figure 1.2: Map of the study area encompassing all the seven rhino bearing areas of India.

Table 1.2: Brief introduction of study area in relation to the density, population size, recovery history and connectivity status of all seven rhino bearing parks of India

State	Park	Population size	Density/ km^2	Recover history	Grassland area (km^2)	Connectivity status	References
Uttar Pradesh	Dudhwa NP	42	1.68	Reintroduced by seven founders. 4 from Chitwan NP of Nepal and 3 from Pobitora WLS, Assam	25(Rhino enclosure)	Isolated	Talukdar et al., 2012
West Bengal	Gorumara NP	52	0.62	Revived from few individuals (14-19) post population crash in mid 90s due to poaching	89.05	Isolated	Bist 1994; WB report 2019
	Jaldapara NP	~250	1.43		165.23	Isolated	
Assam	Manas NP	48	0.28	Reintroduced from 2010-2020 through multiple supplementation from Kaziranga NP (12), Pobitora WLS (10) and CWRC (13)	174	Isolated	Talukdar et al., 2021; Sinha et al., 2022
	Pobitora WLS	101	6.25	54 individuals recorded in 1987 after Wildlife sanctuary declaration. Forest department records shows presence of 14 odd rhinos during 1961-1962	16	Isolated but there are reports of rinos straying out to Orang NP during monsoon	Talukdar 2000; Talukdar et al. 2007 and 2021
	Orang NP	100	1.26	Localised by animals from Kaziranga NP post Laeishmania calamity in 1900	79	Connected via patches of grasslands along Brahmaputra floodplains. Since Orang is highland, many times rhinos of KNP are fled here post monsoon	
	Kaziranga NP	~2500	5.53	Revived from few hundred individuals post population crash in mid 90s due to poaching pressures	432		

1.3 Research questions:

The focus of this study is to use non-invasive genetic tools for population management plans and generate a database to counter alleged rhino crime cases in India. The specific research questions are:

- How the populations of Indian one-horned rhino are genetically structured?
- What is the evolutionary history of the species?
- What population specific genetic parameters are of management interest to devise a scientifically supported genetic rescue/restoration plan?
- How a genetic database can be used to counter rhino poaching threat in India?

1.4 Objectives:

The above mentioned research questions are addressed by following objectives:

- Mitogenome sequencing and optimisation of DNA profiling system for Indian one-horned rhinoceros
- Investigating the phylogeography and evolutionary history of one-horned rhinoceros
- Assessment of rhinoceros genetic health across India
- Developing DNA database for population assignment of rhino contraband

1.5 Thesis structure:

Limited information is available on the genetic status of Indian rhino populations. Considering their future survival is dependent on management plans like translocation and reintroduction, it is critical to assess the genetic health of all populations. Further the reports of increase in IWT due to Covid pandemic and economic boost in south-east Asia (Eikelbloom et al., 2020; Fukushima et al., 2021) demands urgent attention towards implementing new strategies to counter rhino poaching threats. Hence this doctoral study is a first pan India rhino genetic work which aids in devising site specific mitigation plans and generates a forensic standard database for court prosecutions.

Generating landscape level genetic data have become easier because of technological advancements in non-invasive conservation genetic field. Since this thesis aimed to use the database for both population genetics and forensic work , the 1st chapter gives introduction to the types of markers used and detailed methodological outline of their respective standardisation process.

The 2nd chapter deals with phylogeography patterns and evolutionary history of the species, specifically investigation how the populations are structured into maternal clades.

The 3rd chapter deals with the current genetic status of Indian rhino followed by identification of the population/s with poor genetic health that needs urgent management attention.

Finally the 4th chapter deals with the forensic application of this database where the aim is to generate allele frequency map (AFM) to identify the origin of rhino contraband.

In conclusion, the key findings of these thesis is summarised and their implication in conservation plans for one-horned rhinoceros is discussed.

Overall the goal of this thesis is not only restricted to Indian rhino conservation, but it also attempts to highlight the importance of genetic studies for planning long term management plans for fragmented populations. I believe the methodological approach discussed in each chapter will be helpful in attempting similar studies for other threatened large mammals. Lastly, by aiding in rhino conservation, the study will also contribute to the protection of grassland ecosystem as this charismatic mega-herbivore is flagship bearer for the neglected habitat and its dependent species.

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Chapter 1: Mitogenome sequencing and optimisation of DNA profiling system for Indian one-horned rhinoceros

2.1 Introduction:

In India, burgeoning pressures of human interventions have resulted in significant population decline and range contraction of megafauna biodiversity. The situation is worse for grassland dependent species because of severe encroachment pressures (Task Force on Grasslands and Deserts report, 2006). Greater one-horned rhinoceros typifies such endangered grassland species (Karanth et al., 2010; Ripple et al., 2015; Pant et al., 2020) and being trade targeted, their future survival is very much dependent on effective management plans. In this regard, DNA-based technologies come to aid by monitoring the genetic diversity to track the population viability of threatened species (Willi et al., 2022). Further, use of non-invasive samples, specifically faeces, provides more efficient ways to gather information due to large sample size and spatial coverage. However, faeces are prone to degraded DNA quality because of various environmental exposures like sunlight, UV rays and precipitation (Biswas et al., 2019) leading to various types of technical challenges such as non-amplification, erroneous allele calls (genotyping errors), discrepancy in sequence data generation etc. (Pompanon et al., 2005). Therefore, significant attention is required in selection of markers and their optimisations as it is logistically difficult to consider a sample-based standardization approach (Wan et al., 2004; Pompanon et al., 2005). This chapter focuses on standardising a set of mitochondrial (mtDNA) and microsatellite (STR) markers to address questions related to the Indian rhino phylogeography and population genetics.

The above mentioned aspects of population biology (phylogeography and population genetics) can be investigated using a range of different molecular markers (Wan et al., 2004, Thomson et al., 2010). While much newer genomic markers (such as SNP, CNV etc.) have shown to derive strong inferences in population genomic studies (Wan et al., 2004, Thomson et al., 2010), their use in non-invasive samples has been very limited. It was therefore decided to use mitochondrial DNA (mtDNA) and microsatellite markers (STR) to study Indian rhino genetic pool. In particular, entire mtDNA was targeted to assess the genetic variation as earlier studies used them to study their divergence or phylogenetic resolution from African and other Asian rhinos (Xu et al., 1997; Steiner et al., 2017). In addition, it would also help to avoid biases in data interpretation of divergence time, demographic history and phylogenetic relationships (Subramanian et al., 2009; Knaus et al., 2011). Hence in this chapter, one-horned rhino mitogenome data is generated across its Indian distribution to screen all the polymorphic sites for investigating the diversity and evolutionary history in forthcoming chapters.

On the other hand, microsatellites (STR) were chosen as the preferred nuclear marker as they facilitate individual identification, inbreeding status, genetic differentiation etc. along with forensic applications. Given the trend of increasing poaching pressures for rhino horn (Rookmaker et al., 2016), microsatellites have shown great potential to address poaching and trade issues at national as well as international scales (Mondol et al., 2014; Wasser et al., 2015; Harper et al. 2018). The Veterinary Genetics Laboratory, University of Pretoria has effectively employed the Rhino DNA Indexing System (RhoDIS) across South Africa and other African rhino range countries to help in rhino crime case convictions (Harper et al., 2018). While this system is promising in the context of GoH rhinoceros conservation and managing the threats of poaching, it has not been tested or optimised in Indian rhinos. This chapter thus addresses the key methodological issues suggested by International Society for Forensic Genetics (ISFG) guidelines (Linacre et al., 2011; Johnson et al., 2014) to systematically screen dinucleotide microsatellite markers for forensic use in Indian GoH rhinoceros. In addition, DNA extraction protocols from rhino horns were tested based on the protocols described in earlier studies (Harper et al., 2013; Jha et al., 2017; Ewart et al., 2018). Finally, the microsatellite panel was used to match poached carcass samples with seized rhino body parts as scientific evidence in court proceedings.

2.2 Methods:

2.2.1 Sample collection:

All optimisation of laboratory protocols were performed with wild Indian GoH rhinoceros tissue samples collected by trained persons of the respective forest departments. These samples were collected from naturally dead rhinos during post-mortem examinations or during rhino translocation programs. For mtDNA marker selection, 15 tissue samples representing all seven rhino bearing areas of India (more than one individual from each park) were selected. Further, one tissue sample was also received from Valmiki National Park (henceforth NP), Bihar forest department assumed to be representing the wild rhinos of Nepal (Letter/no.-1296 dated 16.10.2020) (Table 2.1). In case of microsatellite standardisation, a total of 96 field collected tissue samples (including earlier 15 samples) and four reference rhino horn samples from Wildlife Institute of India’s (WII) national repository of Wildlife Forensics and Conservation Genetics Cell were used (Table 2.1). Once brought to the laboratory, the samples were catalogued and stored in -20ºC freezer until further processing.

Table 2.1: Distribution details of the samples used in this chapter for mitochondrial (mtDNA) and microsatellite (STR) work

State	**Protected areas**	**Sample distribution**	
		mtDNA	**STR**
Uttar Pradesh	Dudhwa NP	2	4
West Bengal	Gorumara NP	2	2
	Jaldapara NP	2	4
Assam	Manas NP	1	1
	Pobitora WLS	2	11
	Orang NP	3	2
	Kaziranga NP	3	72
Bihar*	Valmiki NP	1	NA
	Total	**16**	**96**

2.2.2 DNA extraction:

DNA was extracted from blood samples using QIAamp DNA Tissue Kit (QIAGEN Inc., Hilden, Germany) following the standard protocol for blood samples. To extract DNA from various quality rhino tissue samples, the protocol described in Mondol et al., (2013) was used. In brief, about 20 mg of tissue was macerated with sterile blade and digested with 30 µL of Proteinase K (20 mg/mL) and 300 µL of ATL buffer (Qiagen Inc., Hilden, Germany) overnight at 56 ºC, followed by Qiagen DNeasy tissue DNA kit extraction protocol. DNA was eluted twice in 100 µL preheated (70 ºC) 1X TE buffer. For every set of samples (n = 11), one extraction negative was included to monitor possible contaminations.

For implementation of this protocol for forensic purpose it was needed to optimise the DNA extraction protocol from rhino horns as the DNA quality from the horns can be variable due to presence of keratin, which acts as a PCR inhibitor (Campos et al., 2012). Problems with downstream processing of rhino horn DNA can further be compounded due to small quantities of starting material (e.g. chopped pieces and chips or powders) received for casework. We tested two different DNA extraction protocols that use Qiagen DNeasy tissue DNA kit (QIAGEN Inc., Hilden, Germany) on four rhino horn samples from WII tissue repository and compared the DNA yield and amplification success for microsatellite markers. The first protocol was exactly the same used for rhino tissue samples (see above) whereas in the second method, a dekeratinizing agent DTT (Dithiothreitol) was used during initial lysis (Campos et al., 2012; Jha et al., 2017; Ewart et al., 2018). The macerated rhino horn base tissue (~20 mg weight) were lysed for 16 h at 56ºC with 30 µL of 1 M DTT, 30 µL of Proteinase K (20 mg/ ml) and 300 µL of ATL buffer (Qiagen Inc., Hilden, Germany) with intermittent vortexing. Post-lysis remaining steps were followed as described in Qiagen DNeasy tissue DNA kit with inclusion of extraction negatives to monitor possible contaminations.

2.2.3 Mitogenome data generation:

2.2.3.1 PCR amplification and sequencing

Mitogenome sequencing was performed using already published 23 overlapping primers (Hassanin et al., 2009). For annealing temperature standardisation, gradient PCR was set in 10 µl reactions containing 4 µl of 2X Qiagen PCR buffer mix (QIAGEN Inc., Hilden, Germany), 1 µl of primer (3 µM), 2 µM BSA (4 mg/ml), 1.4 µl of RNase free water and 5 ng of rhino tissue DNA. PCR conditions included an initial denaturation (95 °C for 15 min); 35 cycles of denaturation (95 °C for 30 s), annealing (50-60 °C gradients for 40 s) and extension (72 °C for 40 s); followed by a final extension (72 °C for 10 min). During each set of reactions, PCR and extraction negatives were included to monitor contamination. Amplified products were visualized with 2% agarose gel, cleaned with Exonuclease (Thermo Scientific, Waltham, USA) and Shrimp Alkaline Phosphatase (Amresco, Solon, USA) mixture and sequenced bidirectionally in an ABI 3500XL bioanalyzer (Applied Biosystems). Out of these 23 primers, two did not show amplification in any samples. The remaining sequences (n=21 from 16 individuals) were aligned with the available one-horned rhino mitogenome (Genbank: X97336, Xu et al., 2016) in Mega v7 (Kumar et al., 2016). Two primers were designed manually in the flanking conserved regions adjacent to the gaps (Table 2.2) and sequences were generated from all the samples (n=16).

The complete mitochondrial sequences (n=16) were aligned and manually screened to identify the segregating sites. Further, a total of 15 primers were designed (multiple primers covering the segregating sites) to amplify all the polymorphic sites as <500 bp fragments to ensure higher success rate from poor quality dung DNA samples. These primers were standardised following same protocol described above.

2.2.3.2 Complete mitogenome annotation and comparative analysis

All rhino sequences (n=16) were aligned in Mega v7 to generate a complete mitogenome sequence and manually checked to identify any nucleotide ambiguities. Annotation was done using MITOS2 web (Bernt et al., 2013) and mitogenome map was created with OGDRAW (Greiner et al., 2019) using default parameters. To ascertain species-wise mitochondrial DNA diversity these sequences were aligned with already published rhino mitogenome sequences from *Diceros bicornis* (n=2, Genbank: FJ905814, NC012682 (Willerslev et al., 2009)), *Ceratotherium simum* (n=2, Genbank: Y07726, NC001808 (Xu et al., 1997)), *Dicerorhinus sumatrensis* (n= 15, Genbank: MF066629-MFO66643 (Steiner et al., 2017) and *Rhinoceros sondaicus* (n=6, Genbank: FJ905815, MK909142, MK909146, MK909148, MK909149, MK909151 (Margaryan et al., 2020)). We calculated number of segregating sites (S), nucleotide (π) and haplotype diversity (Hd) using DnaSP v.5 (Librado et al., 2009) for all genes in the mitogenome.

2.2.4 Microsatellite marker selection:

First, literature review was done on the microsatellite markers that have been used or tested for GoH rhinos earlier (n=34 STR). Among these, 23 are standardised by RhoDIS- South Africa program (Harper et al., 2013) for forensic case work of African rhinos (Harper et al., 2018). Some of these 23 markers (n = 18) have been tested on a few Indian GoH rhinoceros (n=18, Scott 2008), but the entire panel has never been assessed for use in forensic casework for the species in India. Similarly, the remaining 11 primers have been used in GoH rhino population genetic (Das et al. 2015) work but none of them were optimised for forensic work. Thus optimisation of these 34 markers were done based on following criterias: i) marker polymorphism and heterozygosity; ii) amplification success; iii) ease in allele calling and iv) stable allele characteristics to reduce genotyping error rates (Hoffman et al.,2005; Pompanon et al., 2005; Linacre et al., 2011; Johnson et al., 2014). These criteria's were chosen because the final panel will be used for individual identification from dung DNA with anticipation of higher errors owing to degraded DNA quality.

Initially all 34 markers were tested on 30 field-collected rhino tissue samples from different rhino-bearing areas in India (Assam = 21, West Bengal = 5 and Uttar Pradesh = 4 samples, respectively), and the final panel of markers were used for all remaining samples (n = 66) to check data repeatability. The primer selection process involved the following steps:

2.2.4.1 Optimisation of annealing temperatures and DNA concentrations

Before annealing temperature optimisation, DNA concentrations for all 30 samples were quantified in Epoch Microplate Spectrophotometer and found highly variable concentrations (ranging from 10 ng/ µL – 745 ng/µL). Further six samples were selected, representing a gradient of DNA concentrations (~10, 80, 150, 300, 500 and 700 ng/µL) for further optimisations. These were serially diluted to 1X, 1/10X, 1/25X and 1/50X, representing input DNA concentration ranging from 0.2 to 700 ng/µL. To optimise the annealing temperature, PCR reactions were performed for all 34 primers (See Table 1) in 10 µL reactions containing 4 µL of 2X Qiagen multiplex PCR buffer mix (QIAGEN Inc., Hilden, Germany), 0.2 µM labelled forward primer, 0.2 µM unlabelled reverse primer, 4 µM BSA (4 mg/mL) and 2 µL of rhino DNA at respective concentrations calculated above. PCR conditions included an initial denaturation (95ºC for 15 min); 35 cycles of denaturation (95ºC for 30 s), annealing (50-60ºC gradients for 40 s) and extension (72ºC for 40 s); followed by a final extension (72ºC for 20 min). During each reaction set, PCR and extraction negatives were included to monitor contamination. Amplified products were visualized with 2 % agarose gel and further genotyped using HiDi formamide (Applied Biosystems, California, United States) and LIZ 500 size standard (Applied Biosystems, California, United States) in an ABI 3500XL Genetic Analyser (Applied Biosystems, California, United States). The fragment lengths were scored manually using the program GENEMARKER (Softgenetics Inc., Pennsylvania, United States) and allele call bins were created for all the loci. The entire process was repeated three times in two different PCR machines (Eppendorf flexlid and ABI Veriti) and results were analyzed to determine optimal annealing temperatures and DNA concentration and to identify primers that were not amplifying in Indian GoH rhinoceros. One of the tissue samples was selected as a ‘genotyping reference sample’ and has been independently genotyped seven times to confirm the alleles. This sample was used as a standard to reduce genotyping errors from allele shifts among multiple runs in all subsequent genotyping analyses for Indian GoH rhinoceros and designated as reference sample for future process.

2.2.4.2 Peak characteristics, allele calling and marker polymorphism

Following temperature optimisation of all primers and selection of the optimum input DNA concentration (see result section) with six samples, the next set of samples (n = 24) were amplified three independent times to select primers based on allele characteristics (multiple peaks and stutters, peak ratio etc). While allele calling primers with low ‘relative fluorescence units (RFU)’ (less than 100) and multiple peaks were rejected as they can lead to erroneous allele calls (Hoffman et al., 2005; Pompanon et al., 2005). Subsequently, consensus genotypes for all 30 samples were generated using a ‘Quality index’ approach involving all three independent results (Miquel et al., 2006). To check polymorphism, all the markers were amplified with remaining tissue samples (n = 66) followed by exclusion of monomorphic ones from downstream analyses.

2.2.4.3 Stutter-related inconsistencies and multiplexing of primers

To assess stutter-related inconsistencies (particularly in dinucleotide loci) in the selected loci, peak RFU ratios of heterozygote (ratio between short and long allele) and homozygote (ratio between -2R stutter and its homozygote peak) alleles were calculated for all loci (Andreassen et al., 2012). This approach helps in selection of loci with stable stutter character after quantifying inter and intra sample variations in peak ratio. The ratios were independently calculated for all three replicates of polymorphic loci for 30 selected samples followed by estimation of standard deviation in the ratios. Thus the loci with high deviation values were rejected as they can be prone to stochastic errors leading to inconsistent allele calling (Andreassen et al., 2012). The final selected markers were multiplexed based on allele size and dye colour, and then each multiplex panel was checked for data accuracy by replicating the same PCR conditions mentioned above with different number of amplification cycles (20, 25, 30 and 40). Finally all sample (n = 96) were genotyped (for final multiplex panel) in three replicates to ensure consistent results by comparing them with previously generated genotypes.

2.2.4.4 Data analyses

Quantification of allelic dropout and false allele (FA) rates were done manually as error rates per locus including the replicate data. Allelic drop out was estimated as ratio between number of amplifications with loss of one allele and total number of positive heterozygous individual amplification. The false allele was calculated for both homozygotes and heterozygotes as proportion of number of false allele amplification versus total number of amplifications (Broquet et al., 2004; Pompanon et al., 2005). Further, program FreeNA (Chapuis et al., 2007) was used to determine the frequency of null alleles (NA), which estimates the NA frequency using the EM algorithm (Dempster et al., 1977). Program GIMLET (Valière 2002) was used to calculate the PID(sibs) for all the individuals. Following this, any allele having less than 10 % frequency across all amplified loci was rechecked for allele confirmation. ARLEQUIN (Excoffier et al., 2010) was used to calculate overall summary statistics and determine Hardy Weinberg equilibrium and linkage disequilibrium for all the loci.

2.2.4.5 Forensic use of the finalised microsatellite panel

Various legal authorities in India have transferred 17 alleged rhino crime cases to the Institutions forensic laboratory following strict 'Chain of custody' protocols. Of these, 14 samples were from poached rhino carcass and three samples were of seized rhino horn contraband. Following photographic and administrative documentations at the forensic laboratory, DNA was extracted, quantified and species identification was performed by partial cytochrome *b* and 12S rRNA sequences as described in Jha et al., (2017). This species identification protocol was chosen over earlier described ones (for example, Ewart et al., 2018) due to the requirement of India's legal procedure requirements, where ascertainment of species is required to be matched with our country's sequence database. PCR reactions were performed in 10 µl reaction volumes containing 4 µl of 2X Qiagen Hotstart mastermix buffer (QIAGEN

Inc., Hilden, Germany), 0.3 µM of each forward and reverse primer, 4 µM BSA (4 mg/mL) and 1 ng of extracted DNA. PCR conditions included an initial denaturation (95ºC for 15 min); 35 cycles of denaturation (95ºC for 30 s), annealing (55ºC for 40 s) and extension (72ºC for 40 s); followed by a final extension (72ºC for 10 min). The amplified products were electrophoresed, cleaned up with Exo-SAP mixture, sequenced bidirectionally and matched against our Indian forensic database as well as with the Genebank for species confirmation. Confirmed rhino samples were then genotyped three independent times using the final multiplex panels as determined via the optimisation process outlined above. Finally, individual genotypes for the entire panel were matched against the poached rhino carcass data.

2.3 Results:

2.3.1 DNA extraction protocol from rhino horns:

By comparing the efficiency of two DNA extraction protocols it was found that DTT protocol yielded 16 ng/µl- 102 ng/µl, whereas without DTT yielded 0.3 ng/µl- 1 ng/µl from the same samples. Further, DTT-extracted samples amplified all 14 markers in the panel compared to none with the other protocol. Given the stark contrast in amplification success, the DTT protocol was used for all subsequent rhino horn DNA extractions.

2.3.2 Mitogenome data generation across Indian rhino populations

Sequencing with 23 primers (Table 2.2) generated 16,828bp mitogenome (Table 2.3 and Figure 2.1) for wild Indian rhino (n=16, Genbank: MZ736693- MZ736708). Composition analysis revealed AT-skewed mitogenome with 13 protein coding genes, 22 tRNA, 2 ribosomal genes and a non-coding control region (Table 2.3). Comparative analyses with other rhino species (Table 2.4 and Figure 2.2) revealed that the Indian rhinos have low segregating sites ($S_{Java}=15514$, $S_{Africa}=10680$, $S_{Sumatra}=130$, $S_{India}=18$) and nucleotide diversity ($\pi_{Java}=0.56$, $\pi_{Africa}=0.43$, $\pi_{Sumatra}=0.003$, $\pi_{India}=0.0005$) but high haplotype diversity ($Hd_{Sumatra}=0.96$, $Hd_{India}=0.93$, $Hd_{Java}=0.91$, $Hd_{Africa}=0.67$). Both African rhino species (white and black rhino) data were combined for this analyses as no intra-species variation was observed in the available data. Further eight primers (out of 15 designed) were finalised to assess genetic variation by amplifying all 21 polymorphic sites covering 2,531bp sequence of GoH rhino mitogenome (Table 2.2).

Table 2.2: Details of the primers used in sequencing Indian rhino whole mitogenome data and selection of eight primers for phylogeography work

S.No.	Primer	Sequence	Ta (°C)	Amplicon	Targeted genes	Polymorphic sites	Genbank accessions
1	P2*	F: CACTGAAAATGCCTAGATGAG	55	~950	tRNA^{Phe}, rrnS, tRNA^{Val}	0	MZ736693-MZ736708
		R: CTAGGTGTAAACTAGRTGCTT					
2	12S#	F: AGACATAAAAACGTTAGGTC	60	~350	rrnS, tRNA^{Val}	0	
		R: GCTTTGGTTCAAAGTGGTCA					
3	P3*	F: GCACGCACACACCGCCCGTCAC	55	~600	rrnS, tRNA^{Val}, rrnL	0	
		R: CGCTTTCTTAATTGRTGGCTGC					
4	P4*	F: AGCCTGGTGATAGCTGGTTGTCC	55	~650	rrnL	0	
		R: AAGCTCCATAGGGTCTTCTCGTC					
5	P5*	F: CCGTGCAAAGGTAGCATAATC	55	~800	rrnL, tRNA^{Leu}, NADH1	0	
		R: CCTAGNACTTTTCGTTCNACT					
6	P6*	F: GTGGCAGAGCCCGGTAATTG	55	~950	NADH1, tRNA^{Ile}	0	
		R: TTACTCTATCAAAGTAACTC					
7	P7*	F: TYCGAGCATCHTAYCCHCGAT T	55	~800	NADH1, tRNA^{Ile}, tRNA^{Gln}, tRNA^{Met}, NADH2	0	
		R: TGGTTTAGBCCBCCTCAKCCYCC					
8	P8*	F: CACTTYTGAGTNCCAGAAGT	55	~850	NADH2, tRNA^{Trp}, tRNA^{Ala}	0	
		R: TAGGGTRTTTAGCTGTTAAC					
9	P9*	F: AGACCAAGAGCCTTCAAAGC	55	~650	tRNA^{Trp}, tRNA^{Ala},	0	

S.No.	Primer	Sequence	Ta (°C)	Amplicon	Targeted genes	Polymorphic sites	Genbank accessions
		R: GCTTCWACTATDGADGATGC			tRNA^{Asn}, tRNA^{Cys}, tRNA^{Tyr}, COX1		
10	P10*	F: GGNGGNTTYGGHAAYTGACT	55	~750	COX1	1	
		R: GAARATRAAGCCTAGRGCTCA					
11	P11*	F: TTYACHGTHGGAATAGAYGT	55	~800	COX1, tRNA^{Ser}, tRNA^{Asp}, COX2	0	MZ736693-MZ736708
		R: GCRTCTTGRAANCCTARTTG					
12	P12*	F: CCCCCYAYWRYTGGTTTCAAGCCA	55	~500	tRNA^{Asp}, COX2, tRNA^{Lys}, ATP8	0	
		R: GTKGAYGTRTCTAGTTGYGGCAT					
13	P13*	F: CAATGCTCHGARATYTGYGG	55	~950	COX2, tRNA^{Lys}, ATP8, ATP6, COX3	1	
		R: GANARDGCTCCYGTDAGNGGT					
14	P14*	F: GCCTAYGTNTTYACYCTNCTAGT	55	~800	ATP6, COX3, tRNA^{Gly}, NADH3	1	
		R: TGATTGGAAGTCARYTGTAC					
15	P16*	F: AGCYTGYGAAGCAGCACTAGG	55	~950	tRNA^{Arg}, NADH4L, NADH4	0	
		R: GCKGTRGCTCCTATRTARCTTCA					
16	P17*	F: AGCTCHATYTGYYTHCGYCAA AC	55	~700	NADH4, tRNAHis, tRNASer, tRNALeu	1	
		R: CCAATTTTTTGGYTCCTAAGRCC					
17	P18*	F: CCGAAAAAGYAYGCAAGAACTGC	55	~750	tRNASer, tRNALeu, NADH5	0	
		R: GCDGATTTTCCDGTKGCDGCTA					
18	P19*	F: GACGARCAGAYGCHAAYACAGC	55	~700	NADH5	1	

S.No.	Primer	Sequence	Ta (°C)	Amplicon	Targeted genes	Polymorphic sites	Genbank accessions
		R: GTDAKTADDAGGGCTCAGGCG					
19	P20*	F: GGMAGCCTNGCNYTAACAGG	55	~600	NADH6	0	
		R: AGTTTAATGGDHTDGGDGATTG					
20	P21*	F: CCATAACTRTAYAAAGCHGCAA	55	~650	NADH6, tRNAGlu, CYTB	3	
		R: CCTCARAATGATATTTGKCCTCA					
21	P22*	F: CAGGMCTATTCCTRGCHATAC	55	~950	CYTB, tRNAThr	1$21	
		R: CCCTTYTCTGGTTTACAAGACC					MZ736693-MZ736708
22	P23*	F: CATCGGACAACTAGCATCTAT	55	~650	tRNAThr, tRNAPro, CR	10	
		R: CCTGAAGWAAGAACCAGATG					
23	CRintF2#	F: GATTAATATTGCATAGTA	50	~500	CYTB, tRNA-Thr, tRNA-Pro,CR	5(3$23)	
	CRintR3#	R: AATAGTTAATAGAAAGGC					
24	CRF1#	F: CATGCCAGTATTAGTGAC	50	~450	tRNAPhe, CR	6$23	
	CRR1#	R: CAAGGTGTTGTTAGCTAC					
Phylogeography Primers							
25	P10*	F: GGNGGNTTYGGHAAYTGACT	45	431	COX1	1	MZ771364-MZ771458
	COXR#	R: GTAGACTTCAAAATGACC					
26	ATP6#	F: TCTAATACACTTAATCGG	50	379	ATP6, COX3	2	MZ771934-MZ772028
		R: CTTGGAATGTGCTTTCAC					

S.No.	Primer	Sequence	Ta (°C)	Amplicon	Targeted genes	Polymorphic sites	Genbank accessions
27	NADH4#	F: CCAAACACCATGAAGTTA	50	262	NADH4	1	MZ771459-MZ771553
		R: GATCATGAGAATGATGAC					
28	NADH5#	F: AACAAATCTTCACACTTG	50	268	NADH5	1	MZ771554-MZ771648
		R: ATAGGGTGGTGATTGCTC					
29	CYTB1#	F: AAATCTCACCCACTAGTT	50	428	CYTB	2	MZ771649-MZ771743
		R: TTTGTAATGACTGTAGCT					
30	CYTB2#	F: CTACACGAAACAGGGTCC	50	338	CYTB	1	MZ771744 -MZ771838
		R: ATTGGCTTAGGGGTCGGA					
31	CRF1#	F: CATGCCAGTATTAGTGAC	50	281	CR	7	MZ771839-MZ771933
	CRintR2#	R: ATACGCATGTTGACTGGA					MZ771839-MZ771933
32	CRintF2#	F: GATTAATATTGCATAGTA	50	495	CYTB, tRNA-Thr, tRNA-Pro,CR	6	
	CRintR3#	R: AATAGTTAATAGAAAGGC					

*Designed by Hassanin et al. 2009

[#] Designed in this study

[$] Overlapping number of segregating sites with the primer name

Table 2.3: Mitogenome organization of Rhinoceros unicornis. Codons respective to each tRNA are mentioned in parenthesis.

Genes	**Position**		**Gene length**	**Spaces/ overlap**	**Strand**	**Codons**	
	Start	**Stop**				**Start**	**Stop**
tRNA^{Phe} (GAA)	1	69	69	0	H		
rrnS	70	1041	972	-1	H		
tRNA^{Val} (TAC)	1041	1107	67	0	H		
rrnL	1108	2681	1574	3	H		
tRNA^{Leu} (TAA)	2685	2759	75	2	H		
NADH1	2762	3718	957	-1	H	ATG	TAA
tRNA^{Ile} (GAT)	3718	3786	69	-3	H		
tRNA^{Gln} (TTG)	3784	3856	73	2	L		
tRNA^{Met} (CAT)	3859	3927	69	0	H		
NADH2	3928	4971	1044	-2	H	ATA	TAG
tRNA^{Trp} (TCA)	4970	5037	68	5	H		
tRNA^{Ala} (TGC)	5043	5111	69	1	L		
tRNA^{Asn} (GTT)	5113	5185	73	2	L		
OL	5188	5217	30	-1	H		
tRNA^{Cys} (GCA)	5217	5282	66	0	L		
tRNA^{Tyr} (GTA)	5283	5349	67	1	L		
COX1	5351	6895	1545	-3	H	ATG	TAA
tRNA^{Ser} (TGA)	6893	6961	69	11	L		
tRNA^{Asp} (GTC)	6973	7039	67	0	H		
COX2	7040	7723	684	3	H	ATG	TAA
tRNA^{Lys} (TTT)	7727	7793	67	1	H		
ATP8	7795	7998	204	-43	H	ATG	TAA
ATP6	7956	8636	681	-1	H	ATG	TAA
COX3	8636	9420	785	-1	H	ATG	TAa
tRNA^{Gly} (TCC)	9420	9488	69	-3	H		
NADH3	9486	9835	350	0	H	ATA	TAa
tRNA^{Arg} (TCG)	9836	9905	70	0	H		

Genes	**Position**		**Gene length**	**Spaces/ overlap**	**Strand**	**Codons**	
	Start	**Stop**				**Start**	**Stop**
NADH4L	9906	10202	297	-7	H	ATG	TAA
NADH4	10196	11573	1378	0	H	ATG	Taa
tRNA^{His} (GTG)	11574	11642	69	0	H		
tRNA^{Ser} (GCT)	11643	11701	59	1	H		
tRNA^{Leu} (TAG)	11703	11772	70	-9	H		
NADH5	11764	13593	1830	-17	H	ATA	TAA
NADH6	13577	14101	525	3	L	ATG	TAA
tRNA^{Glu} (TTC)	14105	14173	69	4	L		
CYTB	14178	15317	1140	0	H	ATG	AGA
tRNA^{Thr} (TGT)	15318	15386	69	1	H		
tRNA^{Pro} (TGG)	15388	15453	66	268	L		
CR	15454	16828	1388	/	H		

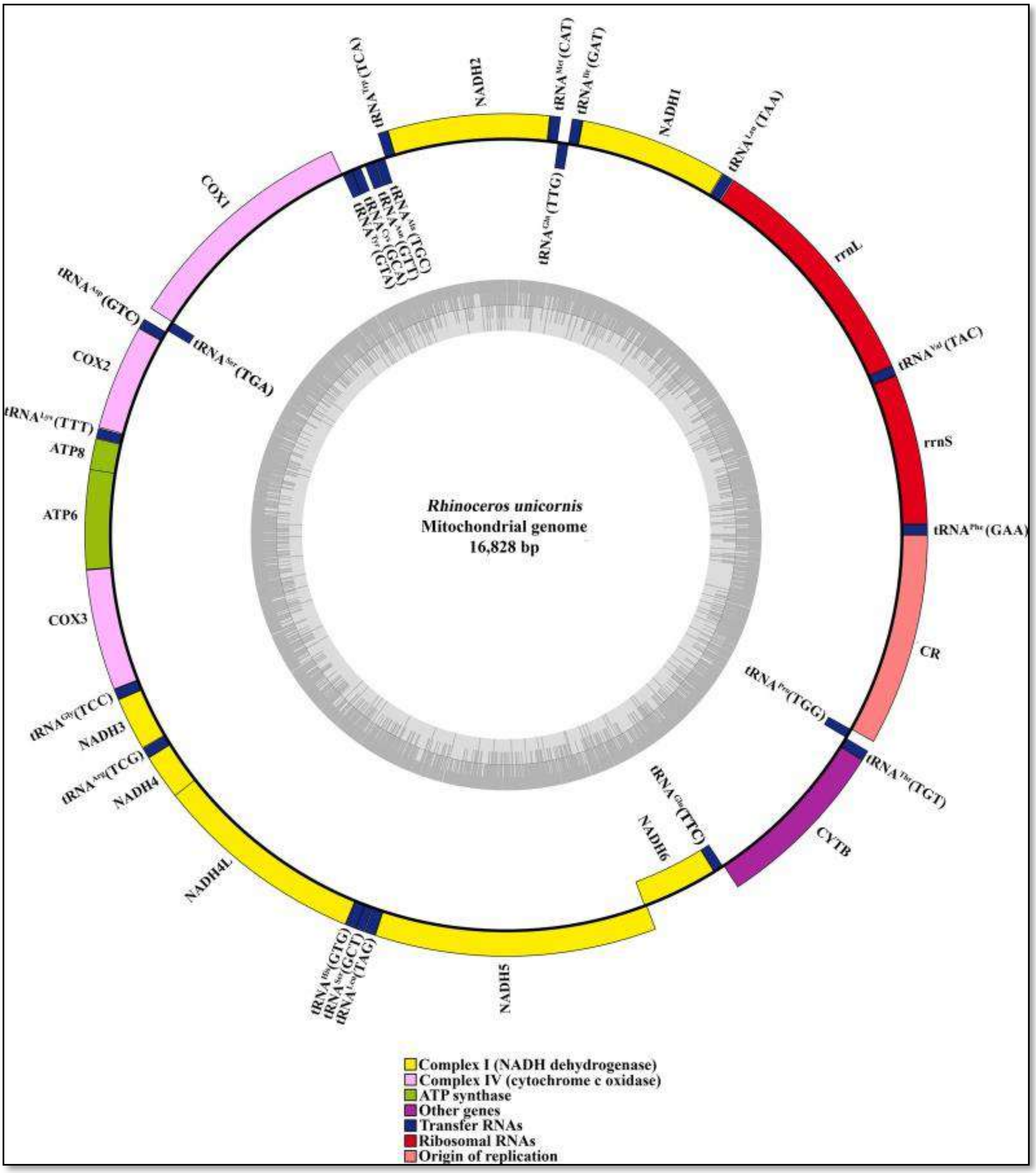

Figure 2.1: Whole mitogenome organisation and annotation of Rhinoceros unicornis.

Table 2.4: Comparative analysis results of genetic diversity indices among five extant rhino species

Mitochondrial genes	**African rhino**			**Sumatran rhino**			**Javan rhino**			**Indian rhino**		
	S	**H_d**	**π**	**S**	**H_d**	**π**	**S**	**H_d**	**π**	**S**	**H_d**	**π**
rrnS	51	0.67	0.0352	0	0	0	1	0.53	0.0006	0	0	0
rrnL	68	0.67	0.0289	7	0.85	0.0014	13	0.73	0.0037	0	0	0
ATP6	64	0.67	0.0627	5	0.69	0.6857	4	0.80	0.8000	1	0.50	0.5000
ATP8	12	0.67	0.0392	1	0.42	0.0021	2	0.60	0.0043	0	0	0
COX1	100	0.67	0.0431	12	0.84	0.0033	11	0.93	0.0029	1	0.50	0.0003
COX2	49	0.67	0.0478	2	0.51	0.0008	4	0.73	0.7333	0	0	0
COX3	69	0.67	0.0587	3	0.54	0.0008	3	0.60	0.0018	1	0.13	0.0002
CYTB	98	0.67	0.0573	14	0.88	0.0050	8	0.73	0.0036	3	0.57	0.0011
NADH1	55	0.67	0.0384	6	0.78	0.0027	4	0.73	0.0020	0	0	0
NADH2	78	0.67	0.0499	7	0.89	0.0022	6	0.73	0.0029	0	0	0
NADH3	28	0.67	0.0540	5	0.81	0.0054	1	0.33	0.0010	0	0	0
NADH4	108	0.67	0.0523	14	0.73	0.0042	7	0.80	0.0023	1	0.33	0.0002
NADH4L	27	0.67	0.0606	2	0.26	0.0009	2	0.53	0.0036	0	0	0
NADH5	144	0.67	0.0527	14	0.81	0.0032	11	0.73	0.0031	1	0.50	0.0003
NADH6	51	0.67	0.0648	1	0.42	0.0008	4	0.73	0.0042	0	0	0
tRNA	65	0.67	0.0290	7	0.82	0.0016	0	0	0	1	0.33	0.0002
CR	185	0.67	0.1395	31	0.93	0.0108	21	0.93	0.0118	9	0.85	0.0045

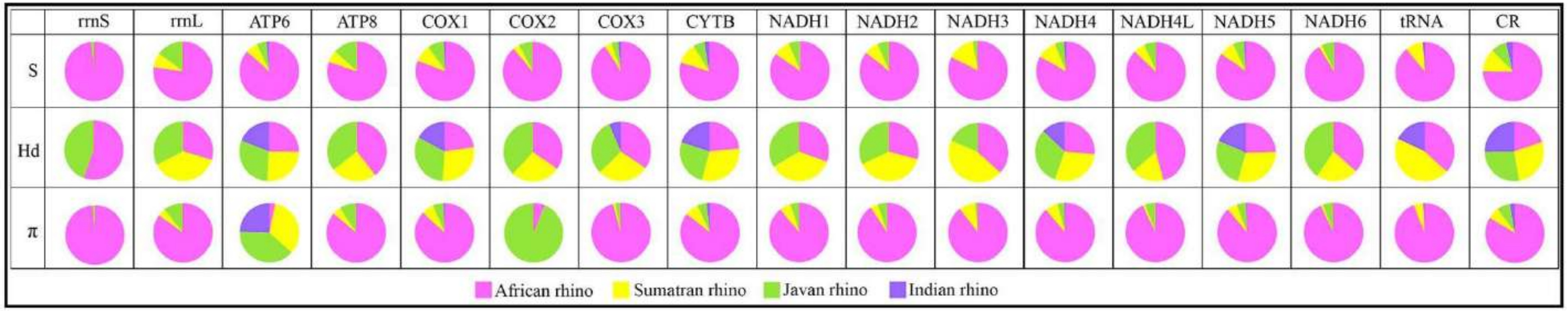

Figure 2.2: Gene-wise comparative analyses of polymorphism indices (S, Hd and π) for all extant rhino species.

2.3.2 Microsatellite panel optimisation for Indian rhino DNA profiling:

2.3.2.1 Screening of rhino marker panel

Systematic exclusions of non-suitable markers were conducted from the initial 34 rhino microsatellites based on amplification success, marker characteristics and polymorphism. During optimisation of the markers 24 different combinations of input DNA concentrations ranging 0.2-700 ng/µl were tried to found the minimum DNA concentration i.e 1 ng/µl is required for reliable microsatellite data generation. We used this DNA concentration for all subsequent PCR amplifications. During the first stage of marker amplification with the reference set of samples (n = 6), three of the 34 initial markers (Rh2, DB52 and B1Rh1C) failed to show any amplification (Table 2.5). These primers were rejected from further processing. In the next step marker suitability was assessed based on peak characteristics (tested with 30 samples) and polymorphism (with 96 rhino samples). Based on peak characteristics three loci were eliminated (Table 2.5) from further testing due to consistent low RFU data (32A and SR268) and multiple peaks (B1Rh1B). The remaining loci (n = 28) were tested for polymorphism and nine monomorphic loci (Table 2.5) were removed from the panel. Finally, data from all the polymorphic loci (n = 19) were analysed for stutter-related inconsistencies across multiple genotyping runs (tested with 30 samples). This resulted in removal of five loci (SR262, 7C, DB66, Rh6, Rh8) with high standard deviation values ranging from ±1 to ±2.3, which may lead to inconsistent allele calling. The remaining 14 loci showed low standard deviation values (±0.04 to ±0.18) (Table 2.5), and could be multiplexed into four panels. During testing these panels with different amplification cycles, PCR reactions with

20 and 25 cycles consistently produced low RFU values for all the loci, whereas reactions with 30 cycles produced low RFU value for larger alleles. While 35 and 40 cycles produced almost identical results, it was decided to continue with 35 cycles for multiplex PCRs in the line of the singleplex reactions. All samples (n = 96) produced identical data with these multiplex panels when compared with the data from singleplex reactions.

2.3.2.2 Summary statistics for panel

None of the selected 14 loci in this panel showed signatures of large-scale allelic dropout. The mean allelic dropout and false allele rates were 0.02 and 0.01, respectively. Average frequency of null allele was 0.05, indicating this panel has low genotyping error rates. Amplification success ranged between 90%-99.6% from different tissue types (blood and tissue, n = 96). These loci showed high to medium levels of polymorphism (Table 2.6). The mean observed heterozygosity was found to be 0.50 ± 0.15 and none of the loci deviated from Hardy-Weinberg equilibrium or showed any evidence for strong linkage disequilibrium between any pair of loci. The cumulative PIDsibs and PIDunbiased values were found to be 1.2×10^{-4} and 1.4×10^{-9}, respectively. Given that the rhino population size across India is ~3700 (Talukdar 2021), this panel provides strong statistical support for unambiguous individual identification in forensic use. All summary statistic measures of polymorphism (number of alleles, allelic size range, heterozygosity etc.) for all loci are presented in Table 2.6.

Table 2.5: Details of the 34 microsatellite markers used for Indian rhinos in this study. The selected markers are listed according to the multiplex panel.

Locus ID	**Dye**	**Ta (°C)**	**Between run precision (S.D)**	**Selected/ Rejected**	**Multiplex PCR sets**
RH1	6-FAM	60	0.09	Selected	MPI
RH3	NED	60	0.04	Selected	MPI
RH10	HEX	60	0.1	Selected	MPI
SR281	6-FAM	60	0.04	Selected	MPI
RH5	HEX	60	0.07	Selected	MPII
RH7	NED	60	0.08	Selected	MPII
IR10	NED	60	0.09	Selected	MPII
SR63	6-FAM	60	0.04	Selected	MPII
IR12	HEX	60	0.05	Selected	MPIII
12F	HEX	60	0.05	Selected	MPIII
7B	FAM	60	0.04	Selected	MPIII
RH4	6-FAM	57	0.18	Selected	MPIV
RH9	PET	57	0.13	Selected	MPIV
RH11	HEX	57	0.15	Selected	MPIV
SR262	6-FAM	-	2.3	Rejected[a]	-
7C	6-FAM	-	1.5	Rejected[a]	-
DB66	6-FAM	-	1.7	Rejected[a]	-
Rh6	PET	-	1	Rejected[a]	-
Rh8	PET	-	1.7	Rejected[a]	-
32F	VIC	-	-	Rejected[b]	-
B1Rh37D	NED	-	-	Rejected[b]	-
SR74	NED	-	-	Rejected[b]	-
DB44	VIC	-	-	Rejected[b]	-
DB23	VIC	-	-	Rejected[b]	-
BR6	NED	-	-	Rejected[b]	-
IR22	VIC	-	-	Rejected[b]	-
Rh12	VIC	-	-	Rejected[b]	-
DB1	PET	-	-	Rejected[b]	-
32A	6-FAM	-	-	Rejected[c]	-
SR268	VIC	-	-	Rejected[c]	-
B1Rh1B	NED	-	-	Rejected[d]	-
Rh2	6-FAM	-	-	Rejected[e]	-
DB52	PET	-	-	Rejected[e]	-
B1Rh1C	PET	-	-	Rejected[e]	-

Primers rejected due to [a] stutter related inconsistency, [b] monomorphism, [c]low RFU, [d] multiple peaks and [e]non-amplification

Table 2.6: Summary statistics of finalised 14 microsatellites arranged in descending order of their informative content

Locus ID	AS (%)	No. of alleles	Allelic size range	Ho	He	Cumulative $P_{ID(unbiased)}$	Cumulative $P_{ID(sibs)}$	ADO	FA	NA
RH10	98.7	7	14	0.67	0.79	7.21E-02	3.79E-01	0.02	0.01	0.06
RH4	96.7	6	24	0.72	0.74	7.36E-03	1.55E-01	0.02	0	0.01
RH5	99.6	5	12	0.56	0.72	9.06E-04	6.55E-02	0	0.02	0.08
SR63	99.6	4	12	0.64	0.72	1.12E-04	2.78E-02	0.02	0.01	0.05
RH3	100	3	34	0.59	0.66	2.08E-05	1.30E-02	0.03	0.01	0.04
RH1	98.4	3	4	0.59	0.63	4.38E-06	6.36E-03	0.04	0	0.03
RH11	94.7	4	12	0.62	0.60	1.03E-06	3.27E-03	0.03	0.02	0.01
IR12	95.1	2	4	0.19	0.49	3.57E-07	1.96E-03	0	0.01	0.20
SR281	97.1	2	2	0.45	0.49	1.34E-07	1.18E-03	0	0.01	0.03
12F	90	4	6	0.39	0.47	5.07E-08	7.22E-04	0.01	0	0.06
7B	93.1	3	6	0.43	0.45	1.91E-08	4.43E-04	0.03	0	0.00
RH9	96.3	3	24	0.37	0.45	7.25E-09	2.77E-04	0.03	0.08	0.06
RH7	99.6	2	2	0.47	0.44	2.94E-09	1.75E-04	0.02	0	0.00
IR10	99.6	2	8	0.31	0.34	1.42E-09	1.23E-04	0.08	0	0.03

Ta- Annealing temperature, AS-Amplification success, Ho and He- observed and expected heterozygosity, ADO- allele drop out, FA- false alleles, NA- null alleles.

2.3.2.3 Individual matching of rhino contraband

Sequencing-based species identification assay confirmed two of the horn chips as Indian GoH rhinoceros, whereas the third sample was identified as water buffalo (*Bubalus bubalis*). Complete 14 loci panel data was generated for all 15 poaching cases (13 carcass and two horn chips).The genotypes from the horn chips matched with two rhino carcasses from West Bengal and Assam, respectively, showing the efficacy of this approach to match seized contraband to their carcass origins. These results were submitted as scientific evidence to concerned authorities for legal proceedings. The genotypes of decomposed rhino carcasses were matched with other evidence such as blood-stained soil, bullets recovered from crime scenes etc. These results showcase the power of such DNA-based approaches in wildlife crime investigations.

2.4 Discussion:

The major aim of this study was to generate mitogenome data for all Indian rhino populations which can be used to assess mitochondrial DNA diversity and evolutionary history along with optimisation of a panel of microsatellites for forensic use. To the best of our knowledge, this is the first report of wild Indian one-horned rhino mitogenome from all the extant populations. Despite relatively similar haplotype diversity of Asian rhinos (India-0.93 (16 samples), Sumatra-0.96 (15 samples), and Java-0.91 (6 samples), respectively), Indian rhino mitogenome showed much lower values for segregating sites and nucleotide diversity. Such mitogenome comparisons may be affected by limited sample size (earlier studies in African rhinos have reported higher diversity based on partial mitogenome data with more samples

(Moodley et al., 2017 & 2018) or representation of historical genetic variations (in Javan rhinos, Margaryan et al. 2020). However, it was surprising to observe that despite similar historical demographic incidences (severe population decline due to habitat shrinkage (Patnaik 2016; Mays et al., 2018) and anthropogenic pressures (Das et al., 2015; Steiner et al., 2017) Indian rhino retain much lower genetic variation than their Sumatran counterpart. This can be potentially attributed to recovery of the Indian species from extremely low founder population (as indicated by high Hd but low π) (Avise 2000; da Silva et al., 2018).

In case of microsatellites, this is the only study apart from the African rhinos (Harper et al., 2013; Dicks et al., 2017; Harper et al., 2018) where a forensic microsatellite marker panel has been standardized to deal with rhino-related crimes. In the study ISFG recommendations were followed during testing and optimisation of the markers such as use of voucher reference samples, various types of rhino samples (blood, tissue, horn chips representing different input DNA concentrations), temperature and PCR cycle optimisation, etc., that is generally lacking in other published GoH rhino population genetic work (Das et al., 2015; Borthakur et al., 2016; Zschokke et al., 2016). Rigorous testing of a large number of microsatellite loci (n = 34) were conducted with field-collected samples before selecting the best ones to develop the panel. Out of the 34 markers initially selected (23 from the African RhoDIS panel (Harper et al., 2018) and 11 tested on Indian GoH rhinoceros (Das et al., 2015), 14 dinucleotide markers w ere finally optimised into four multiplex reactions as a time-saving and economic option. Most of the RhoDIS-Africa markers did not amplify in Indian GoH rhinoceros samples, possibly due to null alleles resulting from evolutionary differences among genus. Such non-amplification of cross-species microsatellites has also been reported earlier in rhinos (Scott 2008; Guerier et al., 2012). Further, it is important to point out that there are general concerns regarding use of dinucleotide markers in forensic studies (Pompanon et al., 2005; Andreassen et al., 2012) but ISFG recommends them if they are widely used in population genetic research and have stable heterozygote balance (Linacre et al., 2011). The final selected panel in this study fulfil these criteria and showed very low genotyping error rates (2 % allelic dropout, 1 % false alleles and 5 % null alleles) and produce a statistically significant PIDsibs value of 1.2×10^{-4}. While using the optimised microsatellite panel to identify the origin of seized horns individual genotype matching was done instead of commonly used random match probability approach for genotype comparisons (Balding et al., 1994) as it requires in-depth understanding of population substructure data (also known as Φ value) (Balding et al., 1994; Johnson et al., 2014). There are seven isolated Indian GoH rhinoceros populations (four in Assam, two in West Bengal and one in Uttar Pradesh) and no population structure data or population-specific allele frequency assessments are available. Moreover, within each of the population the allele frequencies can differ significantly due to variations in population size and histories (in some cases the entire population has been reintroduced using founders from different sources), resulting in possible erroneous interpretations of our results (Balding et al., 1994). This can be replaced by population assignment test with accuracy assessment once significant individual level information from all subpopulations is available. Until the adequate population-specific information is generated, complete genotype matching provides sufficient support in forensic use.

In conclusion, a microsatellite panel is optimised for Indian rhinos to generate DNA profiles and matched with carcasses as scientific evidences in alleged rhino crimes in India.

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Chapter 2: Investigating the phylogeography and evolutionary history of Indian one-horned rhinoceros

3.1 Introduction:

The members of Rhinocerotidae family were once one of the most diverse and widely distributed terrestrial herbivores with complex evolutionary history (Liu et al., 2021). By late Pleistocene, this family was reduced to only nine species (from more than 100 species) spread across Eurasia (seven species) and Africa (two species) (Wan et al., 2017; Liu et al., 2021). Subsequently, early Holocene global warming (after Last Glacial Maxima) triggered their extinction in western Eurasia and southward movement of eastern Eurasian rhinos, leading to their distribution across Southeast Asia (Li et al., 2015; Wan et al., 2017). Further, the range of all Eurasian rhino species (Javan, Sumatran and One-horned rhino) were affected by a combination of natural and anthropogenic factors during Pleistocene-Holocene transition period (15-9 thousand years ago (Kya)) (Alroy 2001; Li et al., 2015; Lal 2016; Mays et al. 2018; Liu et al., 2021); followed by recent events of exploitation of natural resources (during colonial era), industrialisation and poaching (since 17th century) (Menon 1996; Fernando et al., 2006; Das et al., 2015; Steiner et al., 2017). Population size of the most widely distributed Javan rhinos (during Holocene) (Antoine et al., 2012) were greatly reduced during human population expansion since 10,000 years ago (Li et al., 2015), whereas the Sumatran rhino populations became fragmented and isolated (since Holocene) due to submerged Sundaland corridors (late Pleistocene) (Mays et al., 2017). The one-horned rhinos faced climate-change driven habitat shrinkage in late Pleistocene (Patnaik 2016). Currently the Javan and Sumatran rhinos are categorized as Critically Endangered (~60 Javan rhino- (Margaryan et al., 2020) and <100 Sumatran rhinos (Steiner et al., 2017)) and one-horned rhino as Vulnerable by IUCN (~3700 individual (Talukdar et al., 2021). Recovery of these species in their natural habitats requires deeper understanding of demography, ecology and genetics for appropriate conservation measures.

The one-horned rhino, being the only Asian species recovered from severe population decline in the past are critical for the evolutionary potential of this group. With a current population size of ~3700 individuals (increased from few hundred individuals in 1990s), it retains ~96% of the Asian rhino population (Steiner et al., 2017; Margaryan et al., 2020; Talukdar et al., 2021). As majority of the current one-horned rhino bearing areas in India and Nepal are reaching to their carrying capacities (Subedi et al., 2017; Jhala et al., 2021), future conservation efforts are directing towards reintroduction-based programmes. Detailed genetic assessment of the existing rhino populations is critical in this regard since strong historical demographic declines has led to loss of genetic variation in all rhino species (Black rhino- (Moodley et al., 2017), White rhino- (Moodley et al., 2018), Sumatran rhino- (Mays et al., 2018), Javan rhino- (Margaryan et al., 2020)). For example, Liu et al., 2021 suggested low population size and reduced genetic diversity across Rhinocerotidae family for an extended period of time. Similarly, mitogenome-based phylogeography reported low variations in both Sumatran (Steiner et al., 2017) and Javan (Margaryan et al., 2020) rhinos, but no such data is available for one-horned rhinos.

In this chapter the phylogeography and divergence history of one-horned rhinos in India (henceforth Indian rhino) is discussed as it harbours 83% (Kakati et al., 2021) of the global population of this species. All the identified polymorphic sites (identified in Chapter 1) in the Indian rhino mitogenome were sequenced in 111 wild individuals surveyed across seven extant populations covering the states of Assam, West Bengal and Uttar Pradesh. Further, the Evolutionary Significant Units (ESUs) in Indian rhinos were identified and appropriate conservation measures were suggested to secure the evolutionary potential of this species.

3.2 Methods:

3.2.1 Sample collection:

Overall the sampling strategy in this phylogeography study was to select unique rhino individuals from different parts of the species distribution in India. A total of 159 samples (56 tissues and 88 dung) covering three states were collected in addition to 16 tissue samples mentioned in Chapter 1 to assess Indian rhino mitochondrial genetic diversity. The tissue samples of naturally dead rhino were provided by respective forest departments as part of RhoDIS-India protocol (2017-2021). Further dung collection was done to ensure spatial coverage for areas with no representative tissue samples. Rhino dung sampling can be challenging in the wild due to their use of communal latrine system (middens) (Laurie et al., 1986; Johnsingh et al., 2016). In this study, sampling was conducted by foot and vehicle surveys from already known midden sites across six rhino bearing parks (except Kaziranga NP). During sampling, only the fresh bolus from top of the midden was selected and swabbed twice with separate PBS-soaked sterile cotton swabs (Himedia, Mumbai, India). All samples were geo-tagged and transferred to laboratory in -20°C freezer till downstream processing. Park-wise sample collection detail is given in Table 3.1.

3.2.2 DNA extraction:

Tissue DNA was extracted using already established protocol for Indian rhino mentioned in Ghosh et al., 2021. For dung samples, a modified protocol from Biswas et al., 2019 was used. In brief, samples were digested overnight with a combination of 700 µl ATL and 65 µl Proteinase K (20mg/ml) at 56◦C, followed by QIAamp DNA Tissue Kit (QIAGEN Inc., Hilden, Germany) protocol with adjusted volumes. DNA was eluted twice in 100 µl preheated (70 °C) 1X TE buffer and stored in -20°C freezer. Extraction negative was used for each set of extraction (n=23) to monitor possible contamination.

3.2.3 PCR amplification and sequencing:

For all field collected samples (tissue= 56 and dung=88) individual identification was performed using a panel of 14 microsatellites standardised in Chapter 1. After PCR amplification and genotyping (as described in Ghosh et al., 2021), samples with 12-14 loci data were selected for downstream analysis and genetic recaptures were removed. To ensure removal of closely related individuals, one sample from adjacent midden sites was selected. This was followed by generation of sequence data (2531bp covering seven genes) for the selected individuals using 8 finalised primers (Chapter 1) to assess the phylogeography patterns. Post PCR amplification, products were visualized with 2% agarose gel, cleaned with Exonuclease (Thermo Scientific, Waltham, USA) and Shrimp Alkaline Phosphatase (Amresco, Solon, USA) mixture and sequenced bidirectionally in an ABI 3500XL bioanalyzer (Applied Biosystems).

3.2.4 Assessment of genetic diversity in Indian rhinos:

Population-wise basic indices of genetic variations (S, π and Hd) were calculated for concatenated sequence data (2531bp from seven genes) using DnaSP v.5 (Librado et al., 2009) followed by a median joining (Bandelt et al., 1999) haplotype network constructed in PopART v. 1.7 (Leigh et al., 2015). To ascertain any possible population structure a Bayesian approach implemented in BAPS v.5.3 was used as it considers linked loci data (Corander et al., 2007). Pairwise F_{st} and differential hierarchical AMOVA analysis was performed using Arlequin v. 3.0 (Excoffier et al., 2005) to confirm the pattern found in BAPS analysis.

3.2.5 Estimation of clade-specific divergence times:

To identify the clades, Bayesian phylogeny was constructed with MrBayes v. 3.2.7 (Huelsenbeck et al., 2001) using 16 Indian rhino mitogenome (mentioned in Chapter 1) and Javan rhino sequence (outgroup, as they are the sister clade of one-horned rhinoceros) (Harley et al., 2016). Analysis was conducted using GTR+G substitution model determined by jModelTest v2.1.3 (Daribba et al., 2012) (based on Akaike Information Criteria). The MCMC parameters included 2 runs of four chains each of 15 million generations with sampling after 1000 generations till split frequencies were below 0.01. Posterior probabilities were calculated for each node.

To estimate divergence among clades, rate of mutation for Indian rhino was calculated using BEAST v.2.3.6 (Drummond et al., 2007). Analysis was performed with five extant rhino mitogenome (without D-loop) (n=11 sequences, India=7 (haplotypes representing maximum variation in the data), Java=1, Sumatra=1, White=1, Black=1) along with horse (Equus caballus, Genbank: NC001640), donkey (Equus asinus, Genbank: NC001788), Asiatic wild ass (Equus hemionus, Genbank:

NC016061) and zebra (Equus zebra, Genbank: NC018780) as outgroups. GTR+G substitution model was selected through jModelTest v2.1.3 for this multi-species data. Birth-death speciation was considered as tree prior (Drummond et al., 2006; Steiner et al., 2017) along with uncorrelated relaxed log normal clock (Ritchie et al., 2017; Steiner et al., 2017). During analysis, four established internal node calibration points (based on fossil records) with normal distribution priors were employed: (i) Caballine split (4± 0.5 million years ago (Mya)) (Eisenmann 1992; Orlando et al., 2013); (ii) late Oligocene diversification of rhino groups (26± 3.5 Mya) (Hooijer 1946); (iii) split of rhinoceros genus (3± 0.5 Mya) (Liu et al., 2021); (iv) origin of the perissodactyls (55± 3 Mya) (Prothero et al., 1989; Benton et al., 2007). The first three calibration points were considered as monophyletic constraint (Ritchie et al., 2017) as the last point includes both ingroup and outgroup taxa. tMRCA (time to Most Recent Common Ancestor) was inferred using the estimated mutation rate with lognormal distribution under strict molecular clock (intra species data, n=16) (Yoder et al., 2000; Drummond et al., 2006). MCMC runs included 100 million generations, sampled at every 10,000 states with 10% burn-in. Data convergence was checked with Tracer v. 1.5 (Rambaut et al., 2007) and the final tree (with maximum clade credibility) was estimated with TreeAnnotator (Helfrich et al., 2018) and visualised using FigTree v.1.4.2 (Rambaut 2009).

3.3 Results:

3.3.1 Phylogeography of wild Indian rhinos:

This data was generated for additional 95 unique individuals (n= 56 tissue and 39 dung) using 8 primers (Genbank: MZ771364-MZ771458, MZ771459-MZ771553,

MZ771554-MZ771648, MZ771649-MZ771743, MZ771744-MZ771838, MZ771839-MZ771933 and MZ771934-MZ772028) (Table 3.1 and Figure 3.1). Remaining samples could not be used as they were rejected due to low amplification success for microsatellite data (n=12), genetic recaptures (n=13) and individuals from adjacent midden sites (n=24). Sequencing comparison showed that out of the 21 polymorphic sites, two and three sites were specific to West Bengal and Uttar Pradesh, respectively, whereas all others were shared at different levels among the three states (Shared among three states- 10 sites, Assam-Uttar Pradesh- eight sites, Assam-West Bengal- four sites, West Bengal-Uttar Pradesh- 0 sites, Table 3.2). Median joining network (n=111 individuals) showed a total of 30 haplotypes (h) across India. Majority of these haplotypes (93.3%, n=28) were from Assam whereas both West Bengal (one haplotype, n=20) and Uttar Pradesh (one haplotype, n=10) populations were found to be monomorphic (Figure 3.2). The sequence from Bihar rhino individual was identical to the Uttar Pradesh population. Population-wise genetic variation indices (Table 3.3) showed overall highest values for KNP (n= 46; S=18, h=19, π=0.0021, Hd= 0.85), followed by MNP (n=12; S=14, h=6, π=0.0023, Hd= 0.89), ONP (n=12; S=9, h=6, π=0.0016, Hd= 0.89) and PWLS (10; S=2, h=3, π=0.0002, Hd= 0.51). Bayesian genetic clustering corroborated with the earlier pattern (K=3) where samples from West Bengal and Uttar Pradesh formed distinct clusters whereas Assam showed geographically intermixed fixed haplogroups (Figure 3.2). The genetic differentiation (pairwise F_{st}) values among these three clusters were significantly high ranging from 0.68-1 (Table 3.4, indicating highly structured populations). The hierarchical AMOVA analysis using two separate groupings: a) seven populations and b) three states showed higher within population

(50%) and between group variance (45%) (Table 3.4). Such pattern indicates that overall genetic structure is influenced by differentiation at clade level and the amount of diversity present within the Assam clade (Table 3.3 and Table 3.4).

Table 3.1: Sample collection (both tissue and dung) and data generation details of Indian rhinos used in this study

State	**Protected areas**	**Tissue collected**[a]	**Tissue used for whole mitogenome data**	**Dung collected**	**Dung used for phylogeography**[b]
Uttar Pradesh	Dudhwa NP	4	2	13	6
West Bengal	Gorumara NP	2	2	18	8
	Jaldapara NP	4	2	15	6
Assam	Manas NP	3	1	22	9
	Pobitora WLS	9	2	5	1
	Orang NP	3	3	15	9
	Kaziranga NP	46	3	0	0
Bihar*	Valmiki NP	1	1	NA	NA
	Total	**72**	**16**	88	**39**

**Reference sample from non-rhino bearing area*

[a] Total number of tissue samples used in this study. Out of 72, 16 were used for mitogenome data generation as population-wise details given in next column.

[b] Dung details of identified rhino individuals representing six parks to ensure spatial coverage for phylogeography analysis. In total 111 samples were used for this work consisting of 72 tissues and 39 dungs.

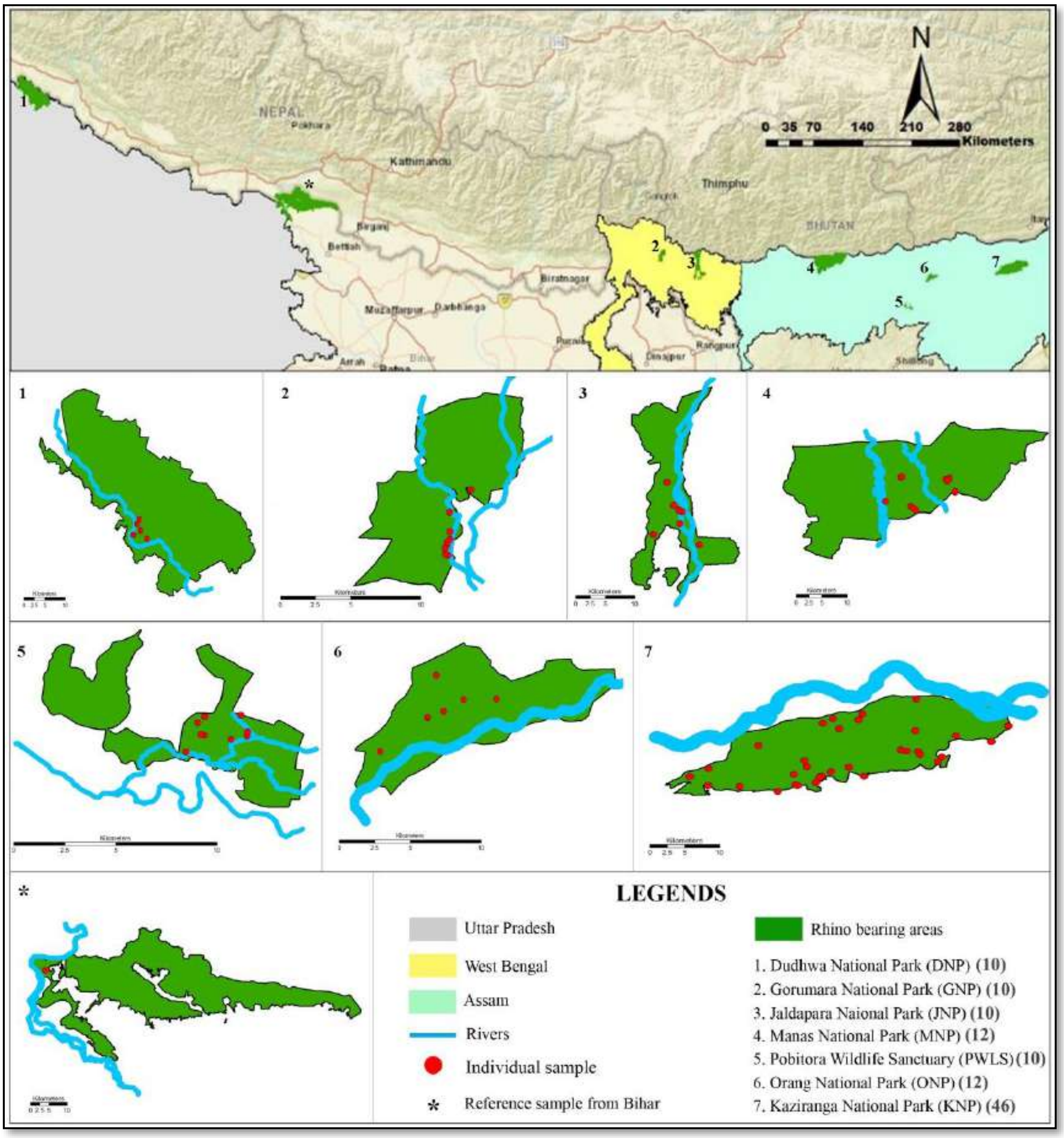

Figure 3.1: Map of the study area and distribution of the final samples used in this study (n=111). The top plate shows the position of the rhino-bearing parks across three Indian states (Uttar Pradesh, West Bengal and Assam). The reference sample of wild rhino received from the state of Bihar is also presented.

Table 3.2: Details of the variable sites based on concatenated sequence of 2531bp of Indian one horned rhino mtDNA haplotypes

Clade	**Gene**		**COX1**	**ATP6**	**COX3**	**NADH4**	**NADH5**	**CYTB**			**CR**												
	Primers		**P10F**	**ATP6F**		**NADH4F**	**NADH5F**	**CYTBF1**		**CYTBF2**	**CRF1**							**CRintF2**					
			COXR	**ATP6R**		**NADH4R**	**NADH5R**	**CYTBR1**		**CYTBR2**	**CRintR2**							**CRintR3**					
	Position		5	8	8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
			9	5	7	1	2	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5
			7	2	9	2	4	2	5	8	3	5	6	6	6	7	7	7	7	8	8	8	8
			8	0	4	6	8	8	5	8	2	5	0	8	8	0	3	7	7	3	6	6	8
	Haplotype	**N**				0	9	5	5	2	2	9	6	5	7	3	0	0	6	5	4	8	4
Uttar Pradesh	H1	10	T	G	T	G	A	G	A	G	C	T	T	G	C	T	G	A	T	C	G	C	T
West Bengal	H2	20	*	*	*	A	*	A	G	A	T	*	C	*	T	*	A	*	*	*	*	T	C
Assam	H3	1	*	*	*	A	*	*	*	*	T	C	*	A	*	*	A	*	*	*	*	*	C
	H4	1	*	*	*	A	*	*	*	*	T	C	*	*	*	C	A	*	*	*	*	*	C
	H5	1	C	T	*	A	G	*	G	*	T	C	*	A	*	*	A	G	*	*	*	T	*
	H6	1	*	*	C	A	*	*	*	*	T	C	*	A	*	*	A	G	*	*	*	T	*
	H7	2	C	T	*	A	G	*	G	*	T	*	*	*	*	C	A	*	*	*	*	*	C
	H8	26	C	T	*	A	G	*	*	*	T	C	*	A	*	*	A	G	*	*	*	T	*
	H9	1	C	T	*	A	G	C	*	A	T	C	*	A	*	*	A	G	*	*	*	T	*
	H10	1	*	*	C	A	*	*	*	*	T	*	C	*	*	*	A	*	*	T	*	T	*
	H11	5	C	T	*	A	G	*	*	A	T	C	*	A	*	*	A	G	*	*	*	T	*
	H12	1	*	*	C	A	*	*	*	A	T	*	C	*	*	*	A	*	*	T	*	T	*
	H13	1	*	*	*	A	*	*	*	A	T	*	*	*	*	*	A	*	*	*	*	*	C
	H14	1	C	T	*	A	G	*	*	*	T	C	*	A	*	*	A	G	*	*	*	*	*
	H15	1	*	*	*	A	*	*	*	*	T	*	*	*	*	*	A	*	C	*	*	T	*
	H16	2	*	T	*	A	G	*	*	*	T	C	*	A	*	*	A	G	*	*	*	T	*
	H17	2	C	*	C	A	*	*	*	*	T	*	C	*	*	*	A	*	C	T	A	T	*
	H18	3	*	*	C	A	*	*	*	*	T	*	C	*	*	*	A	*	C	T	A	T	*
	H19	2	*	*	*	A	*	*	*	*	T	*	*	*	*	C	A	*	*	T	A	T	*
	H20	10	*	*	*	A	*	*	*	*	T	*	*	*	*	C	A	*	*	*	*	*	C
	H21	3	C	*	*	A	*	*	*	*	T	*	*	*	*	C	A	G	*	*	*	T	*
	H22	2	*	*	*	A	*	*	*	*	T	*	*	*	*	C	A	G	*	*	*	T	*
	H23	3	C	*	*	A	*	*	*	*	T	*	*	*	*	C	A	*	*	*	*	*	C
	H24	1	*	*	*	A	G	*	*	*	T	*	*	*	*	C	A	G	*	*	*	*	*
	H25	1	C	T	*	A	*	*	*	*	T	C	*	A	*	*	A	G	*	*	*	T	*
	H26	4	C	T	*	A	G	C	*	*	T	C	*	A	*	*	A	G	*	*	*	T	*
	H27	1	*	*	C	A	*	C	*	*	T	C	*	A	*	*	A	*	*	T	*	T	*
	H28	1	C	*	T	A	*	*	*	*	T	C	*	A	*	*	A	G	*	*	*	T	*
	H29	1	C	T	T	A	G	*	*	*	T	*	*	*	*	C	A	*	*	*	*	*	C
	H30	1	*	T	T	A	G	*	*	*	T	*	*	*	*	C	A	*	*	*	*	*	C

Table 3.3: mtDNA diversity indices of all seven rhino populations in India (n=111). (Bihar sample was included within Uttar Pradesh clade)

Protected areas	Sample size	Segregating sites (S)	Haplotypes (h)	Haplotype diversity (Hd)	Nucleotide diversity (π)
Kaziranga NP	46	18	19	0.85	0.0021
Pobitora WLS	10	2	3	0.51	0.0002
Manas NP	12	14	6	0.89	0.0023
Orang NP	12	9	6	0.87	0.0016
Dudhwa NP	10	0	1	0	0
Valmiki NP	1	NA	NA	NA	NA
Gorumara NP	10	0	1	0	0
Jaldapara NP	10	0	1	0	0
Total	**111**	**21**	**30**	**0.89**	**0.0028**

Table 3.4: Results of Pairwise genetic differentiation and hierarchical AMOVA test (Bihar sample considered under Uttar Pradesh clade).

<table>
<tr><th colspan="5">Pairwise F_{st} among clades (*p<0.05)</th></tr>
<tr><td></td><td>Assam</td><td>Uttar Pradesh</td><td colspan="2">West Bengal</td></tr>
<tr><td>Assam</td><td>0</td><td></td><td colspan="2"></td></tr>
<tr><td>Uttar Pradesh</td><td>0.68*</td><td>0</td><td colspan="2"></td></tr>
<tr><td>West Bengal</td><td>0.73*</td><td>1.0*</td><td colspan="2">0</td></tr>
<tr><th colspan="5">AMOVA test among three clades and seven populations</th></tr>
<tr><th>Source of variation</th><th>d.f.</th><th>Sum of squares</th><th>Fixation index</th><th>Percentage of variation</th></tr>
<tr><td>Among groups</td><td>2</td><td>14.689</td><td>0.45 (F_{ct})</td><td>44.66</td></tr>
<tr><td>Among populations within groups</td><td>4</td><td>3.233</td><td>0.1 (F_{sc})</td><td>5.71</td></tr>
<tr><td>Within populations</td><td>104</td><td>31.267</td><td>0.50 (F_{st})</td><td>49.63</td></tr>
<tr><td>Total</td><td>110</td><td>49.189</td><td>0.60573</td><td></td></tr>
</table>

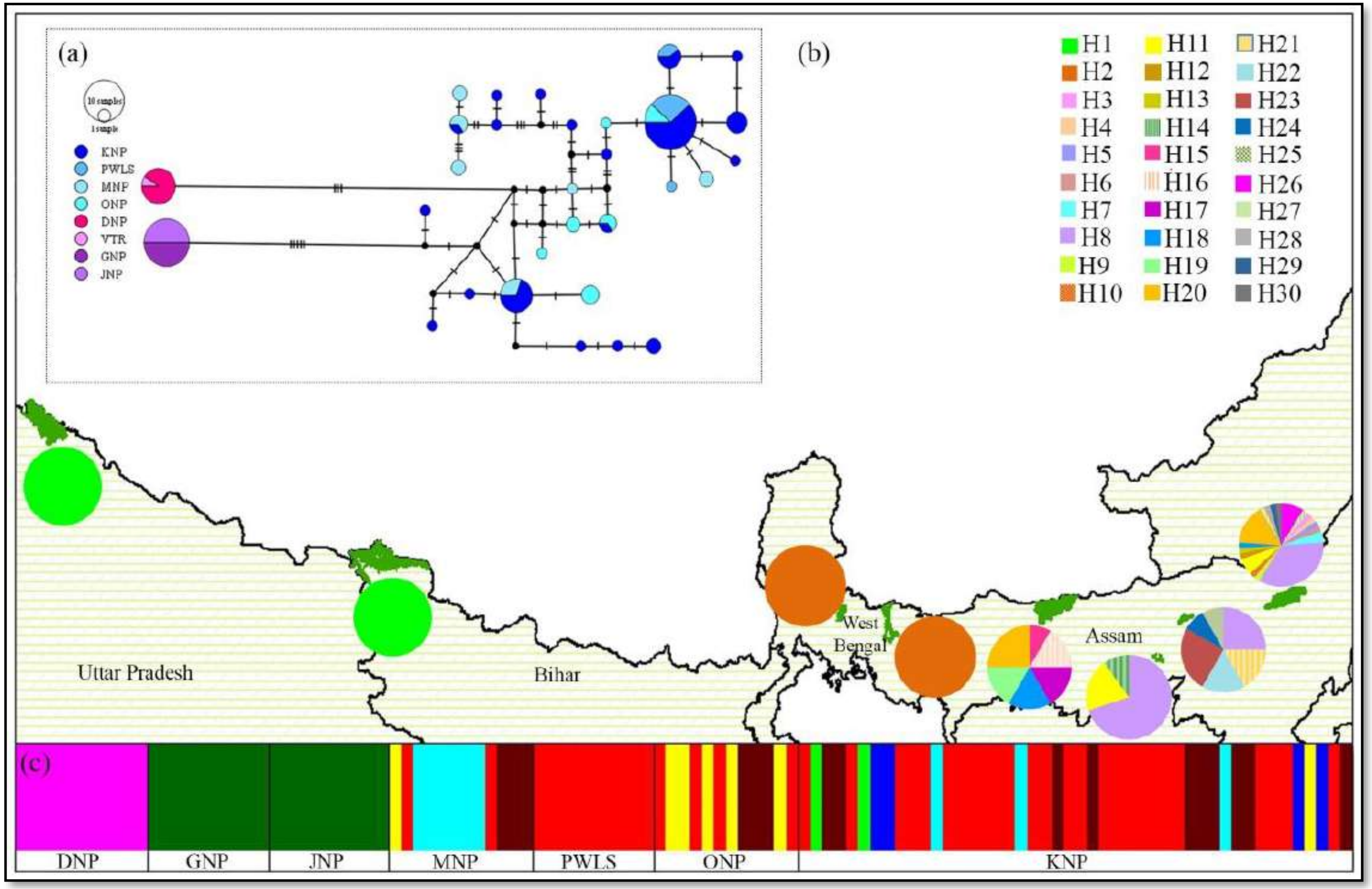

Figure 3.2: Representation of mtDNA variations and genetic structure in Indian rhinos based on 2531 bp concatenated sequence covering all polymorphic sites across seven genes. (a) Median joining network with park-level colour codes; (b) Haplotype frequencies at each of the sampled areas covering all variations (n=30 haplotypes); (c) Bayesian clustering shows monomorphism in Uttar Pradesh (with sample from Bihar, n=11) and West Bengal (n=20) populations and polymorphism in Assam (n=80).

3.3.2 Divergence time of different Indian rhino clades:

The Bayesian phylogeny showed similar pattern of three clades consisting of West Bengal, Assam and Uttar Pradesh (along with the Bihar sample, Figure 3.3). Based on the calibrated root nodes and Indian rhino-specific mutation rate (1.2 x 10-4 mean rate of substitution per site per million years, Figure 3.4), tMRCA analysis suggested a divergence period spanning from 950 (HPD 1360-810 Kya) to 50 (150-10 Kya) Kya (Figure 3.3). Our results indicated the divergence of Indian rhinos ~ 950 Kya (node A, Fig. 3) corresponds to the emergence period of one-horned rhino ancestors in the subcontinent (Lal 2016; Patnaik 2016). Next, the Assam population diverged from the remaining clades at ~500 Kya (HPD 680-330 Kya, nodes B & C, Figure 3.3). This is supported by reports of multiple rhino movements away from Assam (along Siwalik as well to Siva-Malayan region) during this period (van den Bergh et al., 2001; Patnaik 2016). At population level, results suggest a relatively earlier coalescence of Assam ~190 Kya (HPD 300-70 Kya, node D & E, Figure 3.3) compared to West Bengal and Uttar Pradesh (~50 Kya, HPD 150- 10 Mya, node F & G, Figure 3.3). This period (120-10 Kya) is known for confinement of rhinoceros to the north and north-east of India due to monsoon intensification and grassland dominance (Lal 2016; Patnaik 2013 & 2016).

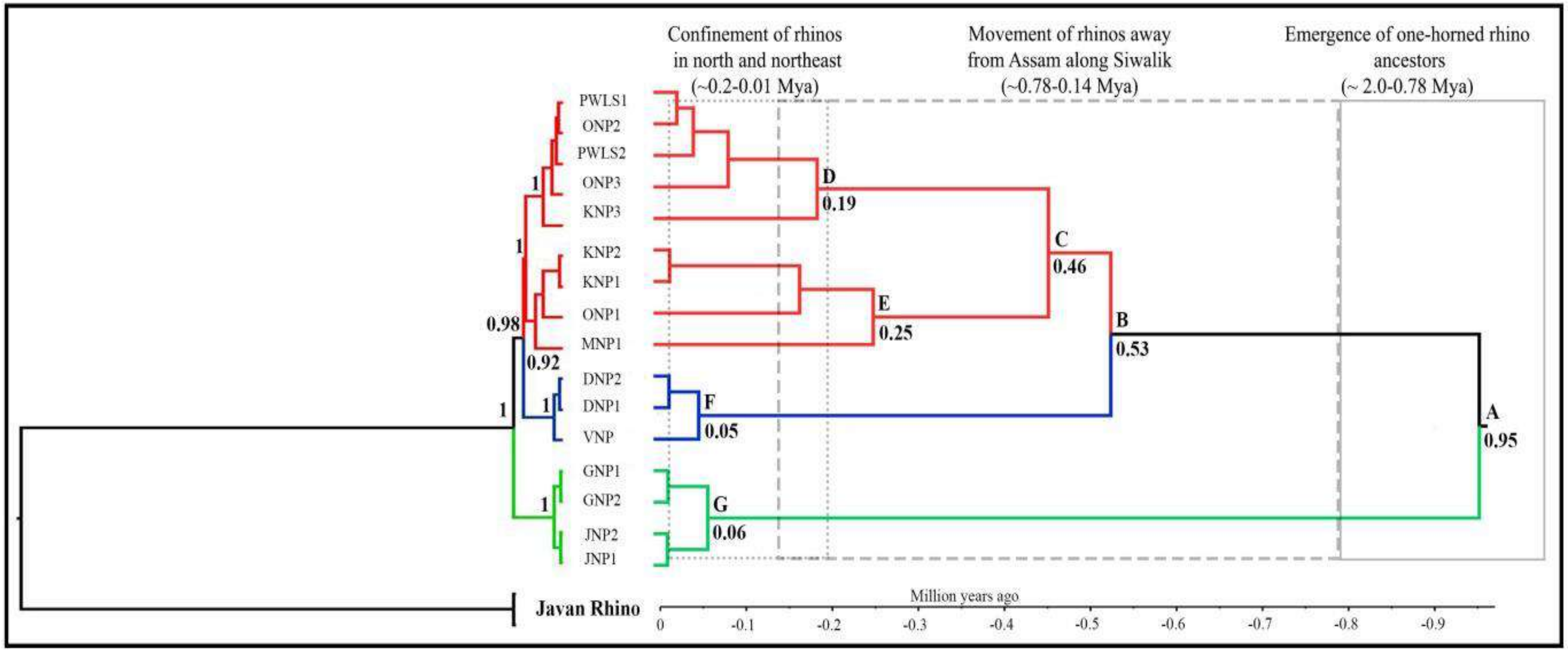

Figure 3.3: Phylogenetic relationship and assessment of divergence time in Indian rhino populations. The left pane shows the basal clade of West Bengal samples (green) and sister clades representing Uttar Pradesh (blue) and Assam (red). Javan rhino sequence was used as outgroup. The posterior probability values (≥0.9) are shown in bold. The right pane indicates the divergence of Indian rhinos, where the Assam population diverged first (~0.5 Mya), followed by parallel divergence of West Bengal and Uttar Pradesh (0.06-0.05 Mya). Node-specific ages are marked (with posterior probability values ≥0.9). The major corroborating paleobiogeographical events are presented above.

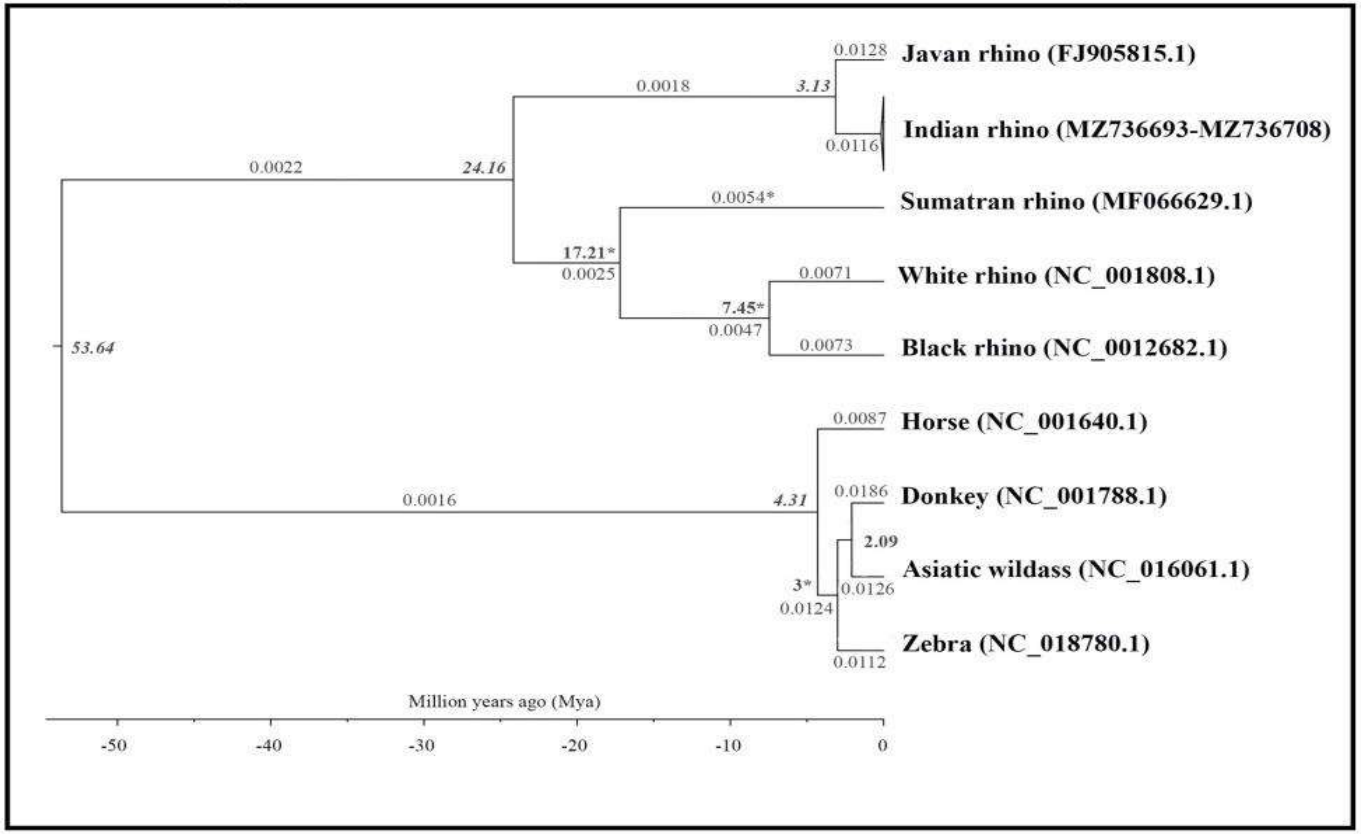

Figure 3.4: Estimation of mitogenome mutation rate for Indian rhino using Caballine (Zebra, Donkey, Asiatic Wild Ass and Horse) as outgroup. Internal node calibration points are presented in italics (bold). Estimated node ages and branch mutation rates with available reference in literature is marked with * (Fernando et al., 2006; Steiner et al., 2011; Steiner et al., 2017; Liu et al., 2021).

3.4 Discussion:

This study presents the most extensive mitochondrial DNA phylogeography of one-horned rhinos across its Indian distribution. Careful considerations involving mitogenome sequencing of representative samples across Indian rhino-bearing areas, identification of all polymorphic regions and their amplification from spatially-covered rhino samples helped us achieving accurate assessment of mtDNA variations. The only other study on one-horned rhino mtDNA variations (based on partial control region sequences, 428 bp) reported 10 haplotypes (Kaziranga National Park, India- 4 and Chitwan National Park, Nepal- 6, respectively) and moderate level of genetic difference (F_{st} value of 0.39 between them) (Zschokke et al.,2011). Careful scrutiny of our data revealed that all the polymorphic sites (or identified segregating sites) were found in fixed positions within one-horned rhino mitogenome (Table 3.2) across India. Given the distribution of polymorphic sites in the sequenced mitogenomes and our sampling coverage, it is likely that these data represent the majority or perhaps all extant mtDNA haplotypes in Indian rhinoceros populations. This claim is also supported by the similar haplotype diversity values from the mitogenome and the phylogeography datasets (0.93 and 0.9, respectively). The clustering analysis of the concatenated rhino sequences showed three distinct genetic clades (corresponding to the states of Assam, West Bengal and Uttar Pradesh) with high F_{st} value (0.68-1), corroborating with the haplotype network patterns. Mantel test (-0.83, p=1) confirmed that such strong genetic structuring is not due to isolation by distance pattern, but driven by lineage-specific evolutionary history (as suggested by AMOVA results). Such pattern of higher within population and between group variance (50% and 45% in Indian rhinos, respectively) indicates that the mitochondrial genetic variation observed in extant Indian rhino is influenced by both evolutionary diversification and retention of diversity at population level only for Assam clade. As two of the clades are monomorphic, they contribute very less proportion of among-population within-group variations (5%). Similar data has also been described in other species such as barking deer- (Martins et al., 2017), dog- (Pang et al., 2009) etc. Interestingly, it was found that the sequence from the Bihar sample (representing samples from Nepal) was identical to the Uttar Pradesh sequences, including the state-specific SNPs. This pattern was expected as the founder animals of the reintroduced Uttar Pradesh population were sourced from Chitwan National Park, Nepal (four dominant breeding females) and Pobitora Wildlife Sanctuary of Assam (dominant breeding male) (Talukdar et al., 2012). Further comparison of 13 partial D-loop sequences from Chitwan National Park, Nepal Zschokke et al. (Zschokke et al., 2011) confirmed this pattern, indicating that the mtDNA signature of the Uttar Pradesh population belongs to Nepal. Given that the entire Uttar Pradesh rhino population showed only one haplotype, future studies need to evaluate the mtDNA variation in the Nepal population.

The phylogenetic analyses reconfirmed the relationship among the existing members of the Rhinocerotidae family (Xu et al., 1996; Steiner et al., 2011 & 2017) where the Sumatran and African rhino formed sister clades, separated from the *Rhinoceros* sp. based on the extant rhino genus/species sequence data only (Woolly rhinoceros sequence was not used). The within species tree topology corroborated with the haplotype network results as Assam and Uttar Pradesh formed phylogenetically closer clades as compared to West Bengal. This observed phylogenetic pattern of

West Bengal being separate clade is influenced by lesser shared polymorphic sites between West Bengal and other two clades (Table 3.2). Combined together, it can be interpreted that one-horned rhino diverged from its recent common ancestors ~950 Kya and different populations (Assam, West Bengal and Uttar Pradesh/Nepal) coalesced ~190-50 Kya time period (Figure 3.3). The molecular dates were comparable to other published literature on rhino evolution (Lal 2016; Patnaik 2013 & 2016) and supported by the paleobiogeography history of the Indian subcontinent (Patnaik 2013 & 2016). For instance, the inward movement of rhinos from Assam along Siwalik (680-330 Kya, node B & C, Figure 3.3) coincides with drop in the sea level which facilitated movement of multiple genera (for example, *Elephas, Panthera*, *Rhinoceros*, *Muntiacus* etc.) through Siva-Malayan route (van de Bergh et al., 2001; Vidya 2016). Report of one-horned and Javan rhino co-existence in Bhutan ~560 Kya (Margaryan et al., 2020) provides further support of such movements. Finally, the coalescence time of the three Indian clades corresponds to Holocene climatic optimum period known for monsoon intensification in north and north-east part of India resulting in range contraction for grassland dependent species (Lal 2016, Patnaik 2016; Kumar et al., 2017). Future efforts should try to include molecular data from historical/ancient samples to tighten the variance associated with divergence estimates (Drummond et al., 2007). Overall, this approach reiterates the critical importance of large datasets (whole mitogenome from multiple individuals in this case), informative prior settings and its assessment with posterior outputs, taxon-specific mutation rate, node calibration points etc. for accurate tMRCA estimation (Yoder et al., 2001; Drummond et al., 2006; Subramanian et al., 2009; Ritchie et al., 2017).

The spatially exhaustive sampling coverage and the patterns of population structure bring out some critical conservation perspectives for the Indian rhinos. The phylogeographic and mitophylogenomic patterns suggest three distinct clades with state-specific evolutionary histories. As these populations are morphologically undistinguishable and interbreed among themselves (Dudhwa, Uttar Pradesh population is genetically mixed (Talukdar et al., 2012)), they should be recognised as 'Evolutionary Significant Units (ESUs)' (Moritz 1994). It is therefore important to use such information towards conservation and management efforts for each of these populations (Moritz 1994; Crandall et al., 2000; Kitchener et al., 2017). Till date genetic information of the species has not been used in translocation planning (possibly due to lack of sufficient data), and the genetic signatures described in this study would be very helpful to increase variation in target populations. For example, the Uttar Pradesh and West Bengal population show state-specific monomorphic haplotypes representing unique but genetically depauperate populations. Based on the data presented here, suitable founder animals from Assam populations can be considered for future translocation programs in these areas, thereby increase the genetic diversity of these populations to combat any potential stochastic events (Crandall et al., 2000; Weeks et al., 2017). However, such efforts would impact the suggested ESU categorizations due to mixing of different gene pools among populations. Another important aspect for management consideration would be better planning for translocation events to any of the existing or new areas (Rookmaker et al., 2016; Jhala et al., 2021). For example, the reintroduced rhino population in Assam (Manas National Park) showed much higher mtDNA variation (six haplotypes), possibly due to periodic supplementation of individuals of varied genetic ancestry across different wild rhino populations (Talukdar et al., 2020) compared to Dudhwa NP (single haplotype) of Uttar Pradesh (single supplementation event). As multiple reintroduction programs are planned in near future, it is suggested that all future efforts should consider selecting genetically variable founder animals, multiple reintroduction events along with evolutionary history (Crandall et al., 2000; Weeks et al., 2017; O'Brien et al., 2017). However it is important to point out that to provide a holistic genetic rescue plan for Indian rhinos, information on nuclear DNA diversity is also critical (Mace et al., 2010; Knaus et al., 2011; Weeks et al., 2017). Finally this current study is the first assessment of range-wide mitogenome diversity in Indian rhinos emphasizing its importance as one of the fundamental information for future population reintroduction/ recovery programs.

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Chapter 3: Assessment of rhinoceros genetic health across India

4.1 Introduction:

The greater one-horned rhinoceros (GoH) is a flagship grassland inhabitant endemic to India and Nepal. It typifies the endangered group of megaherbivore community whose present population size and distribution range is altered by anthropogenic interference (Karanth et al., 2010; Ripple et al., 2015). Extensive poaching pressures accompanied with rapid habitat conversion raised the fear of Indian rhino extinction in early 20th century (Menon 1996; Rookmaker et al., 2016). Since late 1900 subsequent protection measures and management interventions resulted in population recovery from few hundreds to approximately 3500 individuals in India (Talukdar et al., 2021). Although these measures has helped in bringing back the species from brink of extinction, but recovered populations are prone to decreased genetic diversity, amplified population structuring and skewed demographic parameters (Mondol et al., 2013;Moodley et al., 2017; Kolipakam et al., 2019). These concerns are listed in the Convention on Biological Diversity as process of 'genetic erosion', and are suggested to be minimised for future survival of viable populations in wild (Mace et al., 2010; Willi et al., 2022). Thus for a K-selected species like Indian rhinos, genetic assessment is critical to reduce the probable effects of population bottleneck.

Investigating population sub-structuring is first step to assess the impact of anthropogenic pressures on recovered species (Willi et al., 2022). However, determining the genetic sub-populations at landscape level should be done with caution as it is influenced by sampling heterogeneity and presence of hierarchical structuring (Schwartz et al., 2009; Basto et al., 2016). To overcome this issue, integrated multiple clustering approach is preferred over single clustering analysis to avoid under or over estimation of sub-structuring (Ball et al., 2010; Basto et al., 2016). This is followed by assessment of genetic diversity indices focusing on maximisation of heterozygosity and allelic richness (Alvarez et al., 2009; DeWoody et al., 2021) since they are associated with increased fitness, long-term survival and reduced extinction risk (Johnson et al., 2010; Frankham et al., 2016; Taylor et al., 2017; DeWoody et al., 2021). These indices facilitate conservation prioritisation by identifying the populations vulnerable to drift (Mannise et al., 2017; De et al., 2021; Kolipakam et al., 2019). In context of Indian rhinos, this prioritisation is important as currently they are patchily distributed in areas of <20,000km^2 with different density (ranging from 0.2-6.2/ km^2) and recovery history (see table 1.1). Thus populations with immediate need of genetic restoration (i.e. the populations with low diversity (Weeks et al., 2011)) should be identified for focused mitigation plans.

As the future conservation programmes for Indian rhinos are oriented towards translocation-driven population enhancement, it is important to choose genetically diverse and healthy source populations (Weeks et al., 2011; O'Brien et al., 2017). To achieve this, first objective of this chapter is to investigate the number of genetic clusters in Indian rhino populations using multiple clustering approaches. This is followed by estimation of genetic erosion and loss of genetic variability across the landscape. Finally the results will help in providing park specific conservation mitigation plans and identification of source populations for long term persistence of Indian rhinos. The combined information of three ESUs (discussed in chapter 2,Ghosh et al., 2022) and park-wise genetic health assessment (in current chapter) will ensure successful genetic rescue required for the species survival (Weeks et al., 2016; O'Brien et al., 2017; Ralls et al., 2018).

4.2 Methods:

4.2.1 Sample collection:

Sampling design plays a critical role in inferences of population genetic analyses (Schwartz et al., 2009) due to inherent genetic gradient caused by heterogeneous species distribution and their social organisation (Schwartz et al., 2009; Segelbacher et al., 2010). Since rhinos use communal latrines (or middens), the sampling effort for each park was decided based on population size and the distribution of midden sites (clustered or homogenous, Table 4.1). Overall this strategy ensures spatial and fine scale individual coverage across each park. However in case of Kaziranga NP, the spatial coverage was given priority as it harbours ~2500 individuals. A total of 901 samples were collected (tissue=153, dung=748) over a period of four years 2017-2020 (Table 4.1, this also includes the samples mentioned in Chapter 1 and 2). As mentioned earlier in previous chapter, dung DNA was collected by swabbing the upper layer of topmost bolus from known midden sites across all seven rhino parks and tissues were provided by respective forest department.

Table 4.1: Sample effort details for each rhino bearing area of India

State	Park	Population size	Distribution pattern	Samples	
				Dung	Tissue
Uttar Pradesh	Dudhwa NP	38	Clustered	90	4
West Bengal	Gorumara NP	52	Clustered	109	3
	Jaldapara NP	250	Homogenous	99	22
Assam	Manas NP	40	Clustered	75	3
	Pobitora WLS	101	Clustered	207	9
	Orang NP	100	Homogenous	78	2
	Kaziranga NP	2500	Homogenous	90	110

4.2.2 DNA extraction and PCR amplification:

DNA extraction from both tissue and dung was done as discussed in Chapter 2 (Ghosh et al., 2022). For individual identification a panel of 14 microsatellites optimised for Indian one-horned rhinoceros was used (Ghosh et al., 2021). In brief, PCR were performed with 10μl reaction volumes containing 4μl Hotstart Multiplex PCR mix (Qiagen Inc.), 3μl of BSA, 0.2μM of primer mix and 2μl of template DNA. PCR conditions included an initial denaturation (95 °C for 15 min); 40 cycles of denaturation (95 °C for 30 sec); annealing (Ta for 40 sec); extension (72 °C for 40 sec); followed by a final extension (72 °C for 30 min) in an ABI thermocycler. Every set of PCR includes, positive, negative and extraction controls to monitor PCR failure and contamination issue. Amplified PCR products were visualised in 2% agarose gel followed by genotyping using HiDi formamide (Applied Biosystems, California, United States) and LIZ 500 size standard (Applied Biosystems, California, United States) in an ABI 3500XL Genetic Analyser (Applied Biosystems, California, United States). To check between run stochastic errors, the positive reference sample was added in every batch. All the samples were genotyped three times independently and manually scored in GENEMARKER (Softgenetics Inc., Pennsylvania, United States) following the protocol described in Ghosh et al., 2021.

4.2.3 Marker characterisation and individual identification:

To ensure data accuracy for the standardised panel of microsatellites with dung DNA, peak characters and stutters stability was cross checked as described chapter 1 (Ghosh et al., 2021). Consensus genotype was prepared for all samples based on 'Quality index' approach and the markers with stutter issues and low RFUs were not included in downstream analysis as they were prone to genotyping errors (Pompanon et al., 2005; Ghosh et al., 2021).

Post data quality assessment, samples with minimum amplification of 9 loci (based on $\text{PID}_{(\text{sibs})}$ mentioned in Ghosh et al., 2021) were considered for further analysis. Unique genotypes were identified using 'Identity analysis' module of program CERVUS (Kalinowski et al., 2007) with nine minimum loci matching and one fuzzy match. The genotype data for all the pairs of exact and fuzzy match were cross checked for remaining loci. This was followed by re-PCR of samples with ambiguity before removal of genetic recaptures from dataset.

Program GIMLET (Valiere 2002) was used to calculate the probability of identity for siblings ($\text{PID}_{(\text{sibs})}$) and unbiased ($\text{PID}_{(\text{unbiased})}$) for all the populations. Allelic dropout (ADO) and false allele (FA) rates were manually quantified as error rates per locus including the replicate data (Broquet et al., 2004; Pompanon et al., 2005). Similarly amplification success was also estimated as percent of positive PCR amplification for each locus (Broquet et al., 2004). Null allele (NA) was estimated using EM algorithm implemented in program FreeNA (Chapuis et al., 2007). Departure from Hardy Weinberg equilibrium and linkage disequilibrium was checked using ARLEQUIN 3.1 (Excoffier et al., 2005).

4.2.4 Genetic diversity and population structure:

Considering the patchy and isolated distribution of Indian rhino populations (Rookmaker et al., 2016) basic indices of genetic diversity and inbreeding coefficient (Fis) were calculated across all sampled areas using Arlequin (He, Ho) and Fstat (Fis). To identify the number of genetic subpopulations (or K) in Indian rhino, integrated clustering approach was considered because of their i) patchy and isolated distribution, ii) revival from small founder members and iii) presence of two reintroduced populations which may result in hierarchical structuring as observed in case of other studies (Gaggiotti et al., 2009; Hobbs et al., 2011; Vergara et al., 2015; Basto et al., 2016). The consensus solutions of different Bayesian individual-based clustering (IBC) approaches and multivariate analyses were used to infer the K (Ball et al., 2010; Basto et al., 2016; De et al., 2021). The IBC approaches implemented in STRUCTURE, TESS and GENELAND are based on stringent prior assumptions of population genetic models (like Hardy-Weinberg equilibrium (HWE) or linkage equilibrium), thus results in under or overestimation of K due to heterogeneous sampling scheme (Schwartz et al., 2009; Vergara et al., 2015;Basto et al., 2016). To overcome this issue, it has been suggested to corroborate their results with multivariate analysis which does not follow any underlying assumptions (Ball et al., 2010; Vergara et al., 2015; Basto et al., 2016; De et al. 2021). A diagrammatic flow chart of the approach is presented in Figure 4.1.

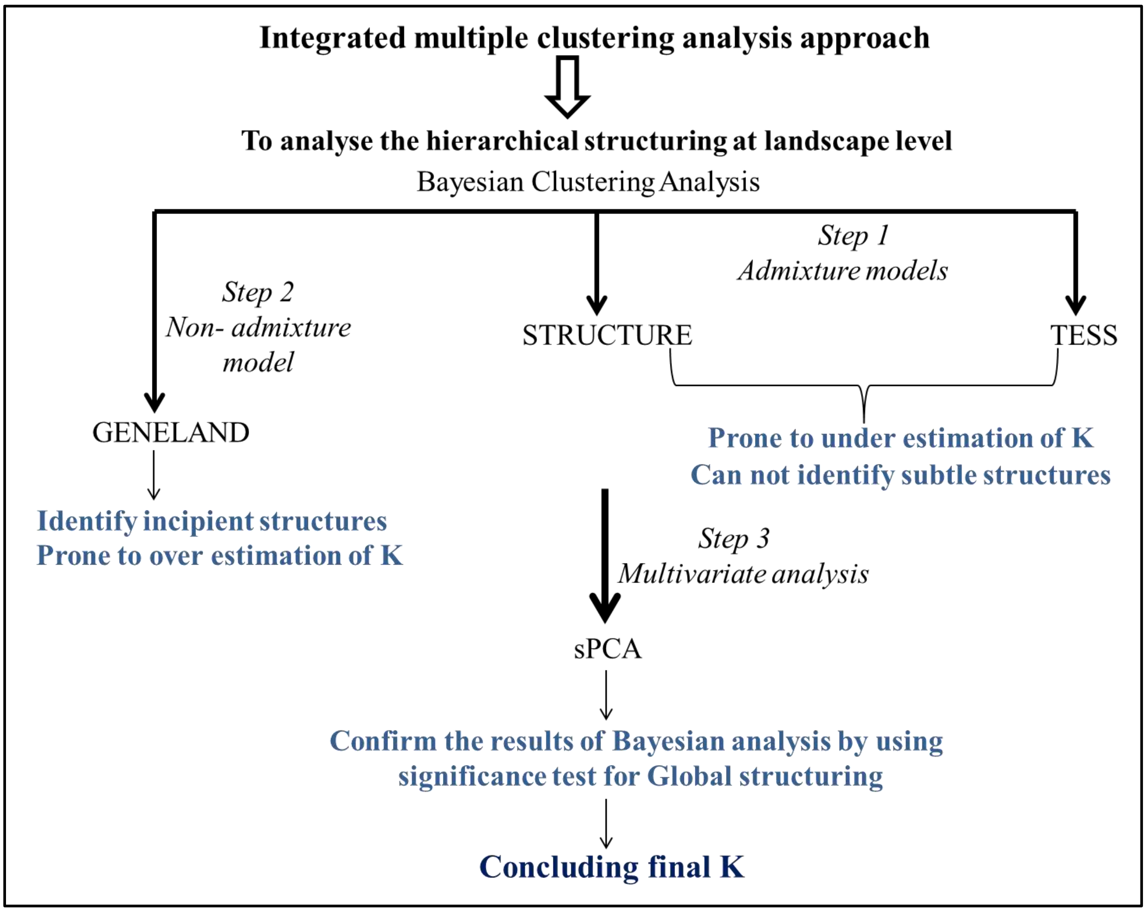

Figure 4.1: A diagrammatic representation of integrated multiple clustering approach to identify the genetic subpopulation at landscape level. The expected outcome of each step is highlighted with bold and blue coloured font.

All the IBC approaches were done using correlated allele frequencies within population (Falush et al., 2003). Following Pritchard et al. 2000, STRUCTURE v2.3 runs were performed using with and without Locprior assumptions. For both models 10 independent runs of each K value (1 to 12) with 4.5 X 10^5 iterations and 5X10^4 burnin were given. The optimal K was selected using the model choice statistics L(K) (Pritchard et al., 2000) after model averaging to get the proportion of admixtures over 12 replicates using CLUMPAK (Kopelman et al., 2015). The spatially explicit algorithm (unlike STRUCTURE) implemented in TESS v2.3.1 was used to detect genetic discontinuities among weekly structured populations (Chen et al. 2007, Vergara et al. 2015). 12 replicate runs of 25000 sweeps after 1200 burn-in were performed to test for the most likely number of clusters (K_{MAX}=2 to 12). Subsequently model averaging was done using CLUMPAK (Kopelman et al., 2015) and K_{MAX} was selected based on the deviance information criterion (DIC) (Durand et al., 2009). Results of both the programme were visualised with STRUCTURE PLOT v2.0 (Ramasamy et al., 2014). This was followed by GENELAND v4.0.8 (non-admixture model, Guillot et al., 2005) to detect subtle genetic structuring which may remain undetected because of assumption of admixture ancestry in both STRUCTURE and TESS (Figure 4.1) (Vergara et al., 2015; De et al., 2021). Simulations of $2X10^5$ iterations and 200 thinning were used without uncertainty on spatial coordinates for K=1 to 10 (with 10 replicates for each K) employing correction for null alleles. Following the suggestion in Guillot et al., 2005, maximum rate of Poisson process was set at 504 and maximum number of nuclei at 1500. The optimal K was decided based on the highest mean posterior density across all iterations followed by generation of individuals assignment map. Finally to find out the true K, sPCA was employed as it accounts for spatial autocorrelation issues associated with neighbour mating and sample distribution (Schwartz et al., 2009; Vergara et al., 2015). It was done in pairs for the populations showing same genetic clusters in STRUCTURE without Locprior but not in the succeeding methods (i.e non-spatial versus spatial IBC). Hence for true inferred clusters (as per IBC methods) a significant global pattern would be indicated by positive Moran’s I index. The analysis was carried out using nearest neighbour as connection network and Monte

Carlo test with 9999 iterations was done to assess the significance of global spatial structure (Jombart 2008).

Post K finalisation, pairwise Fst was estimated among genetic clusters to identify degree of differentiation ranging from little (0) to complete (1) isolation (Hartl and Clark, 1997, De et al. 2021). Subsequently hierarchical analysis of molecular variance (AMOVA) was conducted to compare the variation between genetic clusters, among populations within clusters and within populations. Both of these analyses were done in ARLEQUIN 3.1 (Excoffier et al., 2005) with 10000 permutations and testing for significance at α=0.05.

4.2.5 Assessing genetic health of Indian rhino populations:

Understanding the patterns of (genetic) diversity loss across the geographical distribution of species helps in the identification of genetically vulnerable population/s in need of urgent management intervention (Mannise et al., 2017; Kolipakam et al., 2019; De et al., 2022). In this context, assessment of the relative contribution of each population to the overall genetic pool has been used to assign management priority or conserve breeding lineages in wild and captive populations (Alvarez et al., 2009; Coleman et al., 2013; Mannise et al., 2017). To achieve the same for Indian rhinos, two indices proposed by Caballero and Toro 2002 and Petit et al., 1998 were estimated using MolKin (Guti´errez et al., 2005). The first index, mean genetic distance (GD) is based on maintenance of overall Nei genetic diversity, according to which a population with positive value means less contribution to the gene pool or inherits lower genetic diversity (Caballero and Toro 2002; Alvarez et al., 2009; Mannise et al., 2017; De et al., 2022). In contrast, the second index represents the population's genetic uniqueness based on Hulbert’s rarefacted allelic richness (C) (Petit et al., 1998). Hence a population with positive value means more contribution toward aggregated allelic diversity or retains high allelic richness (Petit et al., 1998; Alvarez et al., 2009; Kolipakam et al., 2019). In addition to these indices, population specific Fst (or Fit), an indicator of genetic erosion (0-1 i.e. no to complete erosion) was also estimated using GESTE (Foll et al., 2006; Gaggiotti et al., 2009). Finally all the three indices were compared to get an overview of genetic health for each rhino population, expecting that the most affected area (with lowest diversity and high erosion) would have positive GD, negative C and higher Fit values (Guti´errez et al., 2005; Foll et al., 2006; Alvarez et al., 2009).

4.3 Results:

4.3.1 Marker characterisation and individual identification:

Out of 14, data from two primers (SR281, 12F) were rejected due to unstable peak characters and low amplification. Thus using 12 loci data we identified 504 individuals from 901 collected samples after removal of 265 poor quality samples and 132 recaptures (details in Table 4.2). Overall low genotyping rates were observed (ADO-0.09, FA- 0.02, NA-0.07) with average amplification success rate of 87%. The cumulative PID(sibs) and PID(unbiased) values (4.6X 10^{-4} and 2.7 X 10^{-8}, respectively) suggest that current set of 12 loci is statistically significant for unambiguous rhino individual identification. Locus wise summary statistics and spatial distribution of identified individuals for each population is given in Table 4.2 and Figure 4.2.

Table 4.2: Details of loci wise summary statistics across all Indian rhino populations

Populations	Loci	Number of Allele	AS (%)	ADO	FA	NA	Individuals identified	Individuals recaptured	Samples rejected	PIDsibs	PIDunbiased
Dudhwa	RH1	3	95	0.08	0.04	0.11	39	26	29	3.00E-03	2.10E-06
	RH3	3	91	0.04	0.03	0.00					
	RH10	5	93	0.03	0.03	0.00					
	IR10	2	93	0.28	0.04	0.02					
	RH7	2	95	0.18	0.07	0.00					
	SR63	3	93	0.14	0.01	0.07					
	RH5	4	96	0.05	0	0.07					
	IR12	3	97	0	0.03	0.08					
	7B	1	75	0	0.01	0.00					
	RH9	1	88	0	0.02	0.00					
	RH4	4	93	0.08	0.03	0.07					
	RH11	3	98	0.01	0.02	0.00					
	Total	**2.83±1.14**	**92**	**0.07**	**0.03**	**0.03**	**39**	**26**	**29**	**3.00E-03**	**2.10E-06**
Gorumara	RH1	2	94	0.00	0.04	0.06	47	28	37	1.20E-02	7.00E-05
	RH3	3	94	0.15	0.05	0.06					
	RH10	4	98	0.05	0.02	0.10					
	IR10	2	96	0.33	0.02	0.00					
	RH7	2	94	0.06	0.02	0.05					
	SR63	3	96	0.04	0.01	0.00					
	RH5	3	91	0.01	0.06	0.02					
	IR12	1	75	0.00	0.00	0.00					
	7B	3	79	0.10	0.05	0.12					

	RH9	1	69	0.00	0.00	0.00					
	RH4	2	93	0.04	0.00	0.00					
	RH11	4	86	0.03	0.00	0.00					
	Total	**2.5±0.96**	**89**	**0.07**	**0.02**	**0.03**	**47**	**28**	**37**	**1.20E-02**	**7.00E-05**
Jaldapara	RH1	3	92.00	0.07	0.07	0.18	83	6	32	4.40E-03	8.10E-06
	RH3	3	94.00	0.05	0.03	0.06					
	RH10	6	94.00	0.03	0.02	0.04					
	IR10	2	95.00	0.06	0.02	0.03					
	RH7	2	93.00	0.04	0.01	0.00					
	SR63	5	90	0.01	0.06	0.04					
	RH5	4	91	0.02	0.04	0.03					
	IR12	2	78	0.00	0.00	0.08					
	7B	2	78	0.27	0.01	0.03					
	RH9	2	86	0.02	0.01	0.07					
	RH4	4	94	0.09	0.02	0.00					
	RH11	3	87	0.03	0.00	0.00					
	Total	**3.17±1.28**	**89**	**0.06**	**0.03**	**0.05**	**83**	**6**	**32**	**4.40E-03**	**8.10E-06**
Manas	RH1	3	90.00	0.29	0.01	0.15	26	23	29	7.60E-04	2.50E-08
	RH3	3	86.00	0.10	0.01	0.03					
	RH10	6	90.00	0.26	0.01	0.11					
	IR10	2	81	0.18	0.02	0.11					

	RH7	2	83	0.16	0.03	0.02					
	SR63	4	68	0.04	0.02	0.11					
	RH5	4	81	0.06	0.05	0.24					
	IR12	3	86	0.27	0.00	0.21					
	7B	3	74	0.17	0.02	0.00					
	RH9	2	86	0.11	0.00	0.10					
	RH4	5	95	0.13	0.00	0.06					
	RH11	4	71	0.10	0.02	0.00					
	Total	**3.42±1.19**	**82**	**0.15**	**0.02**	**0.1**	**26**	**23**	**29**	**7.60E-04**	**2.50E-08**
Pobitora	RH1	3	89	0.26	0.05	0.14	91	42	83	1.90E-03	6.80E-07
	RH3	3	85	0.15	0.02	0.13					
	RH10	6	86	0.23	0.04	0.18					
	IR10	2	89	0.12	0.01	0.00					
	RH7	2	89	0.24	0.01	0.14					
	SR63	4	82	0.20	0.01	0.18					
	RH5	4	79	0.13	0.02	0.20					
	IR12	3	57	0.25	0.01	0.16					
	7B	3	25	0.00	0.00	0.11					
	RH9	3	83	0.12	0.01	0.17					
	RH4	5	92	0.15	0.04	0.12					
	RH11	3	80	0.09	0.04	0.13					
	Total	**3.42±1.12**	**78**	**0.16**	**0.02**	**0.14**	**91**	**42**	**83**	**1.90E-03**	**6.80E-07**
Orang	RH1	3	86	0.10	0.06	0.06	55	6	19	7.80E-04	1.10E-07
	RH3	3	92	0.02	0.05	0.00					
	RH10	7	92	0.05	0.01	0.07					
	IR10	2	86	0.02	0.01	0.07					

	RH7	2	78	0.07	0.03	0.00					
	SR63	4	76	0.07	0.02	0.05					
	RH5	4	85	0.02	0.04	0.09					
	IR12	2	70	0.12	0.02	0.20					
	7B	3	75	0.09	0.01	0.09					
	RH9	2	90	0.07	0.00	0.07					
	RH4	5	95	0.06	0.03	0.03					
	RH11	4	95	0.06	0.01	0.04					
	Total	**3.42±1.44**	**85**	**0.06**	**0.02**	**0.07**	**55**	**6**	**19**	**7.80E-04**	**1.10E-07**
Kaziranga	RH1	3	97	0.08	0.03	0.13	163	1	36	2.90E-04	9.40E-09
	RH3	3	97	0.03	0.03	0.13					
	RH10	7	93	0.04	0.02	0.04					
	IR10	2	91	0.05	0.01	0.06					
	RH7	2	92	0.05	0.01	0.02					
	SR63	4	91	0.03	0.03	0.13					
	RH5	4	93	0.08	0.02	0.09					
	IR12	2	91	0.05	0.00	0.19					
	7B	3	84	0.03	0.02	0.06					
	RH9	3	88	0.03	0.01	0.04					
	RH4	6	95	0.02	0.01	0.06					
	RH11	4	94	0.05	0.01	0.01					
	Total	**3.58±1.50**	**92**	**0.04**	**0.02**	**0.08**	**163**	**1**	**36**	**2.90E-04**	**9.40E-09**

AS- amplification success, ADO- allele drop out, FA- false alleles,

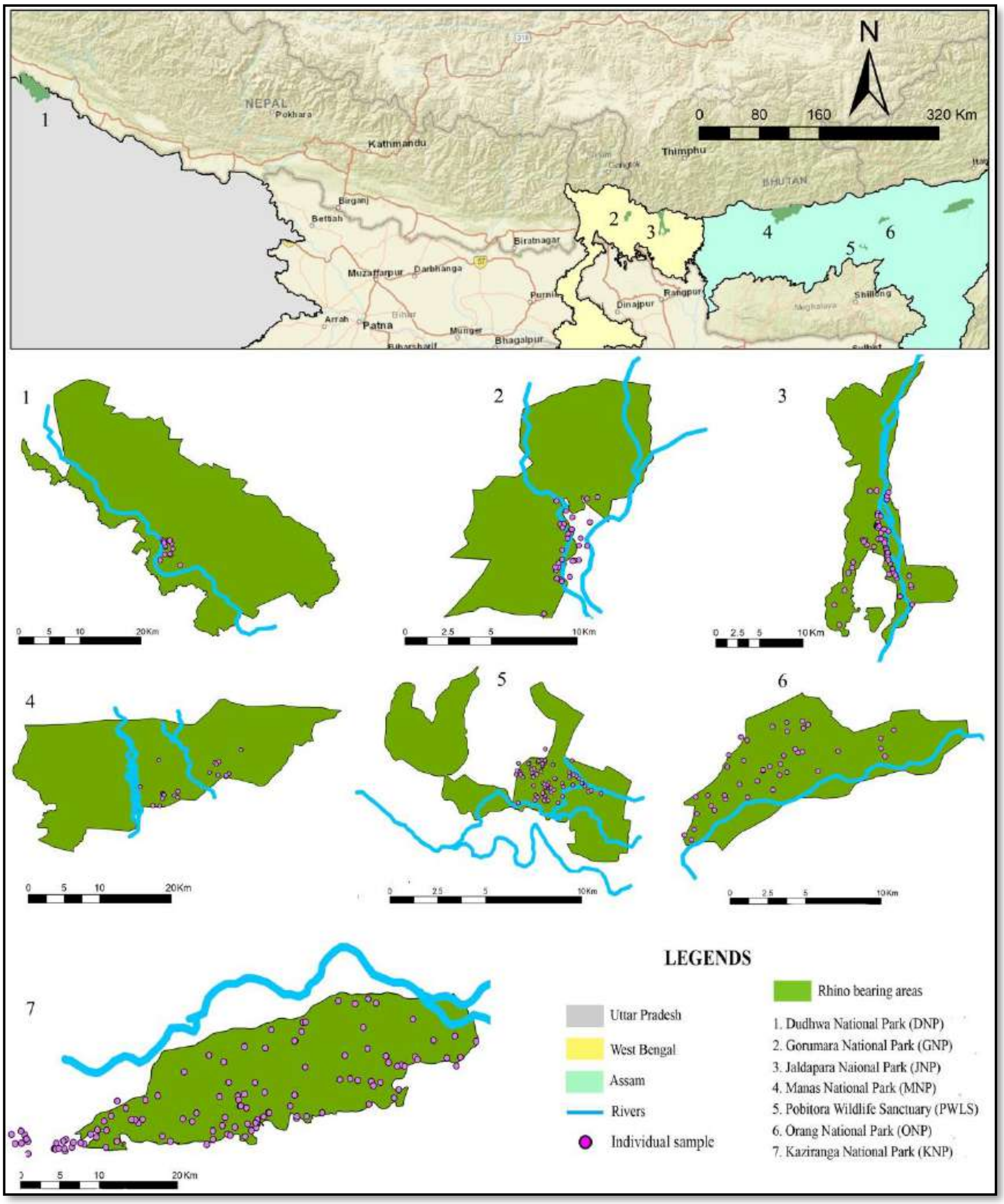

Figure 4.2: Map of the study area representing spatial distribution of 504 identified individuals used in this study. The top plate shows the position of the rhino-bearing parks across three Indian states (Uttar Pradesh, West Bengal and Assam). The individual location outside the boundaries of Gorumara NP and Kaziranga NP represents the population residing in the recent extended areas of these parks.

4.3.2 Genetic diversity and population structure:

The genetic diversity estimates varies among the seven sampled populations with highest heterozygosity (both expected and observed) in Kaziranga NP (0.59 and 0.48 respectively), while the lowest values were found in Gorumara NP (He- 0.34) and Pobitora WLS (Ho-0.24) (Table 4.3). Based on inbreeding coefficient analysis, Pobitora WLS has the highest inbred population (Fis-0.42) followed by Manas NP (0.29).

Table 4.3: Park-wise details of basic genetic diversity indices consisting of Ho and He- observed and expected heterozygosity, Fis-inbreeding coefficient

Population	He	Ho	Fis
DNP	0.43	0.41	0.03
GNP	0.34	0.3	0.07
JNP	0.41	0.36	0.08
MNP	0.52	0.34	0.29
PWLS	0.46	0.24	0.42
KNP	0.59	0.48	0.15
ONP	0.53	0.4	0.2

Among the IBC approaches, non-spatial algorithm implemented in STRUCTURE resulted in K=4 clusters (Figure 4.3). Whereas STRUCTURE with Locprior and spatially explicit TESS analyses inferred K=5 (Figure 4.3). In all of the three scenarios, Gorumara NP, Jaldapara NP, Kaziranga NP and Pobitora WLS belongs to distinct genetic clusters whereas Orang and Manas NP shows mixed signatures. The difference in non-spatial and spatial admixture approach is representation of Dudhwa NP as distinct genetic cluster in the later (Figure 4.3). Further non-admixture approach of GENELAND resulted in K=7 corresponding to the sampling locations

(Figure 4.4). Thus Bayesian analysis resulted in various optimal K (ranging from 4-7) with consensus of 4 distinct clusters corresponding to Gorumara NP, Jaldapara NP of West Bengal and Kaziranga NP, Pobitora WLS of Assam. The cluster compositions for remaining parks (Dudhwa NP, Manas NP and Orang NP) were different across IBC approaches (details in Table 4.4).

Table 4.4: Summary of Bayesian clustering analysis of Indian rhino populations and their respective estimates of inferred genetic clusters

Bayesian IBC analysis	**Models**	**Inferred K**	**Genetic sub-populations**	
			Unique populations	**Mixed populations**
STRUCTURE	Non spatial, Admixture	4	GNP, JNP, KNP and PWLS	DNP=PWLS; MNP=KNP; ONP= mix of KNP and PWLS
	Spatial, Admixture	5	DNP,GNP, JNP, KNP and PWLS	MNP=KNP; ONP= mix of KNP and PWLS
TESS	Spatially explicit, Admixture	5		
GENELAND	Spatially explicit, Non-admixture	7	DNP,GNP, JNP, MNP,PWLS, ONP, KNP	NO

Finally pairwise sPCA approach was taken to identify true population sub-structuring based on significant positive Moran's I index (Figure 4.5 and Table 4.5). A significant global pattern was observed for pairwise comparisons between Dudhwa NP and Pobitora WLS (p=0.001), Orang NP with both Kaziranga NP (p=0.001) and Pobitora NP (p=0.001) and Manas with Pobitora WLS (p=0.001). However, Manas did not show significant structuring with Kaziranga NP (p=0.19) (Table 4.5). Details of pairwise comparison with significance test value, screeplot and graphical presentation of genetic clines is given in Figure 4.5. In conclusion, results of sPCA confirms the presence of six genetic clusters corresponding to Dudhwa NP of Uttar Pradesh, Gorumara NP and Jaldapara NP of West Bengal and Kaziranga NP, Orang NP and Pobitora WLS of Assam along with Manas NP representing signatures predominantly of Kaziranga NP. The pairwise Fst calculation also corroborates with cluster data (Table 4.6) showing less differentiation (Fst<0.05, Hartl and Clark, 1997) among Assam populations contrary to West Bengal ones with Fst values >0.2 (i.e. high differentiation, Hartl and Clark, 1997). Further, hierarchical analysis shows 11% of variation due to 'among groups/clusters' differences while 88% is contributed by 'within population' variations indicating sub-structuring at population level.

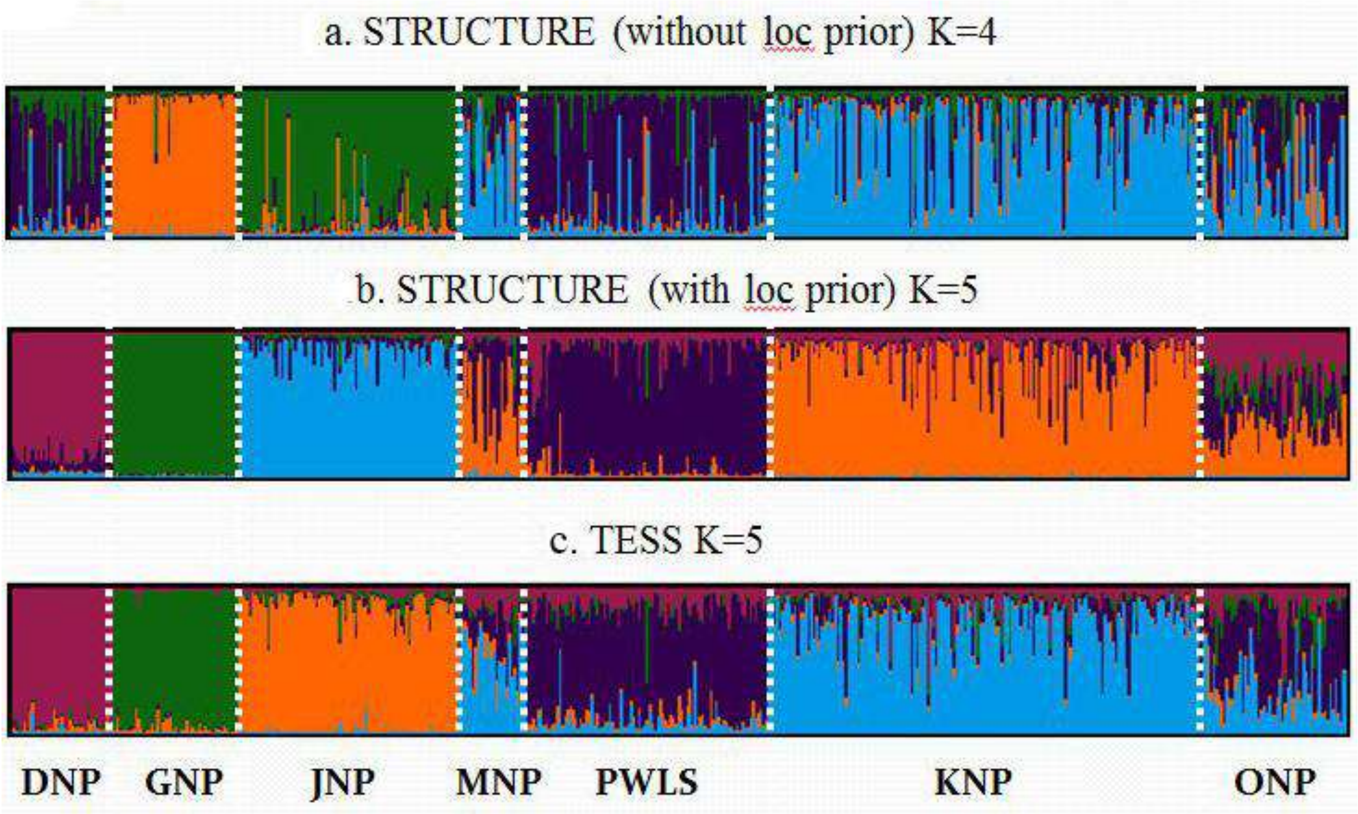

Figure 4.3: Results of admixture based Bayesian clustering analysis. In STRUCTURE optimal K is decided using model choice statistics L(K) which concluded in (a) K=4 for non-loc prior run and (b) K=5 with loc-prior. For TESS Kmax is finalised using the DIC value resulting in K=5. Our findings indicates the presence of 4 consensus clusters corresponding to Gorumara and Jaldapara NP of West Bengal, Pobitora WLS and Kaziranga NP of Assam followed by mixed signatures of Manas and Orang NP. However Dudhwa NP is showing a distinct genetic signatures in spatial IBC analysis (b,c) in contrast to non-spatial one (a).

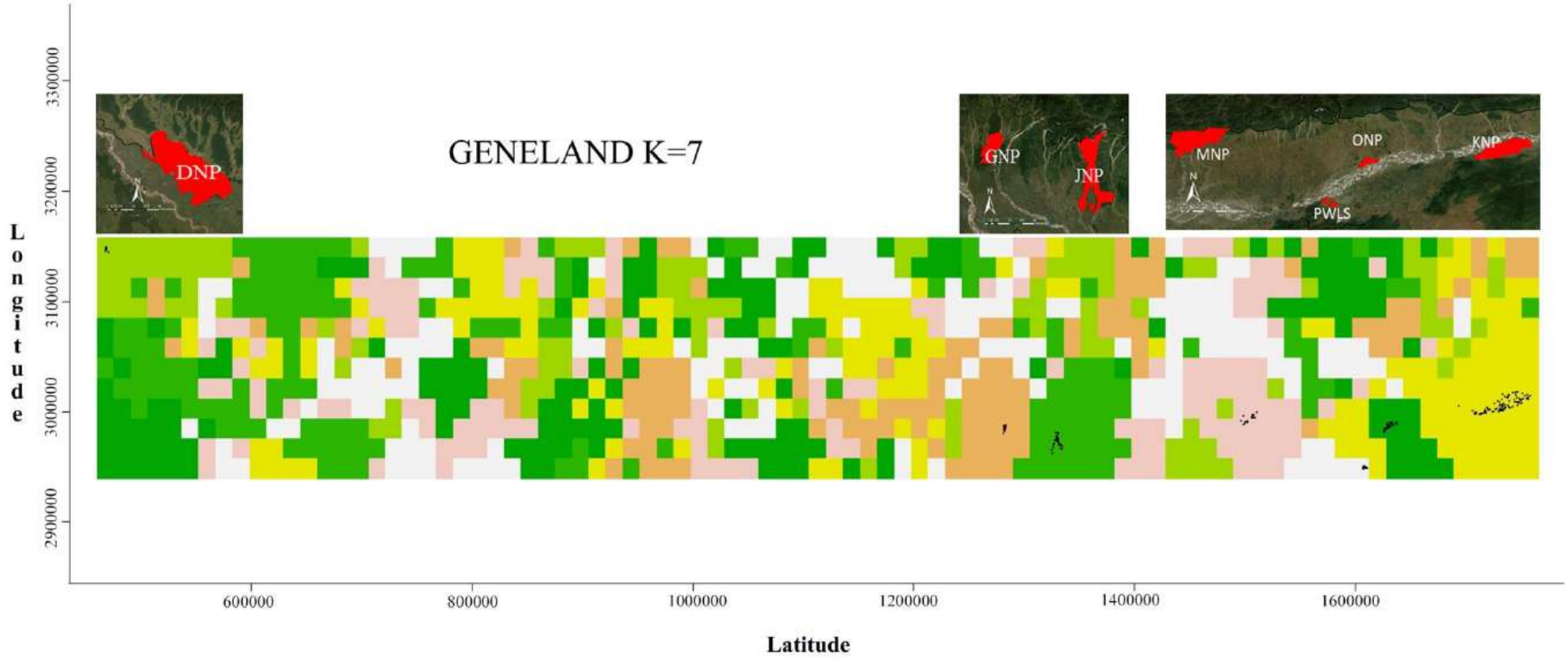

Figure 4.4: Result of non-admixture based Bayesian clustering analysis implemented in GENELAND. It shows that Indian rhino populations are genetically clustered into 7 sub-populations represented by population membership graph. The optimal K was decided based on the highest mean posterior density among each candidate K.

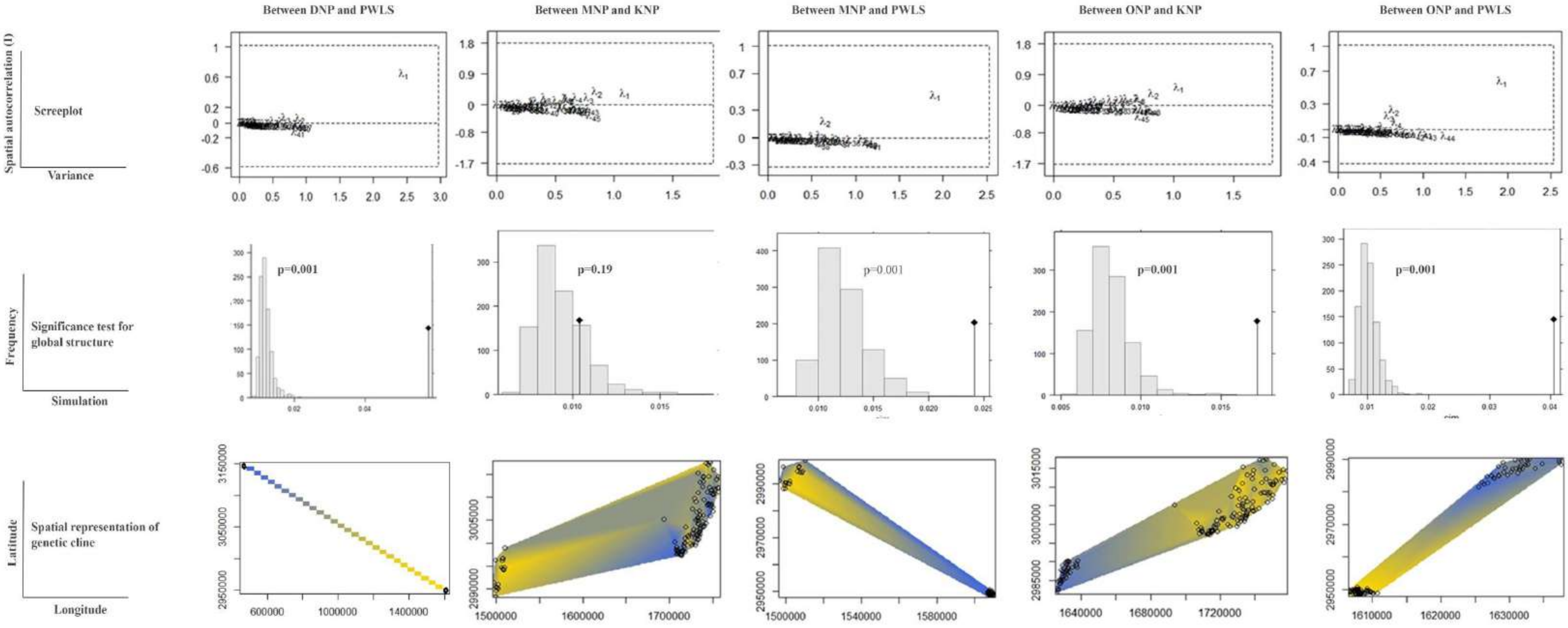

Figure 4.5: Results of sPCA analysis showing pairwise comparison with screeplot, significance test value, and graphical presentation of genetic clines (top to bottom). The result shows that Manas is not a different genetic sub-population (p=0.19) and thus GENELAND was over estimating it. On the other hand Orang is a genetic sub-population which could not be identified using admixture based models thus concluding into six genetic sub-populations in Indian rhinos.

Table 4.5: Result of pairwise sPCA analysis to find out true genetic sub-population

	Population pairs	Structure pattern (pvalue)
1	DNP PWLS	Significant (0.001)
2	ONP KNP	Significant (0.001)
3	ONP PWLS	Significant (0.001)
4	MNP KNP	Non-significant (0.19)
5	MNP PWLS	Significant (0.001)

Table 4.6: Pairwise Fst comparisons (p<0.05) among the identified genetic sub-populations

Genetic sub-populations	Dudhwa	Gorumara	Jaldapara	Manas + Kaziranga	Pobitora	Orang
Dudhwa NP	0					
Gorumara NP	0.29	0				
Jaldapara NP	0.13	0.23	0			
Manas NP + KazirangaNP	0.11	0.21	0.14	0		
Pobitora WLS	0.06	0.27	0.16	0.04	0	
Orang NP	0.09	0.23	0.15	0.04	0.03	0

4.3.3 Assessing genetic health of Indian rhino populations:

Overall the matrices of genetic health (GD, C and Fit) resulted in highest ranking of Kaziranga NP followed by Manas NP with West Bengal populations being the lowest ones (Table 4.7, Jaldapara NP represents the worst scenario). The trend of all three indices across each population (arranged in ascending Fit order) is discussed in following lines (Table 4.7).

I. Kaziranga and Manas NP- Both of these populations have shown –ve GD, +ve C and lowest Fit values which means they are important for maintaining the overall diversity of the Indian rhino species.

II. Orang NP- This population has low Fit (0.08) and positive contribution towards the aggregated diversity (-GD value), but it shows negative value for C (allelic richness). This is possibly because of lesser genetic differentiation of Orang with other Assam rhino populations owing to the rhino movement reports between them (Talukdar 2000; Talukdar et al., 2007).

III. Pobitora WLS- This population despite of having second highest index of allelic richness (C) after KNP shows negative contribution towards overall diversity (+GD value) with moderate level of Fit (0.11). This shows that the population is prone to genetic erosion as observed with highest Fis value (0.43).

IV. Dudhwa NP- This reintroduced population shows +ve GD, +ve C and moderately high Fit (0.24) indicating that the population is facing diversity loss instead of its contribution to allelic richness of Indian rhino gene pool.

This is probably because the Dudhwa rhinos have revived from five founder individuals only (Talukdar et al., 2012).

V. Jaldapara NP- This population needs immediate attention as it shows lowest health indices of genetic diversity and allelic richness along with moderately high genetic erosion.

VI. Gorumara NP- Instead of being the most genetically eroded population (Fit-0.45 and –ve C), it shows a positive contribution towards the overall genetic diversity (-GD). This can be attributed to the unique signatures harboured by this population as a result of which it was the most differentiating population/genetic cluster from the remaining five (see pairwise Fst results in Table 4.5).

Thus based on diversity indices and quantitative analysis of genetic erosion, the populations prioritized for immediate genetic rescue are Pobitora WLS of Assam, Gorumara NP and Jaldapara NP of West Bengal and Dudhwa of Uttar Pradesh. The site specific recommendation is given in details in ‘Discussion’ section.

Table 4.7- Result of genetic health indices across seven Indian rhino populations arranged in ascending order of Fit

Parks	GD	C	Fit
KNP	-	+	0.05
MNP	-	+	0.05
ONP	-	-	0.08
PWLS	+	+	0.11
DNP	+	+	0.24
JNP	+	-	0.25
GNP	-	-	0.45

4.4 Discussion:

This study is the first assessment of one-horned rhino genetic status at pan India level by generating exhaustive individual database covering ~17% of the population size. To achieve this, site-specific sampling strategy was adapted based on ecological and population defining parameters of the species (midden user, density, recovery history etc.). This along with integrated multiple IBC approach and multivariate analysis helped in delineating population clusters at landscape level which is entailed to hierarchical structuring (Ball et al., 2010; Vergara et al., 2015; Basto et al., 2016; De et al., 2021). Based on consensus results, it is concluded that Indian rhino population is structured into 6 genetic clusters/subpopulations. Out of which five are major (consisting of Dudhwa NP, Gorumara NP, Jaldapara NP, Pobitora WLS and Kaziranga NP) as they were distinguishable by admixture analysis implemented in STRUCTURE and TESS. Whereas Orang NP is an incipient genetic subpopulation detected by GENELAND with confirmation from sPCA. This pattern is obvious considering the occasional rhino movement reports from Kaziranga and Pobitora to Orang (Talukdar 2000; Talukdar et al., 2007). Further the genetic signatures of Manas were predominantly shared with Kaziranga, even though individuals from Pobitora were also brought during the reintroduction program. This can be attributed to higher death counts of translocated Pobitora individuals and allocation of more mother-calf pair from Kaziranga (Talukdar et al., 2020). In contrast to Assam, West Bengal populations are the most differentiated ones (Table 4.6) caused by early habitat encroachment and initiation of bounty hunting culture in early 1800s due to tea plantation practices (Bist 1994; Rookmaker et al., 2016). However pairwise Fst calculation shows Jaldapara is comparatively closer to Assam than Gorumara NP. This is possibly due to the connection of Jaldapara with Assam via Sankosh–Rydak–Manas landscape which is known to inhabit rhinos till 1960s and facilitate movements among the states (Bist 1994). Although similar pattern was observed by Das et al., 2015 but this assumption cannot be ascertained due to lack of original Manas population. In Dudhwa NP, contradictory results were observed between clustering analysis and pairwise Fst comparison with respect to Pobitora WLS. In former analyses both the populations belong to different clusters which can be attributed to their evolutionary history as they belong to different ESUs (Chapter2, Ghosh et al., 2022). However in Fst calculation, Dudhwa showed lesser genetic differentiation from Pobitora as compared to other populations (Table 4.6). This is because of the pedigree of reintroduced population where the first three generations were sired by one dominant male of Pobitora WLS (Talukdar et al., 2012). Hence the observed pattern of genetic signatures in Dudhwa NP is the outcome of its recovery from single supplementation event. Overall the integrated approach facilitated the identification of hierarchical structures caused by less differentiated populations (like Assam with pairwise Fst<0.05) and differences in recovery histories (source versus reintroduced populations; single versus multiple supplementation events) as opposed to single IBC analyses used by Das et al., 2015 (Ball et al., 2010; Hobbs et al., 2011; Basto et al., 2016).

In addition to estimation of population differentiation, assessment of genetic health for every park was done by calculating their relative contribution to the overall diversity (Guti´errez et al., 2005). Similar to other studies complimentary results were observed among the quantification indices (GD, C and Fit, Table 4.7) as they are influenced by ecological parameters like demography, connectivity and translocation histories of each area (Caballero et al., 2002; Ollivier et al., 2005; Álvarez et al. 2009, De et al. 2021). Finally the methodology demonstrates population specific concerns and provides management recommendations (as given below) to improve/maintain the genetic diversity important for long term survival of viable Indian rhino population.

4.4.1 Conservation mitigation plans for Indian rhino populations:

1. Kaziranga and Manas NP- Both of these areas represent good genetic health and thus do not need urgent attention. The reintroduced Manas NP owing to its multiple supplementation events from different source populations (Talukdar et al., 2020) with distinct genetic signatures ensured the observed variability (also in mtDNA based diversity, Ghosh et al., 2022). However, one thing needs to be taken care of is the high death rates of translocated males from Pobitora WLS. This will prevent the skewed nuclear genetic signature of Manas towards Kaziranga thus achieving the goal of genetically diverse population.

2. Orang NP- The park plays important role for rhino refuges straying away from Pobitora and sweeping during the flood from Kaziranga in search of highlands. However due to grassland encroachments and poaching pressure outside protected areas these movements are getting restricted thus resulting in incipient structuring of Orang (Talukdar 2000; Talukdar et al., 2007). Hence maintenance of the connectivity by protecting the grassland patches between these three parks is important to improve the allelic diversity (moderately high Fit and –ve C), thus enhancing the

Assam rhino genetic pool at evolutionary scale (since they belong to same ESU, Ghosh et al., 2022).

3. Pobitora WLS- This park harbours more than 100 individuals in less than 16km^2 area of available grassland resulting in inbred population (highest Fis- 0.43). Hence this area needs interventions like grassland restoration for population dispersion ensuring genetic health recovery by reviving the 40km^2 area allocated to the sanctuary boundary. Further this will help in reducing human-wildlife conflict as rhinos often stray out to do crop raiding in adjacent villages (Talukdar et al., 2007).

4. Dudhwa NP- As mentioned in 2nd chapter (Ghosh et al., 2022), this park maternally represents Nepal population thus owing to allelic diversity (+C). However the observed pattern of low diversity contribution is due to its single supplementation event as it is founded by 5 members only, 4 dominant breeding females of Nepal and one dominant male of Pobitora (Talukdar et al., 2012). Hence it can be concluded that future programs should consider multiple supplementation of founder members to maintain genetic diversity in the reintroduced population.

5. Gorumara and Jaldapara NP- The West Bengal populations are in urgent need of genetic restoration to reduce the effect of erosion and thus improving the overall health of this genetic pool (lowest ranking in genetic health indices). To achieve this, translocation of rhino individuals between the parks should be done as it will improve their genetic health indices (observed –GD and + C when recalculated as one population). Further it will also secure the evolutionary history of these populations since they belong to same ESU (Ghosh et al., 2022).

The discussed results helped in understanding the genetic status of Indian rhino pool by identification of populations that went through human induced structuring and in need of conservation attention (Mannise et al., 2017; Kolipakam et al., 2019; De et al., 2021). By following the above mentioned recommendations, one can achieve the balance between genetic diversity and evolutionary history which is the ultimate goal of any genetic rescue plan (Weeks et al., 2016; O'Brien et al., 2017; Willi et al., 2022). The genetic information provided here will assist in identifying appropriate source populations and maintain adequate genetic diversity in the existing (and new) rhino populations, thereby ensuring evolutionary, ecological and demographic stability for the future survival of Indian rhinos.

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Chapter 4: Developing DNA database for population assignment of rhino contrabands in India

5.1 Introduction:

The illegal wildlife trade (IWT) negatively affects the overall welfare of the ecosystem by affecting both trade-targeted and non-targeted species (Mondol et al., 2014a; Cardoso et al., 2012; Morton et al., 2021). In recent years the rampant killing practice has increased because of a surge in demand for wildlife body parts and their derivatives (McNamara et al., 2020; Fukushima et al., 2021). The involvement of organised syndicates and a shift from physical markets to online platforms have created additional challenges in restraining IWT (Lavorgna 2014; Fukushima et al., 2021). The major difficulty in containing these crimes is the lack of information on trade routes required for enforcement agencies to act (Wasser et al., 2007; Ogden et al., 2015). This creates huge gap between conviction rate and profit associated with it, thus hastening the threats of poaching. The DNA-based wildlife forensic comes to aid in these situations. It helps in identification of such routes by assigning the breeding population of seized sample (Mondol et al. 2014b; Wasser et al., 2015), thus facilitating law enforcement in targeted regions (Harper et al., 2018; Wasser et al., 2015 and 2018).

Like all other rhino species, one of the most alarming conservation challenges for GoH rhinoceros populations is poaching for their body parts, especially horns (Emslie et al., 2016; Ellis et al., 2019). Earlier reports from India corroborate this information as 239 rhino poaching incidents have been recorded from 2001–2016 (Das 2019). Recent economic boom in Southeast Asia has increased this demand raising its black market value (Eikelbloom et al., 2020). Further Indian rhino populations are at continuous risk from poaching, primarily due to geographic proximity to the major rhino horn consumer countries, Vietnam and China (Emslie et al., 2016). One way of tackling this problem is by understanding the extent, form and dynamics of this trade at the supply end and focusing on the poaching hotspots in source countries as well as on potential transit routes between source and transit countries (Mondol et al., 2014a; Harper et al., 2018; Wasser et al., 2015 and 2018). Given this, DNA-based forensic tools have been used to address such issues in case of other rhino species (Ewart et al., 2018; Harper et al., 2018). For Indian rhinos, legally authorised forensic protocols are present for species identification (Jha et al., 2017) and individual matching (Ghosh et al., 2021, Chapter 1) but further extensive work is required to test the efficacy of geographic assignment tests to identify the origin of seized rhino contrabands.

The chapter aims to generate an allele frequency map (AFM) for the population assignment analysis of Indian rhino contrabands (Manel et al., 2005). The basic principle behind the geographic origin is to estimate the level of differentiation among the population of the concerned species (results of Chapter 3) and then assign the individual to genetically distinct units (Manel et al., 2002 and 2005; Linacre et al., 2011; Ogden et al., 2015). Although microsatellites (STR) have been proven to be the best choice for such studies (Johnson et al., 2014; Ogden et al., 2015), often it become difficult to assign among less differentiated populations ($F_{st}<0.15$, Table 4.6 in Chapter 3) (Ogden et al., 2015; Moore and Frazier et al., 2019). Hence to avoid such scenarios a two-step approach is proposed here; step 1 involves state level assignment using mitochondrial DNA (mtDNA) based available information on three ESUs (Ghosh et al., 2022). This is followed by population assignment analysis using the data of six Indian rhino genetic sub-populations (Chapter 3) inferred by ISFG standard STR markers (Ghosh et al., 2021). This would ensure double confirmation and in absence of STR amplification (pure DNA quality, if any) it would be possible to atleast inform the state of origin for unknown rhino seized body parts.

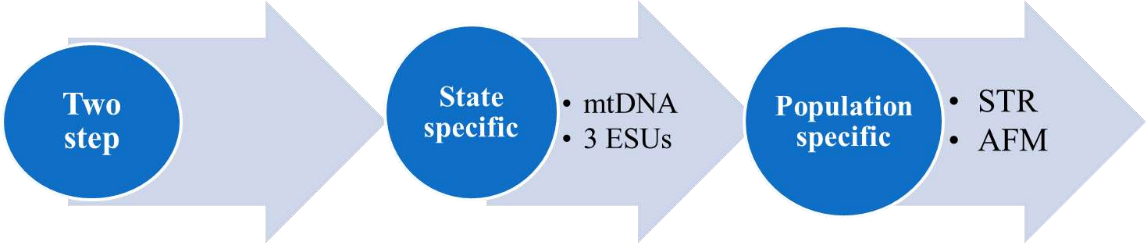

Figure 5.1: Diagrammatic representation of two step approach to assign the population of origin for rhino contraband.

5.2 Methods:

5.2.1 mtDNA based state assignment:

In wildlife forensics mtDNA is mostly used for species identification however they can be used to distinguish between lineages if it corresponds to specific geographic range (Fernandes et al., 2013; Sorenson et al., 2013). Thus based on the available information of three maternal lineages/ESUs in Indian rhino population (Ghosh et al., 2022), the goal is to achieve geographic assignment for rhino bearing states. To attain that mitogenome (Chapter 1; Genbank: MZ736693–MZ736708) was scanned to identify a gene/region which can be used to differentiate between the lineages (corresponding to states) using single PCR approach. Precision and data repeatability of the identified diagnostic site was assessed by generating data from all 504 individuals (identified in Chapter 3) following the PCR details given in Chapter 2

(Ghosh et al, 2022). To check the accuracy of state specific assignment, sequences were aligned in Mega v7 and a haplotype network using median joining algorithm (Bandelt et al., 1999) was constructed using PopART v. 1.7 (Leigh et al., 2015).

5.2.2 STR based population assignment:

Nuclear DNA like STR owing to their high polymorphism can differentiate populations based on allele frequencies and thus are better in geographic assignment for widely distributed species (Manel et al., 2005; Linacre et al., 2011). However the efficacy is largely dependent on the amount of variations observed among the populations and thus identification of unique genetic cluster is the foremost requirement of such procedures (Manel et al., 2002; Ogden et al., 2015; Moore and Frazier et al., 2019). This is followed by assessment of accuracy and precision of the final database following the forensic protocols (Manel et al. 2002 and 2005; Ogden et al., 2015).

Since in last chapter it was concluded that Indian rhino population is structured into six genetic sub-populations, here the aim is to confirm efficacy of this database (Manel et al., 2002; Ogden et al., 2015). Thus to generate a forensic standard database three steps were followed. First Bayesian assignment method implemented in STRUCTURE program was run to confirm whether the existing database of 504 individuals is enough or not for such analysis. POPFLAG option was used along with updated allele frequency in USEPOPINFO model without admixture (Pritchard et al., 2000). The optimal K was fixed at 6 with run lengths and other parameters remaining same as mentioned earlier in Chapter 3 (Figure 5.2). Then the success rate of assignment probability was calculated based on number of individuals assigned to the predefined clusters with q>0.7 (Kopatz et al., 2014 and 2021; Ginja et al., 2017). This success rate was estimated by running POPFLAG model with 25%, 50%, 75% (randomly chosen individuals) and 100% of reference database. In the second step, individuals with mixed genetic signatures (q<0.7) were removed to attain a reference database of forensic standards i.e. value of q should be >0.9 (Manel et al., 2002; Manel et al., 2005; Ogden et al., 2015). It was checked by re-running STRUCTURE using progressive clustering method within the states. Finally in third step, the precision of this database was estimated by calculating the probability of exclusion (Manel et al., 2002 and 2005). It was done using GeneClass (Piry et al., 2004) with the threshold of 0.01 (Manel et al., 2005; Biello et al., 2021) and using Monte Carlo resampling simulation of 100000 (Patekau et al., 2004).

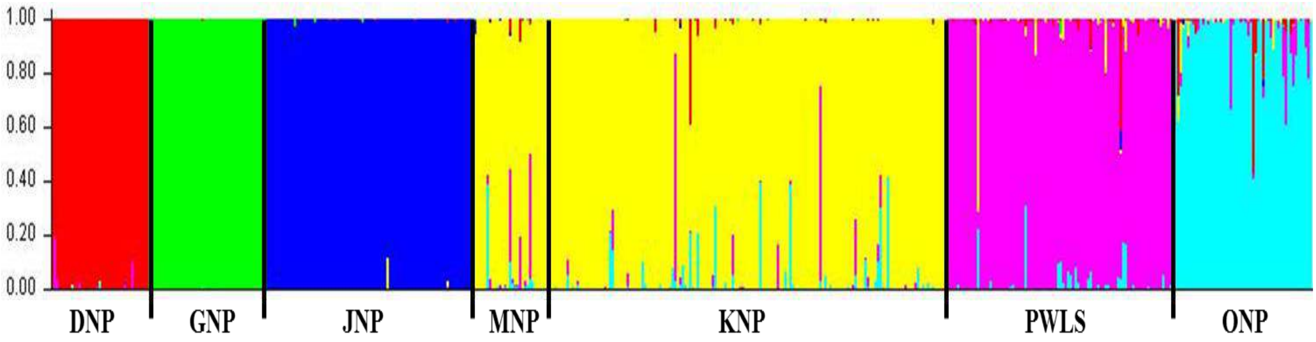

Figure 5.2: Structure plot of identified six genetic clusters in Indian rhino populations using POPFLAG model.

5.2.3 Population assignment of seized rhino contraband:

As mentioned in Chapter 1, various legal authorities in India have transferred 17 alleged rhino crime cases to the Institutions forensic laboratory following strict ‘Chain of custody’ protocols. Of these, five samples were provided with suspected poached rhino carcass (n=1 from West Bengal and n= 4 from Assam) and 12 samples without any reference (n=1 from West Bengal, one from Manipur and rest from Assam forest department). Following photographic and administrative documentations at the forensic laboratory, species identification was done following the India's legal procedure requirements (details given in Chapter 1). Confirmed rhino samples were sequenced with the finalised mtDNA primer for state assignment followed by population assignment analysis within the identified state using POPFLAG (parameters settings as mentioned above). Also the exclusion test was done to ensure the precision of assignment analysis for rhino contrabands.

5.3 Results:

5.3.1 mtDNA based state assignment:

Whole mitogenome screening resulted in identification of two West Bengal and three Uttar Pradesh specific SNPs. However no Assam specific SNP was found across mitogenome (details of segregating sites in Table 3.2, Chapter 2; Ghosh et al., 2022). For forensic purpose a region of 241bp within control region was selected as diagnostic site to distinguish among three states. This amplicon of 241bp consists of one West Bengal and one Uttar Pradesh specific SNPs and for assigning Assam exclusion principle was adapted. Finally the PCR resulted in amplification of 499 individuals from 504 with no amplification from five dung samples. Haplotype network construction resulted in similar pattern of three clades (as observed in Chapter 2) corresponding to Assam, West Bengal and Uttar Pradesh (Figure 5.3). Hence ensuring that finalised primer pair CRF1 & CRintR2 can be used to assign state to rhino contraband of unknown origin with success rate of 99%.

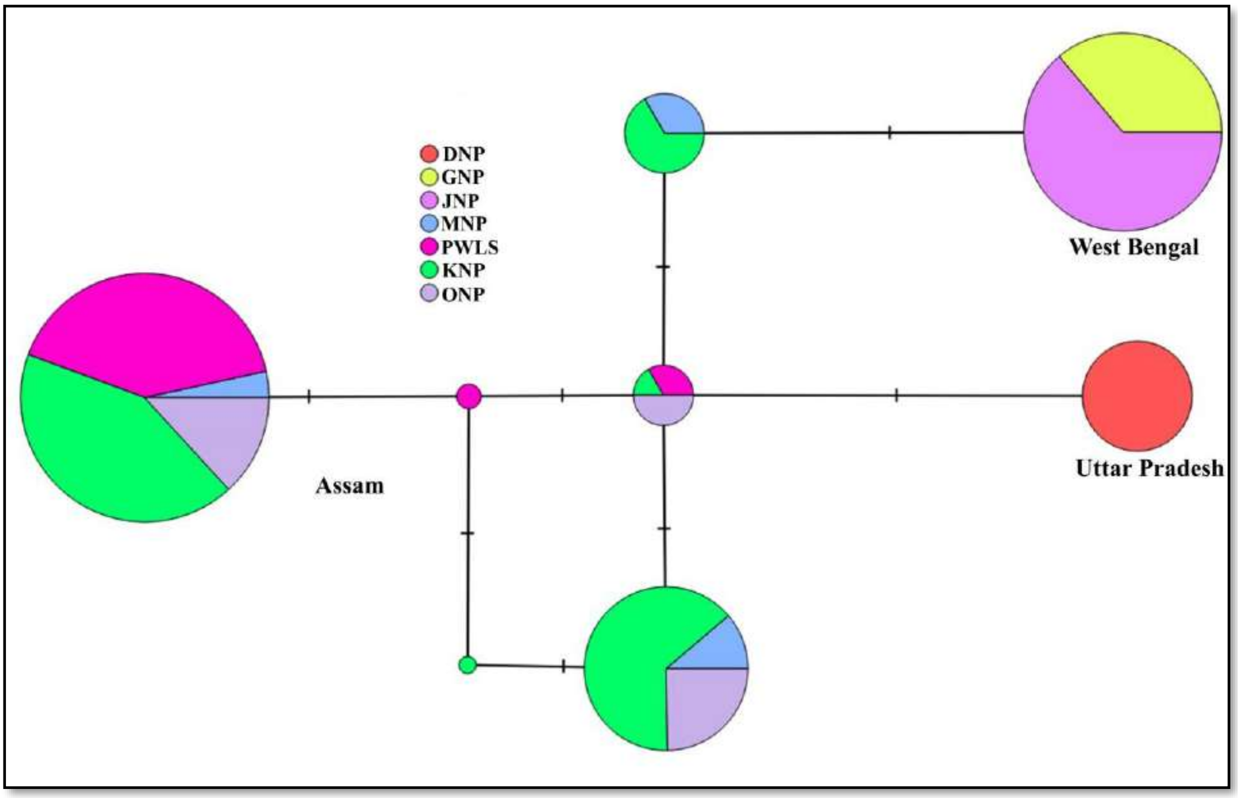

Figure 5.3: Haplotype network analysis (n=499 individuals) based on single diagnostic site representing distinct signatures corresponding to Assam, West Bengal and Uttar Pradesh.

5.3.2 STR based population assignment:

The PopFlag model with prior population information (step 1, K=6) helped in detection of 11 genotypes with mixed ancestry coefficient i.e q<0.7. All these genotypes belong to four sampled populations of Assam, 5 from Orang NP, three from Manas NP, 2 from Pobitora WLS and 1 from Kaziranga. The highest proportion of mixed genotypes (9%) in Orang NP resulted in lowest success rate of 91% with entire dataset (see table 5.1). The experiment done using different amount of reference database (25-100%, Table 5.1) shows that for Assam population minimum of ~70 individual data is required to attain success rate of 95%. Whereas the West Bengal populations shows consistent results of 100% success rate with sample size of ~12-20 individuals. Similarly the Dudhwa population also shows 100% assignment success from 20 individual onwards (Table 5.1). However, the removal of the mixed 11 genotypes and re-running the dataset with Bayesian progressive clustering approach (step 2) resulted in 100% success rate in Assam populations along with others. Overall the edited database of 493 individuals improved the average q value from 0.7 to 0.9, thus ensuring the discussed threshold in wildlife forensics (Manel et al., 2005; Milions et al., 2006). Finally the exclusion test of the revised database (step 3) correctly assigned 485 individuals to their predefined clusters, whereas 5 were wrongly assigned and 3 could not be assigned to any clusters. Thus the precision of revised database consisting of 493 individuals is 98% which is quite significant based on the threshold decided (Manel et al., 2005; Milions et al., 2006).

Table 5.1: Success rate of assigned individuals with q>0.7 across six identified genetic clusters. Number of individuals used in each PopFlag run is given in parentheses.

	%data	**DNP (n=inds)**	**GNP (n=inds)**	**JNP (n=inds)**	**KNP_MNP (n=inds)**	**PWLS (n=inds)**	**ONP (n=inds)**
Percentage of individuals assigned	25	44 (9)	100 (12)	100 (21)	0 (47)	0 (23)	0 (14)
	50	100 (19)	100 (24)	100 (41)	95 (95)	77 (46)	50 (28)
	75	100 (29)	100 (35)	100 (63)	97 (142)	94 (69)	90 (42)
	100	100 (39)	100 (47)	100 (83)	98 (189)	98 (91)	91 (55)

5.3.3 Population assignment of confiscated rhino contrabands:

Species identification assay confirmed 16 samples as GoH rhinoceros, whereas the one horn chip was identified as water buffalo (*Bubalus bubalis*) (also discussed in Chapter 1). The state assignment analysis shows that out of 16 rhino crime cases, 11 belongs to Assam and four to West Bengal and one from Uttar Pradesh. Further the population assignment analysis with the threshold of q>0.9 reveals that within Assam populations all the rhino contrabands get assigned to Kaziranga NP. In West Bengal both the parks were assigned with two rhino seized parts with q>0.95. Interestingly the sample from Manipur get assigned to Dudhwa NP, however no case of rhino poaching has been addressed from this park in past years. Thus, it can be an indication of poaching from Chitwan NP, Nepal as Dudhwa shares its signatures (Ghosh et al., 2022). Details of park wise assignment is given in Table 5.2.

Table 5.2: Assignment details of 16 confirmed one-horned rhino crime cases in India. The results shows that out of 13 suspected seizures from Assam, two belongs to West Bengal populations (one each to Gorumara NP and Jaldapara NP) thus showcase the power of DNA-based population assignment test.

Seizure states	No. of one-horned rhino seizures	State assigned	Park assigned
Assam	13	11	KNP=11
West Bengal	2	4	GNP=2; JNP=2
Manipur	1	1	DNP=1

5.4 Discussion:

The purpose of this chapter is to assess the potential of the current genetic database (Chapter 3 result) as a useful tool for finding the origin of confiscated rhino contrabands. Overall 100% success rate in mtDNA based state assignment was achieved with amplification success of 99% (five dung did not amplify). In case of microsatellite data removal of 11 individuals (mixed ancestry, $q<0.7$) resulted in database of 439 individuals (with $q>0.9$) achieving 100% success and 98% precision which is significant considering the thresholds accepted in wildlife forensics (Manel et al., 2002; Milions et al., 2006; Lorenzini et al., 2011; Ogden et al., 2015). This dataset also ensure high accuracy for populations with lower genetic differentiation (e.g Assam populations with Fst of 0.03-0.05, see Table 4.6 in Chapter 3), in contrast to published literatures where the assignment tests were done among populations with differentiation of >0.15 (Manel et al., 2002; Lorenzini et al., 2011; Wasser et al., 2015; Biello et al., 2021). Further the experiment done with different amount of reference database for POPINFO model (Table 5.1) gives brief idea about the minimum sample size required for establishing a forensic database for any large mammal with wide distribution. The results indicated that for populations with high genetic differentiations (>0.15) minimum of 20 individuals is enough to attain 100% assignment success (similar to other simulation based studies Manel et al., 2002; Milions et al., 2006). On the other hand populations with lower differentiation and incipient genetic structuring (Orang NP) require atleast 40-45% of population size to achieve threshold of $q>0.9$ (no study available on lower Fst populations). However it needs to be highlighted that populations with same genetic signatures (like Kaziranga and Manas NP) could not be differentiated for assignment tests. Nevertheless the forensic application of this data resulted in geographic allotment of 11 seized rhino contrabands for which no suspected carcass or individual reference data was available.

DNA-based geographic assignment analysis is resource intensive and requires comprehensive knowledge of a species life history at population level across the entire distribution range (Manel et al., 2005; Linacre et al., 2011; Johnson et al., 2014; Ogden 2015; Moore and Frazier et al., 2019). Due to this, there are just a few studies on large mammals at the landscape level where the genetic information has been transcended into a forensic database (BobCats-Millions et al., 2006; African elephants- Wasser et al., 2018; African rhinos- Harper et al., 2018). The fact that accuracy of the present database is significant enough for forensic application demonstrates the crucial importance of sampling strategy and identification of hierarchical genetic sub-structuring at landscape level (Linacre et al., 2011; Johnson et al., 2014; Moore and Frazier et al., 2019). Also use of mitochondria based state identification facilitates use of progressive clustering for population assignment with higher precision. Finally use of the database for alleged rhino crime cases shows the presence of trading route between West Bengal and Assam (as two Assam seizures were assigned to West Bengal populations, Table 5.2) for transportation of rhino horns and other body parts other than the known route via Myanmar (Emslie et al. 2016). Given the scale of monetary profits and administrative support these rhino horn syndicates has reached, such updated information on trading routes will be critical to disrupt the value of illegal market (Wasser et al., 2007; Eikelbloom et al., 2020). Although the data has ensured successful court prosecutions in alleged 18 Indian rhino crime cases, but it really needs to combine with higher law and order agencies like International revenue offices, Interpol group on wildlife crime to ensure the genetic monitoring of the seizures at international scale (Wasser et al., 2007 and 2015; Harper et al., 2018). Further it is imperative to extend the database to adjoining Nepal boundaries as it holds the next largest one-horned rhino population to achieve the trans-boundary protocol for surveillance of rhino illegal trading. Finally it can be concluded that this study addresses the key issues related to geographic assignment analysis of large mammals especially for populations with Fst<0.15, which is often the case (Ogden 2015; Moore and Frazier et al., 2019). Hence it is believed that the current methodology can be used as a model to generate database for similar trade-targeted megafauna species across the globe.

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Key findings and conservation implications

6.1 Conclusion:

One-horned rhino was found throughout the Indo-Gangetic plains during the early 20th century (Rookmaker 1980, Rookmaker et al., 2016) but faced drastic reductions in distribution and population size (including local extinctions) due to anthropogenic pressures (Menon 1996; Rookmaker et al., 2016). However, strong conservation and management initiatives since late 1900s resulted in one of the most successful species recovery (in terms of increase in population size) in wild (Rookmaker et al., 2016). Despite a relatively long history of effective management efforts, there is dearth of information on negative population trends (e.g. restricted gene-flow due to fragmentation, inbreeding etc.) often observed in recovered wild populations (Hobbs et al., 2011; Mondol et al., 2013; Moodley et al., 2017 and 2018). Thus a holistic conservation strategy requires identification of natural versus human-induced genetic structuring to ensure focused mitigation plans (Mace et al., 2010; Whiteley et al., 2015; Willi et al., 2022). Further, increasing demands for rhino horns in Chinese traditional medicine have put the existing populations under continuing threats, and large profits and low conviction rates make poaching difficult to contain (Ewart et al., 2018; Eikelbloom et al., 2020). In these contexts very limited information was available on one-horned rhinoceros (discussed in Table 1.1, Introduction). This work is the first assessment of range-wide genetic diversity in Indian rhinos emphasizing the importance of evolutionary history (Ghosh et al., 2022) and genetic health indices as fundamental information for future reintroduction/ recovery programs (as suggested in case of other species like Florida panther (Hostetler et al., 2010; Johnson et al., 2010), Cheetah (O’Brien et al., 2017), North American Fisher (Knuas et al., 2011) etc.). In addition, establishment of DNA profiling system and allele frequency map of forensic standards (ISFG) facilitates court prosecutions to combat rhino poaching in India (Ghosh et al., 2021). Hence this doctoral study emphasizes on shifting the management orientation from doubling the rhino numbers to mitigating the human induced population structure/decline by addressing the discussed issues.

The key findings and management recommendations from this doctoral thesis are discussed below.

1. One of the major objectives of this study is investigating genetic consequences of range and population decline in Indian rhinoceros. These patterns are influenced by evolutionary and contemporary history of landscape features and species colonisation along with their social behavioural construct (Chakraborty et al. 2014; Vergara et al., 2015; Basto et al., 2016; Moodley et al., 2018). Therefore by comprehending these spatio-temporal factors affecting the genetic structuring of the species, one can recommend mitigation plans for conservation action (Weeks et al., 2016; Basto et al., 2016; O'Brien et al., 2017; Willi et al., 2022). This study recommends preservation of evolutionary units as immediate measure for improving the overall genetic diversity at park level (Discussion of 3rd chapter). It was concluded based on identification of three ESUs corresponding to Uttar Pradesh, West Bengal and Assam (Ghosh et al., 2022, Chapter 2) which were found to be further structured into six genetic clusters because of various anthropogenic pressures (Chapter 3). For instance, securing the evolutionary potential of Assam includes maintenance of connectivity of Orang NP with Kaziranga NP and Pobitora WLS by protecting the

grassland patches along Brahmaputra. Establishing the metapopulation dynamics between these areas will resolve the current issue of carrying capacity (also seen in simulation study by Jhala et al., 2021) and reduce the effects of genetic erosion by facilitating gene flow. Similarly West Bengal populations are found to be in urgent need of genetic restoration to revert back the severity of drift effect (Table 4.7 of 3rd chapter). As no natural corridor exists between these two areas, the immigration of new alleles has to be done via translocation programmes among them. This will ensure preservation of evolutionary potential and increase in overall diversity and allelic richness of these populations (detailed discussion in Chapter 3). However a word of caution needs to be considered while making genetic restoration plans by translocating individuals between two genetically depauperated populations (Gorumara NP and Jaldapara NP of West Bengal) (Tallmon et al., 2004). As concluded by the results of mitochondrial (unique and single haplotype, Ghosh et al., 2022) and microsatellite analysis (high Fst values >0.2 in respect to other populations, Table 4.6), both the populations are prone to extinctions as a result of 'uniqueness' (as observed in case of five Australian mammals Weeks et al., 2016). Thus translocation between them may not ensure improved survival fitness even with an apparent increase in genetic diversity of the populations (Tollman et al., 2004). Thus regular genetic monitoring will be important to estimate the aftermath of translocation programme (Benson et al., 2011; Scott et al., 2020). In case of no improvement in genetic health (e.g. decrease in Fit index), further introducing individuals from diverse and healthy source population likes Kaziranga NP will be important for long term survival of these West Bengal populations. Even though it will affect the suggested ESU categorisation but according to many recent studies this paradigm shift in conservation biology practices is necessary for preventing threatened species from further extinction risks (Ralls et al., 2017; Weeks et al., 2017; Willi et al., 2022).

2. Second important aspect of the thesis is forensic application of genetic database to combat rhino poaching in India. Currently, global rhino populations face major illegal killing due to increase in demands and price of rhino horn derivatives (Rookmaker et al., 2016; Ewart et al., 2018). To reduce this pressure, there have been arguments on legalizing rhino horn sales from existing stockpiles, but recent studies suggested against it and recommended strict law enforcement at the source and consumer regions (Eikelbloom et al., 2020). In such scenarios, wildlife forensic genetics assists in better court prosecutions by generating evidence using scientific analytical methods. This approach deals with three main aspects of illegal trading/IWT; the species identification of seized sample, its individual matching with a suspected carcass or any circumstantial evidence like blood-stained bullet pellet and finally, detecting the geographic origin of seizure to identify the poaching site (Linacre et al., 2011; Johnson et al., 2013). While there was already a legalised protocol for one-horned rhino species confirmation (Jha et al., 2017), the 1st and 4th chapter of this thesis dealt with next two aspects. By established forensic standard DNA profiling system (Ghosh et al, 2021) and allele frequency map for Indian rhinos (Chapter 4), it will be possible to track the dynamics of this trade (Ogden et al., 2015; Harper et al., 2018; Wasser et al., 2018). For example, identification of smuggling route between Assam and West Bengal suggest how illegal rhino body parts are moved within India (Chapter 4 results). Further DNA profile match between

different seizures suggests separate transportation route of same individuals' body part to shipping location, an indication of export cartel involvement (similar findings in ivory trading, Wasser et al., 2018). Thus by generating such systematic data (for longer period) on size and distribution of seizures, homogeneity of origin and proximity of seizure from shipping site will ensure targeted law enforcement (Wasser et al., 2007 and 2018; Fukushima et al., 2022). In conclusion, this will help in decreasing the number of seizures from entering global market which will subsequently reduce the poaching pressures on Indian rhino populations.

3) Finally the critical aspect of this study is the methodological design taken to tackle the human-induced threats towards the extant population of Indian rhinoceros. A holistic approach for marker selection (both mitochondria and microsatellite, Chapter 1) was used to achieve the goal of using same data for both population genetics and forensic work, a problem specifically faced in field of wildlife forensic studies (Johnson et al., 2014; Moor and Frazier 2019). In Chapter 2nd and 3rd consideration of sampling heterogeneity and hierarchical approach facilitated in identifying park specific mitigation issues (Schwartz et al., 2009; Segelbacher et al., 2010; Hobbs et al., 2011), which were not simultaneously addressed in earlier landscape level studies on Indian mega fauna (Vidya 2016; Kolipakam et al., 2019; Modi et al., 2021; De et al., 2021 etc.). In Chapter 4th rigorous data accuracy assessment resulted in two-step confirmatory tests for geographical assignment of unknown rhino horn seizures. Moreover, identification of hierarchical structuring (mtDNA= three ESU and STR= six clusters) assured a forensic standard database for less differentiated populations ($F_{st}<0.05$), a concern in wildlife forensics for large

mammals (Manel et al., 2005; Ogden 2015; Moore and Frazier et al., 2019). Thus by addressing the limitations discussed in earlier studies, the inferences generated herein provide critical details for planning well-informed rhino conservation action.

6.2 Conservation implication:

This entire work was done under the premise of a government-endorsed program of the Ministry of Forest, Environment and Climate Change, Government of India (MoEF&CC) titled 'RhoDIS-India' (or Rhino DNA Indexing System-India). The findings of this thesis have resulted in few contributions towards rhino conservation as listed below.

- The reference database facilitated successful court prosecutions of 18 alleged rhino crime cases by providing scientific evidence in terms of DNA profile match and pointing out the trading routes.
- The detailed genetic assessment resulted in identification of populations for conservation investment. The populations with immediate intervention requirements are Pobitora WLS of Assam, Dudhwa NP of Uttar Pradesh and both the parks of West Bengal. (The park specific recommendation is given in Chapter 3)
- Findings of this study also indicate the importance of multiple supplementation events (Manas NP versus Dudhwa NP) in reintroduction programmes from varied source populations to maintain the overall diversity.
- The genetic database was used by forest department of West Bengal during rhino census 2019 to ensure better individual coverage especially from the

sites where less direct observations were recorded. It was also used to measure the genetic relatedness between the four individuals of existing rhino enclosure in Dudhwa NP, Uttar Pradesh before translocating them to a new grassland area within the park in 2018.

- The results will facilitate decision making process by managers during source selection in reintroduction program since the conservation action plans for Indian rhinos are currently oriented towards establishing new habitats and translocation-driven population enhancement (Rookmaker et al., 2016; Jhala et al., 2021).

Overall this work brings into focus the requirement of genetic monitoring of recovered populations. Despite of multiple studies showing application of genetic data in conservation, it is often ignored in planning species recovery program (Mace et al., 2010; O'Brien et al., 2017; Ralls et al., 2017; O'Brien et al., 2017; Weeks et al., 2017; Garner et al., 2020; Willi et al., 2022). The implication of this work is not limited to rhino conservation but it encompasses other endangered species in India who are facing the brunt of increasing human population, rapid economic and infrastructure development. Unfortunately for grassland dependent species situation is worse since they are the marginalised and most neglected ecosystem in India (Task Force on Grasslands and Deserts report, 2006). Thus conservation of a charismatic megaherbivore like rhino will ensure an overall protection of this ecosystem. Hence, in global scenario, when 60% of megaherbivores are extinction prone (Ripple et al., 2015), I believe, the approach, results and inferences of the doctoral study are relevant for other conservationists.

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ANNEXURE- I

Permits and ethical clearance:

Data generated in this study is part of a collaborative programme title “Implementing Rhino DNA Indexing System to counter rhino poaching threat and aid population management in India”. Biological sampling from all the three rhino bearing states was permitted by Ministry of Environment, Forests and Climate Change (MoEF&CC), Government of India (Letter No. 4-22/2015/WL). Permission for dung sampling was provided by state forest departments of Assam (Letter No. A/GWL/RhoDIS/2017/913, 3653/WL/2W-525/2018, WL/FE.15/22), West Bengal (Letter No. 3967/WI/2W-525/2018) and Uttar Pradesh (Letter No. 1978/23-2-12 (G)). In addition one tissue sample from Valmiki National Park, Bihar forest department assumed to be representing the wild rhinos of Nepal (Letter/no.-1296 dated 16.10.2020) was received.

ANNEXURE-II

Conference certificates:

BRITISH ECOLOGICAL SOCIETY

Festival of Ecology

This certificate confirms that:

Tista Ghosh

Attended the above British Ecological Society virtual conference

From **14 December 2020** to **18 December 2020**
and presented the below virtual talk:

DNA detective: Combating rhino poaching in India using DNA based approach

Amy Everard

A Everard

Events Manager
British Ecological Society

British Ecological Society
42 Wharf Road, London, N1 7GS, United Kingdom
Tel: +44 (0)20 3994 8245

Society for Conservation Biology

CERTIFICATE OF PRESENTATION

THIS CERTIFICATE IS AWARDED TO

Tista Ghosh

For presenting: A mixture of uniqueness and diversity: Recipe for genetic rescue of Greater one-horned rhinoceros of India

on December 16, 2021
at the 2021 International Congress for Conservation Biology

Tony Lynam
President, Society for Conservation Biology

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This is to certify that

Tista Ghosh

has attended the Student Conference on Conservation Science, 29-31 March 2022,

at the University of Cambridge, Downing Street, Cambridge, UK.

Administrator……………………………………………………

Date: 28 March 2022

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Research paper

Optimisation and application of a forensic microsatellite panel to combat Greater-one horned rhinoceros (*Rhinoceros unicornis*) poaching in India

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ABSTRACT

The Greater one-horned (GoH) rhinoceros is one of the most charismatic endemic megaherbivores of the Indian subcontinent. Threatened by poaching, habitat loss and disease, the species is found only in small areas of its historical distribution. Increasing demands for rhino horns in chinese traditional medicine has put the existing population under continuing threat, and large profits and low conviction rates make poaching difficult to contain. DNA forensics such as the RhoDIS-Africa program has helped in combating illegal rhino horn trade, but the approach is yet to be optimised for Indian GoH rhinoceros. Here we followed the International Society for Forensic Genetics (ISFG) guidelines to establish a 14 dinucleotide microsatellite panel for Indian GoH rhinoceros DNA profiling. Selected from a large initial pool ($n = 34$), the microsatellite markers showed high polymorphism, stable peak characteristics, consistent allele calls and produced precise, reproducible genotypes from different types of rhino samples. The panel also showed low genotyping error and produced high statistical power during individual identification (P_{IDsibs} value of $1.2*10^{-4}$). As part of the official RhoDIS-India program, we used this panel to match poached rhino carcass with seized contraband as scientific evidence in court procedure. This program now moves to generate detailed allele-frequency maps of all GoH rhinoceros populations in India and Nepal for development of a genetic database and identification of poaching hotspots and trade routes across the subcontinent and beyond.

1. Introduction

The Greater one-horned (GoH) rhinoceros (*Rhinoceros unicornis*) is one of the most iconic, obligate grassland-dwelling megaherbivores endemic to India and Nepal [1]. Once distributed throughout the northern part of the Indian subcontinent covering all the major river basins from Pakistan to Indo-Myanmar borders during the 1600s, the species is currently found in 12 fragmented, protected regions occupying >2000 km^2 area in India and Nepal [1]. With a global population size of ~3700 individuals, the species is categorized as 'Vulnerable' by IUCN [1] and is listed in Appendix I of CITES and Schedule I of the Wildlife Protection act of India (1972) [1]. The species was on the brink of extinction around the early 1900s but strong conservation measures have resulted in population recovery of Indian GoH rhinoceros population [2,3]. All extant GoH rhino populations are currently found exclusively within protected areas [1]. The major threats for the species are poaching, decline in habitat quality, disease risks, resource depletion and occasional human-animal conflicts [1,4–7].

Like all other rhino species, one of the most alarming conservation challenges for GoH rhinoceros populations is poaching for their body parts, especially horns [1,7]. Poaching threats on the existing rhino populations are continuously increasing due to high demands of their horns for traditional chinese medicine and other purposes in the illegal wildlife markets globally [8,9]. Recent reports from India corroborate this information as 239 rhino poaching incidents (~10 % of the current population size) have been recorded between 2001–2016 [10]. The large profits and low conviction rates in illegal wildlife trade has further hastened the threats of poaching by organized syndicates. One way of tackling this problem is to focus on preventing poaching at the source, where its containment would be most disruptive to these criminal networks. It is also important to understand the extent, form and dynamics of this trade at the supply end and focus on the poaching hotspots in source countries as well as on potential transit routes between source and transit countries [11–13]. Given the trend of increasing poaching pressures for rhino horn [3], DNA-based forensic tools are critical to counter this threat as such tools have shown great potential to address

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poaching and trade issues at national as well as international scales [11–13]. The Veterinary Genetics Laboratory, University of Pretoria has effectively employed the Rhino DNA Indexing System (RhoDIS) across South Africa and other African rhino range countries to help in rhino crime case convictions [13]. This program is one of the rare and exceptional examples of collaboration between forest authorities, scientific organizations and legal authorities to provide scientific evidences in cases of alleged poaching events [13]. While this system is promising in the context of GoH rhinoceros conservation and managing the threats of poaching, the system has not been tested or optimised in India. For GoH rhinoceros, species identification protocols are well tested and standardized for forensic use [9,14], but the microsatellite markers have not been optimised in the line of the African-RhoDIS program and has only been used in population genetic research [15–18].

In this paper, we addressed key methodological issues related to optimisation of microsatellite markers for rhino forensic use. We followed International Society for Forensic Genetics (ISFG) guidelines [19, 20] to systematically screen dinucleotide microsatellite markers for forensic use in Indian GoH rhinoceros (RhoDIS-India). While there is a general concern regarding use of dinucleotide markers in forensic research, they have been widely used in wildlife forensics [19,20], including rhinos [21,22]. In addition, we tested DNA extraction protocols from rhino horns based on the protocols described in earlier studies [9,14,21]. Finally, we used the microsatellite panel to match poached carcass samples with seized rhino body parts as scientific evidence in court proceedings. We believe that this approach can be used as a model to tackle wildlife crime issues in other trade-target species.

2. Materials and methods

2.1. Permission and ethical considerations

The RhoDIS-India program is a Ministry of Environment, Forests and Climate Change, Government of India led rhino conservation project with a goal to control rhino poaching at national and regional scale. All relevant permissions to implement field sampling have been received from Government of India (Letter No. 4-22/2015/WL). All tissue sampling from naturally dead or poached rhinos were conducted during post-mortem examinations performed by trained officials of the respective State Forest Departments of Assam, West Bengal and Uttar Pradesh and did not require any other ethical clearances.

2.2. Sample collection

All optimisation of laboratory protocols was performed with field-collected wild Indian GoH rhinoceros tissue samples. A total of 96 reference rhino tissue samples (6 blood samples and 90 muscle tissues) were provided to us from forest department authorities of Assam (n = 86), West Bengal (n = 6) and Uttar Pradesh (n = 4). These samples were collected from naturally dead rhinos (n = 92) during post-mortem examinations as well as during rhino translocation programs (n = 4). All field-sampling was conducted by trained persons of the respective forest departments. Once brought to the laboratory, the samples were catalogued and stored in −20 °C freezer until further processing. In addition, we have also used four reference rhino horn samples for initial laboratory protocol optimisation. These samples were part of Wildlife Institute of India's (WII) national repository of Wildlife Forensics and Conservation Genetics Cell. About 200 mg of small horn pieces were cut using sterile blades and stored in −20 °C freezer for protocol optimisation in the laboratory.

2.3. DNA extraction

DNA was extracted from blood samples using QIAamp DNA Tissue Kit (QIAGEN Inc., Hilden, Germany) following the standard protocol for blood samples. To extract DNA from various quality rhino tissue samples from the field we used the protocol described in Mondol et al. [24]. In brief, about 20 mg of tissue was macerated with sterile blade and digested with 30 μL of Proteinase K (20 mg/mL) and 300 μL of ATL buffer (Qiagen Inc., Hilden, Germany) overnight at 56 °C, followed by Qiagen DNeasy tissue DNA kit extraction protocol. DNA was eluted twice in 100 μL preheated (70 °C) 1X TE buffer. For every set of samples (n = 11), one extraction negative was included to monitor possible contaminations.

For implementation of RhoDIS-India program we needed to optimise the DNA extraction protocol from rhino horns as the DNA quality from the horns can be variable due to presence of keratin, which acts as a PCR inhibitor [23]. Problems with downstream processing of rhino horn DNA can further be compounded due to small quantities of starting material (e.g. chopped pieces and chips or powders) received for casework. We tested two different DNA extraction protocols that use Qiagen DNeasy tissue DNA kit (QIAGEN Inc., Hilden, Germany) on four rhino horn samples from WII tissue repository and compared the DNA yield and amplification success for microsatellite markers. The first protocol was exactly the same used for rhino tissue samples (see above) whereas in the second method we used a dekeratinizing agent DTT (Dithiothreitol) during initial lysis [9,14,21,23]. The macerated rhino horn base tissue (~20 mg weight) were lysed for 16 h at 56 °C with 30 μL of 1 M DTT, 30 μL of Proteinase K (20 mg/ ml) and 300 μL of ATL buffer (Qiagen Inc., Hilden, Germany) with intermittent vortexing. Post-lysis remaining steps were followed as described in Qiagen DNeasy tissue DNA kit. DNA was eluted twice in 100 μL preheated (70 °C) 1X TE buffer [24]. For each extraction, extraction negatives were included to monitor possible contaminations. PCR amplifications are performed as described below.

2.4. Primer selection

The RhoDIS program (both in South Africa [13] and the targeted Indian program) aimed to provide legal aid for rhino poaching and trade cases at a global scale. Achieving this goal is only possible through a same panel of microsatellite markers along with reference samples to ensure data uniformity across laboratories. The Veterinary Genetics Laboratory, University of Pretoria has successfully standardized a panel of 23 microsatellite markers for use in forensic case work of African rhinos [21]. Some of these 23 markers (n = 18) have been tested on a few Indian GoH rhinoceros [25], (n = 18) but the entire panel has never been assessed for use in forensic casework for the species in India. Thus, we optimised the same markers for the RhoDIS-India program to select the best ones based on the criteria including i) marker polymorphism and heterozygosity; ii) amplification success; iii) ease in allele calling and iv) stable allele characteristics [19,20,26,27]. In addition, we have also added 11 loci earlier used on Indian GoH rhinoceros [16]. This was done anticipating that some African rhino markers might not amplify or produce erroneous results with the Indian rhinos [25,28]. Initially we tested all 34 markers on 30 field-collected rhino tissue samples from different rhino-bearing areas in India (Assam = 21, West Bengal = 5 and Uttar Pradesh = 4 samples, respectively), and the final panel of markers were used for all remaining samples (n = 66). The primer selection process involved the following steps:

2.4.1. Optimisation of annealing temperatures and DNA concentrations

Before annealing temperature optimisation, we quantified DNA concentrations for all 30 samples in Epoch Microplate Spectrophotometer and found highly variable concentrations (ranging from 10 ng/μL – 745 ng/μL). Further we selected six samples representing a gradient of DNA concentrations (~10, 80, 150, 300, 500 and 700 ng/μL) for further optimisations. We serially diluted these DNA concentrations to 1X, 1/10X, 1/25X and 1/50X, representing input DNA concentration ranging from 0.2 to 700 ng/μL. To optimise the annealing temperature, PCR reactions were performed for all 34 primers (See Table 1) in 10 μL reactions containing 4 μL of 2X Qiagen multiplex PCR buffer mix

Table 1

Details of the 34 microsatellite markers used for GoH rhinos in this study. The selected markers are listed according to their informative content.

Locus ID	Repeat type	Dye	Ta	Amplification success (%)	No. of allele	Allelic size range	Ho	He	Cumulative P_{ID} (unbiased)	Cumulative P_{ID} (sibs)	ADO	FA	NA	Between run precision (S.D)	Selection criteria	Multiplex PCR sets
RH10	Di	HEX	60	98.7	7	14	0.67	0.79	7.21E-02	3.79E-01	0.02	0.01	0.06	0.1	Selected	MPI
RH4	Di	6-FAM	57	96.7	6	24	0.72	0.74	7.36E-03	1.55E-01	0.02	0	0.01	0.18	Selected	MPIV
RH5	Di	HEX	60	99.6	5	12	0.56	0.72	9.06E-04	6.55E-02	0	0.02	0.08	0.07	Selected	MPII
SR63	Di	6-FAM	60	99.6	4	12	0.64	0.72	1.12E-04	2.78E-02	0.02	0.01	0.05	0.04	Selected	MPII
RH3	Di	NED	60	100	3	34	0.59	0.66	2.08E-05	1.30E-02	0.03	0.01	0.04	0.04	Selected	MPI
RH1	Di	6-FAM	60	98.4	3	4	0.59	0.63	4.38E-06	6.36E-03	0.04	0	0.03	0.09	Selected	MPI
RH11	Di	HEX	57	94.7	4	12	0.62	0.60	1.03E-06	3.27E-03	0.03	0.02	0.01	0.15	Selected	MPIV
IR12	Di	HEX	60	95.1	2	4	0.19	0.49	3.57E-07	1.96E-03	0	0.01	0.20	0.05	Selected	MPIII
SR281	Di	6-FAM	60	97.1	2	2	0.45	0.49	1.34E-07	1.18E-03	0	0.01	0.03	0.04	Selected	MPI
12F	Di	HEX	60	90	4	6	0.39	0.47	5.07E-08	7.22E-04	0.01	0	0.06	0.05	Selected	MPIII
7B	Di	FAM	60	93.1	3	6	0.43	0.45	1.91E-08	4.43E-04	0.03	0	0.00	0.04	Selected	MPIII
RH9	Di	PET	57	96.3	3	24	0.37	0.45	7.25E-09	2.77E-04	0.03	0.08	0.06	0.13	Selected	MPIV
RH7	Di	NED	60	99.6	2	2	0.47	0.44	2.94E-09	1.75E-04	0.02	0	0.00	0.08	Selected	MPII
IR10	Di	NED	60	99.6	2	8	0.31	0.34	1.42E-09	1.23E-04	0.08	0	0.03	0.09	Selected	MPII
SR262	Di	6-FAM	60	–	–	–	–	–	–	–	–	–	–	2.3	Stutter related inconsistency (rejected)	–
7C	Di	6-FAM	60	–	–	–	–	–	–	–	–	–	–	1.5	Stutter related inconsistency (rejected)	–
DB66	Di	6-FAM	60	–	–	–	–	–	–	–	–	–	–	1.7	Stutter related inconsistency (rejected)	–
Rh6	Di	PET	60	–	–	–	–	–	–	–	–	–	–	1	Stutter related inconsistency (rejected)	–
Rh8	Di	PET	60	–	–	–	–	–	–	–	–	–	–	1.7	Stutter related inconsistency (rejected)	–
32F	Di	VIC	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
B1Rh37D	Di	NED	57	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
SR74	Di	NED	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
DB44	Di	VIC	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
DB23	Di	VIC	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
BR6	Di	NED	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
IR22	Di	VIC	57	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
Rh12	Di	VIC	57	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
DB1	Di	PET	60	–	–	–	–	–	–	–	–	–	–	–	Monomorphic (rejected)	–
32A	Di	6-FAM	60	–	–	–	–	–	–	–	–	–	–	–	Low RFU (rejected)	–
B1Rh1B	Di	NED	60	–	–	–	–	–	–	–	–	–	–	–	Multiple peak (rejected)	–
SR268	Di	VIC	60	–	–	–	–	–	–	–	–	–	–	–	Low RFU (rejected)	–
Rh2	Di	6-FAM	–	–	–	–	–	–	–	–	–	–	–	–	Did not amplify (rejected)	–
DB52	Di	PET	–	–	–	–	–	–	–	–	–	–	–	–	Did not amplify (rejected)	–
B1Rh1C	Di	PET	–	–	–	–	–	–	–	–	–	–	–	–	Did not amplify (rejected)	–

Ta- Annealing temperature, Ho and He- observed and expected heterozygosity, ADO- allele drop out, FA- false alleles, NA- null alleles.

(QIAGEN Inc., Hilden, Germany), 0.2 μM labelled forward primer, 0.2 μM unlabelled reverse primer, 4 μM BSA (4 mg/mL) and 2 μL of rhino DNA at respective concentrations calculated above. PCR conditions included an initial denaturation (95 °C for 15 min); 35 cycles of denaturation (95 °C for 30 s), annealing (50–60 °C gradient for 40 s) and extension (72 °C for 40 s); followed by a final extension (72 °C for 20 min). During each reaction set, PCR and extraction negatives were included to monitor contamination. Amplified products were visualized with 2 % agarose gel and further genotyped using HiDi formamide (Applied Biosystems, California, United States) and LIZ 500 size standard (Applied Biosystems, California, United States) in an ABI 3500XL Genetic Analyser (Applied Biosystems, California, United States). The fragment lengths were scored manually using the program GENEMARKER (Softgenetics Inc., Pennsylvania, United States) and allele call bins were created for all the loci. The entire process was repeated three times in two different PCR machines (Eppendorf flexlid and ABI Veriti) and results were analyzed to determine optimal annealing temperatures and DNA concentration and to identify primers that were not amplifying in Indian GoH rhinoceros. One of the tissue samples was selected as a 'genotyping reference sample' and has been independently genotyped seven times to confirm the alleles. This sample was used as a standard to reduce genotyping errors from allele shifts among multiple runs in all subsequent genotyping analyses for Indian GoH rhinoceros and designated as reference sample for RhoDIS-India program.

2.4.2. Peak characteristics, allele calling and marker polymorphism

Following temperature optimisation of all primers and selection of the optimum input DNA concentration (see result section) with six samples, we amplified the next set of samples (n = 24) three independent times to select primers based on allele characteristics (multiple peaks and stutters, peak ratio etc). While allele calling we identified primers with low 'relative fluorescence units (RFU)' (less than 100) and multiple peaks. These primers were rejected from further analyses as they can lead to erroneous allele calls [26,27]. Subsequently, we prepared consensus genotypes for all 30 samples using a 'Quality index' approach involving all three independent results [29]. To check polymorphism in all the markers, we amplified them with remaining tissue samples (n = 66). All monomorphic markers were then excluded from downstream analyses.

2.4.3. Stutter-related inconsistencies and multiplexing of primers

To assess stutter-related inconsistencies (particularly in dinucleotide loci) in our selected loci, we calculated peak RFU ratios of heterozygote (ratio between short and long allele) and homozygote (ratio between -2R stutter and its homozygote peak) alleles for all loci [30]. This approach helped us to select loci with stable stutter character after quantifying the inter and intra sample variations in peak ratio. The ratios were independently calculated for all three replicates of all polymorphic loci for 30 selected samples. Subsequently, we calculated the standard deviation in the ratios, and the loci with high standard deviation values were rejected as they can be prone to stochastic errors leading to inconsistent allele calling [30].

The selected markers were multiplexed based on allele size and dye colour, and then each multiplex panel was checked for data accuracy by replicating the same PCR conditions mentioned above with different number of amplification cycles (20, 25, 30 and 40). Finally all sample (n = 96) were genotyped (for final multiplex panel) in three replicates to ensure consistent results by comparing them with previously generated genotypes.

2.5. Data analyses

We quantified allelic dropout and false allele (FA) rates manually as error rates per locus including the replicate data. Allelic drop out was quantified as ratio between number of amplifications with loss of one allele and total number of positive heterozygous individual amplification. The false allele was calculated for both homozygotes and heterozygotes as proportion of number of false allele amplification versus total number of amplifications [27,31]. Further, we used the program FreeNA [32] to determine the frequency of null alleles (NA), which estimates the NA frequency using the EM algorithm [33]. Program GIMLET [34] was used to calculate the $P_{ID(sibs)}$ for all the individuals. Following this, any allele having less than 10 % frequency across all amplified loci was rechecked for allele confirmation. ARLEQUIN [35] was used to calculate overall summary statistics and determine Hardy Weinberg equilibrium and linkage disequilibrium for all the loci.

2.6. Forensic use of the RhoDIS-India marker panel

We received 16 rhino poaching cases from various legal authorities in India. The case properties were transferred to our laboratory following strict 'Chain of custody' protocols. Of these, 13 samples were from poached rhino carcass and three samples were of seized rhino horn contraband. Some of the case properties from rhino carcasses had multiple samples for individual matching (n = 7). Following photographic and administrative documentations at the forensic laboratory, DNA was extracted, quantified and species identification was performed by partial cytochrome *b* and 12S rRNA sequences as described in Jha et al. (2017). This species identification protocol was chosen over earlier described ones (for example, see [9]) due to the requirement of India's leagl procedure requirements, where ascertainment of species is required to be matched with our country's sequence database. PCR reactions were performed in 10 μl reaction volumes containing 4 μl of 2X Qiagen Hotstart mastermix buffer (QIAGEN Inc., Hilden, Germany), 0.3 μM of each forward and reverse primer, 4 μM BSA (4 mg/mL) and 1 ng of extracted DNA. PCR conditions included an initial denaturation (95 °C for 15 min); 35 cycles of denaturation (95 °C for 30 s), annealing (55 °C gradient for 40 s) and extension (72 °C for 40 s); followed by a final extension (72 °C for 10 min). The amplified products were electrophoresed, cleaned up with Exo-SAP mixture, sequenced bidirectionally and matched against our Indian forensic database as well as with the Genebank for species confirmation. Confirmed rhino samples were then genotyped three independent times using the final multiplex panels as determined via the optimisation process outlined above. Finally, individual genotypes for the entire panel were matched against the poached rhino carcass data.

3. Results

3.1. Selection of rhino marker panel

We conducted systematic exclusions of non-suitable markers from the initial 34 rhino microsatellites based on amplification success, marker characteristics and polymorphism (see methods for details). During optimisation of the markers we tried 24 different combinations of input DNA concentrations ranging 0.2–700 ng/μl and found that minimum 1 ng/μl DNA is required for reliable microsatellite data generation. We used this DNA concentration for all subsequent PCR amplifications.

During the first stage of marker amplification with the reference set of samples (n = 6) we found that three of the 34 initial markers (Rh2, DB52 and B1Rh1C) failed to show any amplification (see Table 1). These primers were rejected from further processing. In the next step we assessed the marker suitability based on peak characteristics (tested with 30 samples) and polymorphism (with 96 rhino samples). When looking for peak characteristics, we found that two loci (32A and SR268) consistently produced very low RFU and one locus (B1Rh1B) showed multiple peaks (Table 1). These primers were eliminated from further testing. The remaining loci (n = 28) were tested for polymorphism and we identified nine monomorphic loci (Table 1) which were removed from the panel. Finally, data from all the polymorphic loci (n = 19) were analysed for stutter-related inconsistencies across multiple genotyping runs (tested with 30 samples). We found that five loci (SR262, 7C, DB66, Rh6, Rh8) showed high standard deviation values ranging from ±1 to ±2.3, which may lead to inconsistent allele calling in dinucleotide loci. The remaining 14 loci showed low standard deviation values (±0.04 to ±0.18) (see Table 1), and could be multiplexed into four panels. During testing these panels with different amplification cycles we found that PCR reactions with 20 and 25 cycles consistently produced low RFU values for all the loci, whereas reactions with 30 cycles produced low RFU value for larger alleles. While 35 and 40 cycles produced almost identical results, we decided to continue with 35 cycles for multiplex PCRs in the line of the singleplex reactions. All samples ($n = 96$) produced identical data with these multiplex panels when compared with the data from singleplex reactions (See Supplementary Fig. 1 for representative electropherograms).

3.2. *Summary statistics for the RhoDIS-India marker panel*

None of the selected 14 loci in this panel showed signatures of large-scale allelic dropout. The mean allelic dropout and false allele rates were 0.02 and 0.01, respectively. Average frequency of null allele was 0.05, indicating this panel has low genotyping error rates. Amplification success ranged between 90 %–99.6 % from different tissue types (blood and tissue, $n = 96$). These loci showed high to medium levels of polymorphism (Table 1). The mean observed heterozygosity was found to be 0.50 ± 0.15 and none of the loci deviated from Hardy-Weinberg equilibrium or showed any evidence for strong linkage disequilibrium between any pair of loci. The cumulative $\mathrm{PID}_{\mathrm{sibs}}$ and $\mathrm{PID}_{\mathrm{unbiased}}$ values were found to be 1.2×10^{-4} and 1.4×10^{-9}, respectively. Given that the rhino population size across India is ~3000 [1], this panel provides strong statistical support for unambiguous individual identification in forensic use. All summary statistic measures of polymorphism (number of alleles, allelic size range, heterozygosity etc.) for all loci are presented in Table 1.

3.3. *DNA extraction protocol from rhino horns*

We compared the efficiency of two DNA extraction protocols and found that with DTT protocol yielded 16 ng/μl- 102 ng/μl, whereas without DTT yielded 0.3 ng/μl- 1 ng/μl from the same samples. Further, DTT-extracted samples amplified all 14 markers in the panel compared to none with the other protocol. Given the stark contrast in amplification success, we used the DTT protocol for all subsequent rhino horn DNA extractions.

3.4. *Individual matching of rhino contraband*

Sequencing-based species identification assay confirmed two of the horn chips as Indian GoH rhinoceros, whereas the third sample was identified as water buffalo (*Bubalus bubalis*). We generated complete 14 loci panel data for all 15 poaching cases (13 carcass and two horn chips) received as part of the RhoDIS-India program. The genotypes from the horn chips matched with two rhino carcasses from West Bengal and Assam, respectively, showing the efficacy of this approach to match seized contraband to their carcass origins. These results were submitted as scientific evidence to concerned authorities for legal proceedings. We have also matched genotypes of decomposed rhino carcasses with other evidence such as blood-stained soil, bullets recovered from crime scenes etc. These results showcase the power of such DNA-based approaches in wildlife crime investigations.

4. Discussion

Our major aim in this study was to optimise a panel of microsatellites for forensic use in Indian GoH rhinoceros. To the best of our knowledge, this is the only study apart from the African rhinos [13,21,22] where a forensic microsatellite marker panel has been standardized to deal with rhino-related crimes. In the study we followed the ISFG recommendations during initial testing and optimisation of the markers such as use of voucher reference samples, various types of rhino samples (blood, tissue, horn chips representing different input DNA concentrations), temperature and PCR cycle optimisation, etc., that is generally lacking in other published GoH rhino population genetic work [16–18]. We have conducted rigorous testing of a large number of microsatellite loci ($n = 34$) with field-collected samples before selecting the best ones to develop the panel. Out of the 34 markers initially selected (23 from the African RhoDIS panel [21] and 11 tested on Indian GoH rhinoceros [16], we finally optimised 14 dinucleotide markers into four multiplex reactions as a time-saving and economic option. Most of the RhoDIS-Africa markers did not amplify in Indian GoH rhinoceros samples, possibly due to null alleles resulting from evolutionary differences among genus. Such non-amplification of cross-species microsatellites has also been reported earlier in rhinos [25,28]. Further, it is important to point out that there are general concerns regarding use of dinucleotide markers in forensic studies [27,30] but ISFG recommends them if they are widely used in population genetic research and have stable heterozygote balance [19]. The final selected panel in this study fulfil these criteria and showed very low genotyping error rates (2 % allelic dropout, 1 % false alleles and 5 % null alleles) and produce a statistically significant $\mathrm{PID}_{\mathrm{sibs}}$ value of 1.2×10^{-4}. However, addition of an allelic ladder [19,36] and further development of a single multiplex reaction (such as the *Meow-Plex* for felids [37], *UrsaPlex* for ursids [38], *DogFiler* for canids [39], *SkydancerPlex* for hen harrier [40] etc.) will greatly benefit inter-laboratory data generation and comparisons in rhino forensics. In addition, further validation work in terms of sequencing of the alleles for all the loci in the final panel, cross-species amplification testing, allele nomenclature designation etc. (for example, see [22] for black rhinoceros) are required for wide use of this optimised panel in forensic case work. In future, we plan to conduct these tests rigorously with extensive sampling of all Indian GoH rhinoceros populations for the RhoDIS-India program.

While using the optimised microsatellite panel to identify the origin of seized horns we performed individual genotype matching instead of commonly used random match probability approach for genotype comparisons [41]. We decided to use this approach as the random match probability requires in-depth understanding of population substructure data (also known as Φ value) [20,41]. There are seven isolated Indian GoH rhinoceros populations (four in Assam, two in West Bengal and one in Uttar Pradesh) and no population structure data or population-specific allele frequency assessments are available. Moreover, within each of the population the allele frequencies can differ significantly due to variations in population size and histories (in some cases the entire population has been reintroduced using founders from different sources), resulting in possible erroneous interpretations of our results [41]. We believe that until we generate adequate population-specific information complete genotype matching provides sufficient support in forensic use. This can be replaced by random match probability once significant individual level information from all sub-populations is available. Current advances in non-invasive tools (for example, faeces) make it easier to develop such a database.

5. Conclusion

In conclusion, we optimised a microsatellite panel for Indian GoH rhinoceros and used it to generate rhino DNA profiles and matched with carcasses as scientific evidences in alleged rhino crimes in India. This work is the first step towards generating an allele frequency map of GoH rhinoceros across its distribution in India. In future, this data would be used to determine origins of Indian GoH rhinoceros contraband and identifying poaching hotspots [11–13]. Currently, global rhino populations face major poaching threats as the demands and prices of rhino horn derivatives are at the highest level. To reduce poaching pressure there have been arguments on legalizing rhino horn sales from existing stockpiles, but recent studies suggested against it [42] and recommended strict law enforcement at the source and consumer regions. The Indian GoH rhinoceros populations are at continuous risk from poaching, primarily due to geographic proximity to the major rhino horn consumer countries (Vietnam and China) and transit country (Myanmar) [7]. We hope that this work, being a government-endorsed program of the Ministry of Forest, Environment and Climate Change, Government of India would improve the court prosecution rate, reduce the time required in prosecutions and in turn will work as a deterrent towards rhino poaching incidences. Long-term accumulation of such information would also provide useful data to identify rhino poaching hotspots and trade routes, thereby help in breaking the wildlife trade chains. Finally, similar approach can also be used for other trade-target species globally.

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CRediT authorship contribution statement

Tista Ghosh: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Amit Sharma:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing - original draft, Writing - review & editing. **Samrat Mondol:** Conceptualization, Methodology, Resources, Writing - original draft, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsigen.2021.102472.

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RESEARCH

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Consideration of genetic variation and evolutionary history in future conservation of Indian one-horned rhinoceros (*Rhinoceros unicornis*)

Tista Ghosh[1*], Shrewshree Kumar[1], Kirtika Sharma[1], Parikshit Kakati[2], Amit Sharma[2] and Samrat Mondol[1*]

Abstract

Background: The extant members of the Asian rhinos have experienced severe population and range declines since Pleistocene through a combination of natural and anthropogenic factors. The one-horned rhino is the only Asian species recovered from such conditions but most of the extant populations are reaching carrying capacity. India currently harbours ~83% of the global wild one-horned rhino populations distributed across seven protected areas. Recent assessments recommend reintroduction-based conservation approaches for the species, and implementation of such efforts would greatly benefit from detailed genetic assessments and evolutionary history of these populations. Using mitochondrial data, we investigated the phylogeography, divergence and demographic history of one-horned rhinos across its Indian range.

Results: We report the first complete mitogenome from all the extant Indian wild one-horned rhino populations ($n=16$ individuals). Further, we identified all polymorphic sites and assessed rhino phylogeography (2531 bp mtDNA, $n=111$ individuals) across India. Results showed 30 haplotypes distributed as three distinct genetic clades (F_{st} value 0.68–1) corresponding to the states of Assam ($n=28$ haplotypes), West Bengal and Uttar Pradesh (both monomorphic). The reintroduced population of Uttar Pradesh showed maternal signatures of Chitwan National Park, Nepal. Mitochondrial phylogenomics suggests one-horned rhino diverged from its recent common ancestors ~950 Kya and different populations (Assam, West Bengal and Uttar Pradesh/Nepal) coalesce at ~190–50 Kya, corroborating with the paleobiogeography history of the Indian subcontinent. Further, the demography analyses indicated historical decline in female effective population size ~300–200 Kya followed by increasing trends during ~110–60 Kya.

Conclusion: The phylogeography and phylogenomic outcomes suggest recognition of three 'Evolutionary Significant Units (ESUs)' in Indian rhino. With ongoing genetic isolation of the current populations, future management efforts should focus on identifying genetically variable founder animals and consider periodic supplementation events while planning future rhino reintroduction programs in India. Such well-informed, multidisciplinary approach will be the only way to ensure evolutionary, ecological and demographic stability of the species across its range.

Keywords: Megaherbivore, Paleobiogeography events, Evolutionary significant units (ESUs), Rhinocerotidae family, Reintroduction program, Founder effect

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Background

The members of Rhinocerotidae family were once one of the most diverse and widely distributed terrestrial herbivores with complex evolutionary history [1]. By late Pleistocene, this family was reduced to only nine species (from more than 100 species) spread across Eurasia (seven species) and Africa (two species) [1, 2]. Subsequently, early Holocene global warming (after Last Glacial Maxima) triggered their extinction in western Eurasia and southward movement of eastern Eurasian rhinos, leading to their distribution across Southeast Asia [2, 3]. Further, the range of all Eurasian rhino species (Javan, Sumatran and One-horned rhino) were affected by a combination of natural and anthropogenic factors during Pleistocene-Holocene transition period [15–9 thousand years ago (Kya)] [1, 3–6], followed by recent events of exploitation of natural resources (during colonial era), industrialisation and poaching (since seventeenth century) [7–10]. Population size of the most widely distributed Javan rhinos (during Holocene) [11] were greatly reduced during human population expansion since 10,000 years ago [3], whereas the Sumatran rhino populations became fragmented and isolated (since Holocene) due to submerged Sundaland corridors (late Pleistocene) [6]. The one-horned rhinos faced climate-change driven habitat shrinkage in late Pleistocene [12]. Currently the Javan and Sumatran rhinos are categorized as Critically Endangered (~60 Javan rhino—[13] and <100 Sumatran rhinos—[10]) and one-horned rhino as Vulnerable by IUCN (~3700 individual, [14]). Recovery of these species in their natural habitats requires deeper understanding of demography, ecology and genetics for appropriate conservation measures.

The one-horned rhino, being the only Asian species recovered from severe population decline in the past are critical for the evolutionary potential of this group. With a current population size of ~3700 individuals (increased from few hundred individuals in 1990s), it retains ~96% of the Asian rhino population [10, 13, 14]. As majority of the current one-horned rhino bearing areas in India and Nepal are reaching to their carrying capacities [15, 16], future conservation efforts are directing towards reintroduction-based programmes. Detailed genetic assessment of the existing rhino populations is critical in this regard since strong historical demographic declines has led to loss of genetic variation in all rhino species (Black rhino—[17], White rhino—[18], Sumatran rhino—[6], Javan rhino—[13]). For example, Liu et al. [1] suggested low population size and reduced genetic diversity across Rhinocerotidae family for an extended period of time. Similarly, mitogenome-based phylogeography reported low variation in both Sumatran [10] and Javan [13] rhinos, but no such data is available for one-horned rhinos.

In this paper, we investigated the phylogeography and evolutionary history of one-horned rhinos in India (henceforth Indian rhino) as it harbours 83% [19] of the global population of this species. We sequenced the polymorphic sites in the Indian rhino mitogenome in 111 wild individuals surveyed across seven extant populations covering the states of Assam, West Bengal and Uttar Pradesh. Further, we identified the Evolutionary Significant Units (ESUs) in Indian rhinos and suggested appropriate conservation measures to secure the evolutionary potential of this species. We believe that the results will provide the most exhaustive genetic information for Indian rhinos that would be useful in future reintroduction and population management efforts.

Results

Rhino mitogenome data and comparative analyses

Sequencing with 23 primers (Additional file 1: Table S1) generated 16,828 bp mitogenome (Additional file 2: Fig. S1) for wild Indian rhino (n=16, Genbank: MZ736693–MZ736708, Additional file 1: Table S2). Comparison with the available one-horned rhino mitogenome data (Genbank: X97336) showed identical patterns of gene annotations. Composition analysis revealed AT-skewed mitogenome with 13 protein coding genes, 22 tRNA, 2 ribosomal genes and a non-coding control region (Additional file 1: Table S3). Comparative analyses with other rhino species (Additional file 1: Table S4 and Additional file 2: Fig. S2) revealed that the Indian rhinos have low segregating sites ($S_{Java}=15{,}514$, $S_{Africa}=10{,}680$, $S_{Sumatra}=130$, $S_{India}=18$) and nucleotide diversity ($\pi_{Java}=0.56$, $\pi_{Africa}=0.43$, $\pi_{Sumatra}=0.003$, $\pi_{India}=0.0005$) but high haplotype diversity ($Hd_{Sumatra}=0.96$, $Hd_{India}=0.93$, $Hd_{Java}=0.91$, $Hd_{Africa}=0.67$). Both African rhino species (white and black rhino) data were combined for this analyses as no intra-species variation was observed in the available data.

Phylogeography of wild Indian rhinos

Out of 15 primers designed to assess genetic variation, eight were finally used (Additional file 1: Table S1) to amplify all 21 polymorphic sites (covering 2531 bp sequence) of rhino mitogenome. This data was generated for additional 95 unique individuals (n=56 tissue and 39 dung, Additional file 1: Table S2) (Genbank: MZ771364–MZ771458, MZ771459–MZ771553, MZ771554–MZ771648, MZ771649–MZ771743, MZ771744–MZ771838, MZ771839–MZ771933 and MZ771934–MZ772028). Remaining samples could not be used as they were rejected due to low amplification success for microsatellite data (n=12), genetic recaptures (n=13) and individuals from adjacent midden sites (n=24). Sequencing comparison showed that out of the 21 polymorphic sites, two and three sites were specific to West Bengal and Uttar Pradesh, respectively, whereas all others were shared at different levels among the three states (shared among three states—10 sites, Assam–Uttar Pradesh—eight sites, Assam–West Bengal—four sites, West Bengal–Uttar Pradesh—0 sites, Additional file 1: Table S5). Median joining network (n = 111 individuals) showed a total of 30 haplotypes (h) across India. Majority of these haplotypes (93.3%, n = 28) were from Assam whereas both West Bengal (one haplotype, n = 20) and Uttar Pradesh (one haplotype, n = 10) populations were found to be monomorphic (Fig. 1). The sequence from Bihar rhino individual was identical to the Uttar Pradesh population. Population-wise genetic variation indices (Table 1) showed overall highest values for KNP (n = 46; S = 18, h = 19, π = 0.0021, Hd = 0.85), followed by MNP (n = 12; S = 14, h = 6, π = 0.0023, Hd = 0.89), ONP (n = 12; S = 9, h = 6, π = 0.0016, Hd = 0.89) and PWLS (10; S = 2, h = 3, π = 0.0002, Hd = 0.51). Bayesian genetic clustering corroborated with the earlier pattern (K = 3) where samples from West Bengal and Uttar Pradesh formed distinct clusters whereas Assam showed geographically intermixed fixed haplogroups (Fig. 1). The genetic differentiation (pairwise F_{st}) values among these three clusters were significantly high ranging from 0.68 to 1 (Table 2, indicating highly structured populations). The hierarchical AMOVA analysis using two separate groupings: (a) seven populations and (b) three states showed higher within population (50%) and between group variance (45%) (Table 2). Such pattern indicates that overall genetic structure is influenced by differentiation at clade level and the amount of diversity present within the Assam clade (Tables 1 and 2).

Divergence time of different rhino clades and demographic history

The Bayesian phylogeny showed similar pattern of three clades consisting of West Bengal, Assam (nodes C–E) and Uttar Pradesh (along with the Bihar sample, Fig. 2). Based on the calibrated root nodes and Indian rhino-specific mutation rate (1.2×10^{-4} mean rate of substitution per site per million years, Additional file 2: Fig. S3), tMRCA analysis suggested a divergence period spanning

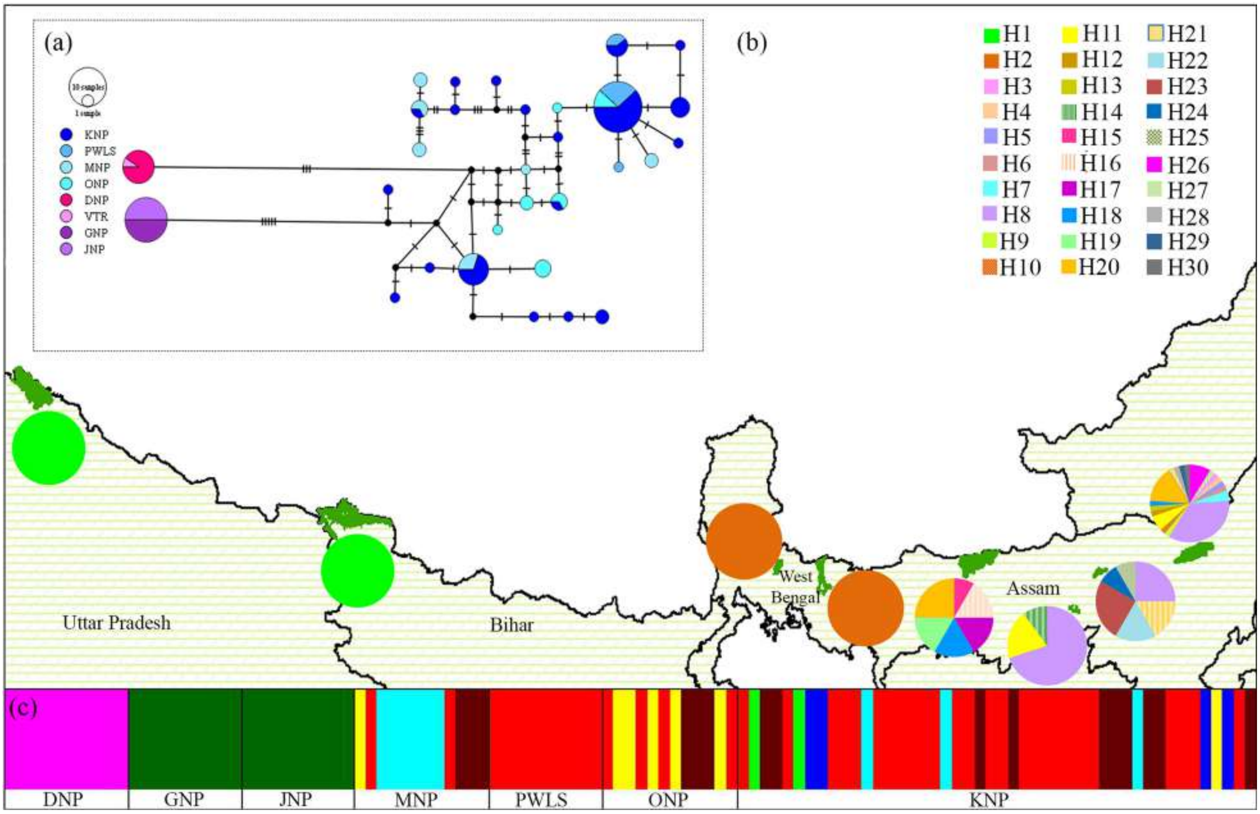

Fig. 1 Representation of mtDNA variations and genetic structure in Indian rhinos based on 2531 bp concatenated sequence covering all polymorphic sites across seven genes. **a** Median joining network with park-level colour codes; **b** Haplotype frequencies at each of the sampled areas covering all variations (n = 30 haplotypes); **c** Bayesian clustering shows monomorphism in Uttar Pradesh (with sample from Bihar, n = 11) and West Bengal (n = 20) populations and polymorphism in Assam (n = 80)

Table 1 mtDNA diversity indices of all rhino populations in India (n = 111)

Protected areas	Sample size	Segregating sites (S)	Haplotypes (h)	Haplotype diversity (Hd)	Nucleotide diversity (π)
Kaziranga National Park, Assam	46	18	19	0.85	0.0021
Pobitora Wildlife Sanctuary, Assam	10	2	3	0.51	0.0002
Manas National Park, Assam	12	14	6	0.89	0.0023
Orang National Park, Assam	12	9	6	0.87	0.0016
Dudhwa National Park, Uttar Pradesh	10	0	1	0	0
Valmiki National Park, Bihar	1	NA	NA	NA	NA
Gorumara National Park, West Bengal	10	0	1	0	0
Jaldapara National Park, West Bengal	10	0	1	0	0
Total	**111**	**21**	**30**	**0.89**	**0.0028**

Table 2 Results of pairwise genetic differentiation and hierarchical AMOVA test (Bihar sample considered under Uttar Pradesh clade)

Pairwise F_{st} among clades (*$p < 0.05$)

	Assam	Uttar Pradesh	West Bengal
Assam	0		
Uttar Pradesh	0.68*	0	
West Bengal	0.73*	1.0*	0

AMOVA test among three clades and seven populations

Source of variation	d.f	Sum of squares	Fixation index	Percentage of variation
Among groups	2	14.689	0.45 (F_{ct})	44.66
Among populations within groups	4	3.233	0.1 (F_{sc})	5.71
Within populations	104	31.267	0.50 (F_{st})	49.63
Total	110	49.189	0.60573	

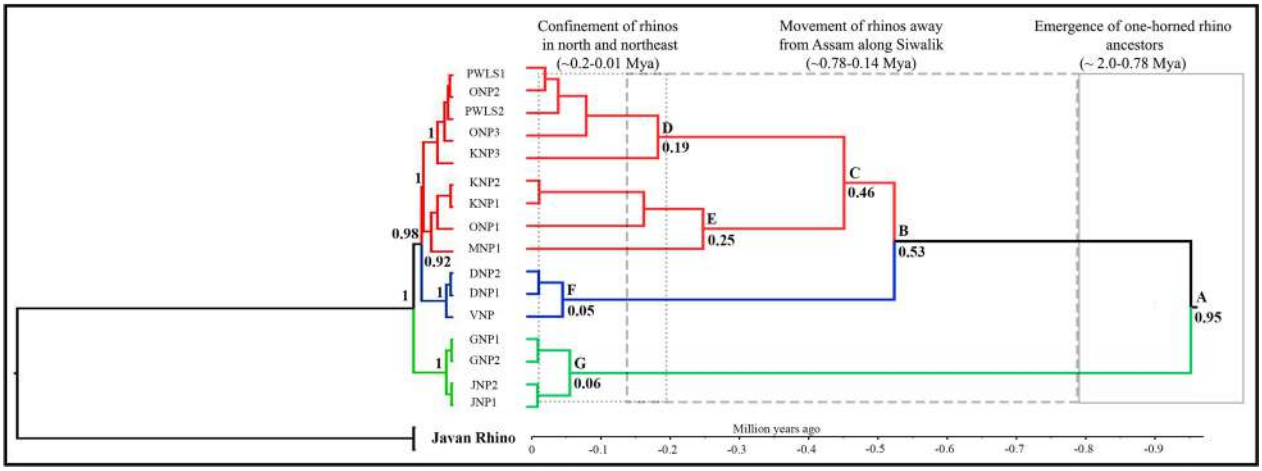

Fig. 2 Phylogenetic relationship and assessment of divergence time in Indian rhino populations. The left pane shows the clustering of three maternal clades of West Bengal samples (green), Uttar Pradesh (blue) and Assam (red). Javan rhino sequence was used as outgroup. The posterior probability values (≥ 0.9) are shown in bold. The right pane indicates the divergence of Indian rhinos ~ 0.95 Mya, where the Assam population coalesce first (~ 0.19 Mya), followed by divergence of West Bengal and Uttar Pradesh (0.06–0.05 Mya). Node-specific ages are marked (with posterior probability values ≥ 0.9). The major corroborating paleobiogeographical events are presented above

from 950 (HPD 1360–810 Kya) to 50 (150–10 Kya) Kya (Fig. 2). Our results indicated the divergence of Indian rhinos ~950 Kya (node A, Fig. 2) corresponds to the emergence period of one-horned rhino ancestors in the subcontinent [5, 12]. Next, the Assam population diverged from the remaining clades at ~500 Kya (HPD 680–330 Kya, nodes B & C, Fig. 2). This is supported by reports of multiple rhino movements away from Assam (along Siwalik as well to Siva-Malayan region) during this period [12, 20]. At population level, results suggest a relatively earlier coalescence of Assam ~190 Kya (HPD 300–70 Kya, node D & E, Fig. 2) compared to West Bengal and Uttar Pradesh (~50 Kya, HPD 150–10 Mya, node F & G, Fig. 2). This period (120–10 Kya) is known for confinement of rhinoceros to the north and north-east of India due to monsoon intensification and grassland dominance [5, 12, 21].

All four BSP analyses showed similar population trends for overlapping time periods where the combined data identified trends at a deeper coalescence period compared to the clade-specific data (Fig. 3). The Assam clade showed a steep increase in female effective population size ~110 Kya followed by constant population size from ~90 Kya (Fig. 3a) whereas West Bengal and Uttar Pradesh clades showed similar demographic trends of stable populations from ~60 Kya (Fig. 3b, c). The combined dataset showed a steep decline in population size ~300–200 Kya followed by a gradual increase ~120 Kya and steep rise ~60 Kya (Fig. 3d).

Discussion

This study presents the most extensive mitochondrial DNA phylogeography of one-horned rhinos across its Indian distribution. Careful considerations involving mitogenome sequencing of representative samples across Indian rhino-bearing areas, identification of all polymorphic regions and their amplification from spatially-covered rhino samples helped us achieving accurate assessment of mtDNA variations. To the best of our knowledge, this is the first report of wild Indian one-horned rhino mitogenome from all the extant populations. Despite relatively similar haplotype diversity of Asian rhinos [India—0.93 (16 samples), Sumatra—0.96 (15 samples), and Java—0.91 (6 samples), respectively], Indian rhino mitogenome showed much lower values for segregating sites and nucleotide diversity (Additional file 1: Table S4). Such mitogenome comparisons may be affected by limited sample size (earlier studies in African rhinos have reported higher diversity based on partial mitogenome data with more samples [17, 18]) or

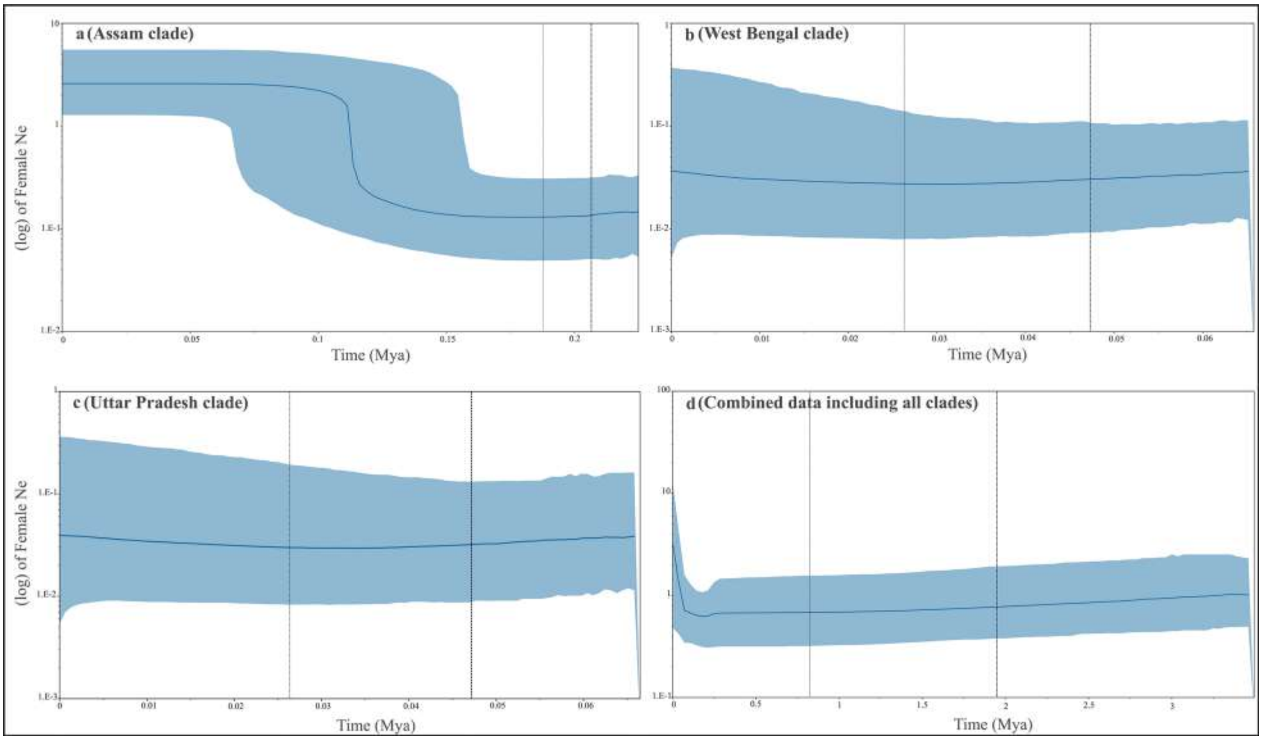

Fig. 3 Bayesian skyline plot analysis (BSP) to determine the changes in female effective population size across three clades, **a** Assam, **b** West Bengal, **c** Uttar Pradesh and **d** combined dataset of Indian rhinos. The vertical lines represent the HPD intervals of the given divergence time for each analysis whereas the shaded horizontal area is the HPD of the median effective size value

representation of historical genetic variations (in Javan rhinos, [13]). However, it was surprising to observe that despite similar historical demographic incidences (severe population decline due to habitat shrinkage [6, 12] and anthropogenic pressures [9, 10]) Indian rhino retain much lower genetic variation than their Sumatran counterpart. This can be potentially attributed to recovery of the Indian species from extremely low founder population (as indicated by high Hd but low π) [22, 23].

As expected, the phylogeography data (2531 bp mtDNA, n = 16 samples) revealed higher number of haplotypes than the mitogenome data (n = 30 haplotypes) (due to large sample size). The only other study on one-horned rhino mtDNA variations (based on partial control region sequences, 428 bp) reported 10 haplotypes (Kaziranga National Park, India—4 and Chitwan National Park, Nepal—6, respectively) and moderate level of genetic difference (F_{st} value of 0.39 between them) [24]. Careful scrutiny of our data revealed that all the polymorphic sites (or identified segregating sites) were found in fixed positions within one-horned rhino mitogenome (Additional file 1: Table S5) across India. Given the distribution of polymorphic sites in the sequenced mitogenomes and our sampling coverage, it is likely that these data represent the majority or perhaps all extant mtDNA haplotypes in Indian rhinoceros populations. This claim is also supported by the similar haplotype diversity values from the mitogenome and the phylogeography datasets (0.93 and 0.9, respectively). Our study also shows that the Indian rhinos have the highest number of haplotypes compared to the other genus/species reported so far [10, 13, 17, 18]. The clustering analysis of the concatenated rhino sequences showed three distinct genetic clades (corresponding to the states of Assam, West Bengal and Uttar Pradesh) with high F_{st} value (0.68–1), corroborating with the haplotype network patterns. Mantel test (− 0.83, p = 1) confirmed that such strong genetic structuring is not due to isolation by distance pattern, but driven by lineage-specific evolutionary history (as suggested by AMOVA results). Such pattern of higher within population and between group variance (50% and 45% in Indian rhinos, respectively) indicates that the mitochondrial genetic variation observed in extant Indian rhino is influenced by both evolutionary diversification and retention of diversity at population level only for Assam clade. As two of the clades are monomorphic, they contribute very less proportion of among-population within-group variations (5%). Similar data has also been described in other species such as barking deer—[25], dog—[26] etc. Interestingly, we found that the sequence from the Bihar sample (representing samples from Nepal) was identical to the Uttar Pradesh sequences, including the state-specific SNPs. This pattern was expected as the founder animals of the reintroduced Uttar Pradesh population were sourced from Chitwan National Park, Nepal (four dominant breeding females) and Pobitora Wildlife Sanctuary of Assam (dominant breeding male) [27]. Further comparison of 13 partial D-loop sequences from Chitwan National Park, Nepal Zschokke et al. [24] confirmed this pattern, indicating that the mtDNA signature of the Uttar Pradesh population belongs to Nepal. Given that the entire Uttar Pradesh rhino population showed only one haplotype, future studies need to evaluate the mtDNA variation in the Nepal population.

The phylogenetic analyses reconfirmed the relationship among the existing members of the Rhinocerotidae family [10, 28, 29] where the Sumatran and African rhino formed sister clades, separated from the *Rhinoceros* sp. based on the extant rhino genus/species sequence data only (Woolly rhinoceros sequence was not used). The within species tree topology corroborated with the haplotype network results as Assam and Uttar Pradesh formed phylogenetically closer clades as compared to West Bengal. We believe that the observed phylogenetic pattern of West Bengal being separate clade is influenced by lesser shared polymorphic sites between West Bengal and other two clades (Additional file 1: Table S5). Combined together, we interpret that the one-horned rhino diverged from its recent common ancestors ~ 950 Kya and different populations (Assam, West Bengal and Uttar Pradesh/Nepal) coalesce around ~ 190–50 Kya time period (Fig. 2). The molecular dates were comparable to other published literature on rhino evolution [5, 12, 21] and supported by the paleobiogeographic history of the Indian subcontinent [12, 21]. For instance, the inward movement of rhinos from Assam along Siwalik (680–330 Kya, node B & C) coincides with drop in the sea level which facilitated movement of multiple genera (for example, *Elaphas*, *Panthera*, *Rhinoceros*, *Muntiacus* etc.) through Siva-Malayan route [20, 30]. Report of one-horned and Javan rhino co-existence in Bhutan ~ 560 Kya [13] provide further support of such movements. Finally, the coalescence time of the three Indian clades (~ 190–50 Kya, Fig. 2) corresponds to Holocene climatic optimum period known for monsoon intensification in north and north-east part of India resulting in range contraction for grassland dependent species [5, 12, 31]. We feel that our approach of using taxon-specific mutation rate and fossil data for node calibration has resulted in achieving such meaningful estimates of tMRCA. Future efforts should try to include molecular data from historical/ancient samples to tighten the variance associated with divergence estimates [32]. Overall, this approach reiterates the critical importance of large datasets (whole mitogenome from multiple individuals in this case), informative prior settings and its assessment with posterior outputs, taxon-specific mutation rate, node calibration points etc. for accurate tMRCA estimation [33–37].

The BSP results with different datasets (combined vs. three individual clades) showed similar patterns of changes at different evolutionary timescale. The combined data indicate a historical decline in maternal effective population size ~300–200 Kya, followed by increasing tends during ~110–60 Kya (coinciding with Holocene climatic optimum period, also seen in the Assam clade analysis, Fig. 3). This pattern is similar to earlier findings (described in [1] based on whole genome data) with a difference in the Ne values arising from lower effective size in mtDNA [38]. The West Bengal and the Uttar Pradesh clades did not show any changes in population trajectories owing to the monomorphic data. It is noteworthy to point out that such mitochondrial DNA-based analyses would only capture the demographic history at longer evolutionary time scale, and use of suitable nuclear markers (microsatellites, SNPs etc.) could provide much powerful demographic inferences [38].

The spatially exhaustive sampling coverage and the patterns of population structure brings out some critical conservation perspectives for the Indian rhinos. The phylogeographic and mitophylogenomic patterns suggest three distinct clades with state-specific evolutionary histories. As these populations are morphologically undistinguishable and interbreed among themselves (Dudhwa, Uttar Pradesh population is genetically mixed, [27]), we suggest that they should be recognised as 'Evolutionary Significant Units (ESUs)' [39]. It is therefore important to use such information towards conservation and management efforts for each of these populations [39–41]. Despite strong recoveries across all existing populations since late 1990s, recent analyses suggest high extinction probability of the species [42], and further conservation efforts are mostly concentrated on translocation activities [16, 43]. Till date genetic information of the species has not been used in translocation planning (possibly due to lack of sufficient data), and the genetic signatures described in this study would be very helpful to increase variation in target populations. For example, the Uttar Pradesh and West Bengal population show state-specific monomorphic haplotypes representing unique but genetically depauperate populations. Based on the data presented here, suitable founder animals from Assam populations can be considered for future translocation programs in these areas, thereby increase the genetic diversity of these populations to combat any potential stochastic events [40, 41]. However, such efforts would impact the suggested ESU categorizations due to mixing of different gene pools among populations. Another important aspect for management consideration would be better planning for translocation events to any of the existing or new areas [16, 43]. For example, the reintroduced rhino population in Assam (Manas National Park) showed much higher mtDNA variation (six haplotypes), possibly due to periodic supplementation of individuals of varied genetic ancestry across different wild rhino populations [44] compared to Dudhwa NP (single haplotype) of Uttar Pradesh (single supplementation event). As multiple reintroduction programs are planned as per the 'National Conservation Strategy for the Indian One horned rhinoceros (*Rhinoceros unicornis*), Government of India, Ministry of Environment Forest and Climate Change, 2021' objectives (in the states of Uttar Pradesh, Bihar, West Bengal and Assam) in near future, we suggest that all future efforts should adopt the Manas NP model with consideration of selecting genetically variable founder animals, multiple reintroduction events etc. [16, 45].

Conclusion

The one-horned rhino was found throughout the Indo-Gangetic plains during the early twentieth century [43, 46] but faced drastic reductions in distribution and population size (including local extinctions) [47, 48], followed by one of the most successful species recovery (increase in population size) in wild across the world [7, 43, 48]. We present the first assessment of range-wide mitogenome diversity in Indian rhinos where we emphasize the importance of large data, spatial sampling coverage of populations and evolutionary history as fundamental information for future population reintroduction/recovery programs (as suggested in case of other species [49–52]). Our results are important for Indian rhino conservation because they suggest higher genetic diversity than earlier reported [24]. However, the existing habitats are small, disjunct, isolated and reaching their respective carrying capacities [16, 48] and conservation options are becoming limited except establishing new habitats and translocation-driven population enhancement [16]. We believe that the genetic information provided here will assist in identifying appropriate source populations and maintain adequate genetic diversity in the existing (and new) rhino populations, thereby ensuring evolutionary, ecological and demographic stability for their future survival.

Methods

Permission and ethical considerations

Data generated in this study is part of a collaborative programme titled "Implementing Rhino DNA Indexing System to counter rhino poaching threat and aid population management in India" (henceforth RhoDIS-India). Biological sampling from all the three rhino bearing states was permitted by Ministry of Environment, Forests and Climate Change (MoEF&CC), Government of India (Letter No. 4-22/2015/WL). Permission for dung sampling was provided by state forest departments of Assam (Letter No. A/GWL/RhoDIS/2017/913, 3653/WL/2W-525/2018, WL/FE.15/22), West Bengal (Letter No. 3967/WI/2W-525/2018) and Uttar Pradesh [Letter No. 1978/23-2-12 (G)]. We have also received one tissue sample from Valmiki National Park (henceforth NP), Bihar forest department assumed to be representing the wild rhinos of Nepal (Letter/no.-1296 dated 16.10.2020). No ethical permissions were required for tissues as they were collected from naturally dead rhinos as well as for dung samples.

Study area

During the 1600s, the one-horned rhinos were distributed throughout the northern Indian subcontinent covering all the major river basins from Pakistan to Indo-Myanmar borders. The species has lost most of its habitat and population size due to a range of anthropogenic interventions (habitat loss, hunting, poaching etc.) [7, 9, 14] and are currently distributed across only 12 protected areas covering >2000 km^2 area in India and Nepal [14]. This study on the Indian rhino was conducted across all extant rhino-bearing parks (n = 7) found across the states of Assam (n = 4 parks), West Bengal (n = 2 parks) and Uttar Pradesh (n = 1 park) situated in Terai-Duars region of north and north-east India (Fig. 4). Assam currently hosts the largest population of Indian rhinos (~ 80% of the total population), which are found across four isolated parks: Kaziranga NP, Orang NP, Pobitora Wildlife Sanctuary (WLS) and Manas NP. All of these populations have experienced severe hunting and poaching pressures during early-late 1990s, leading to local population sizes ranging from 50-few hundreds and local extinction in Manas NP, but have revived to their current population sizes [7, 14]. Between 2010 and 2020 Manas received 35 translocated rhinos from Kaziranga NP and Pobitora WLS part of the population recovery program [44]. The most recent population estimates of these parks are as follows: Kaziranga NP: ~ 2500; Orang NP: 100; Pobitora WLS: 101; and Manas NP: 42 [14].

The state of West Bengal currently retains ~ 350 rhino individuals distributed between two parks, Gorumara NP (52 individuals) and Jaldapara NP (>250 individuals). This population has recovered from a severe population decline of ~20 individuals during early 1900s (due to severe habitat loss) [9]. The rhino population in Uttar Pradesh was locally extinct along with the entire Terai in mid 1990s (mostly due to habitat loss and hunting). During 1984–85, rhinos were reintroduced in Dudhwa NP (Uttar Pradesh) from Chitwan NP, Nepal and Pobitora WLS, Assam and currently this park hosts ~ 40 rhinos [27]. Apart from this, wild rhinos are occasionally reported from Valmiki NP, Bihar (adjacent to Chitwan NP, Nepal in the Indian part of Terai). These rhinos are either swept down by natural flooding or use the grasslands along the river Gandak within Valmiki NP during monsoon seasons.

Biological sampling

Overall the sampling strategy in this phylogeography study was to select unique rhino individuals from different parts of the species distribution in India. A total of 160 samples (72 tissues and 88 dung) covering four states (Fig. 4) were used to assess rhino mitochondrial genetic diversity. The tissue samples of naturally dead rhino were provided by respective forest departments as part of RhoDIS-India protocol (2017–2021). Further dung collection was done to ensure spatial coverage for areas with no representative tissue samples. Rhino dung sampling can be challenging in the wild due to their use of communal latrine system (middens) [53, 54]. In this study, sampling was conducted by intensive foot and vehicle surveys from already known midden sites across six rhino bearing parks (except Kaziranga NP). During sampling, only the fresh bolus from top of the midden was selected and swabbed twice with separate PBS-soaked sterile cotton swabs (Himedia, Mumbai, India). All samples were geotagged and transferred to laboratory in − 20 °C freezer till downstream processing.

DNA extraction

Tissue DNA was extracted using already established protocol for Indian rhino mentioned in Ghosh et al. [55]. For dung samples, a modified protocol from Biswas et al. [56] was used. In brief, samples were digested overnight with a combination of 700 µl ATL and 65 µl Proteinase K (20 mg/ml) at 56 °C, followed by QIAamp DNA Tissue Kit (QIAGEN Inc., Hilden, Germany) protocol with adjusted volumes. DNA was eluted twice in 100 µl preheated (70 °C) 1X TE buffer and stored in − 20 °C freezer. Extraction negative was used for each set of extraction (n = 23) to monitor possible contamination.

PCR amplification and sequencing

To assess genetic variation of the extant rhino populations, complete mitogenome data was generated for representative samples from each park (n = 15, see Additional file 1: Table S2 for details) and one from the Valmiki National Park, Bihar. These samples were selected based on their geographic locations representing the farthest samples within each park to ensure inclusion of potentially unrelated individuals. Mitogenome sequencing was performed using already published 23

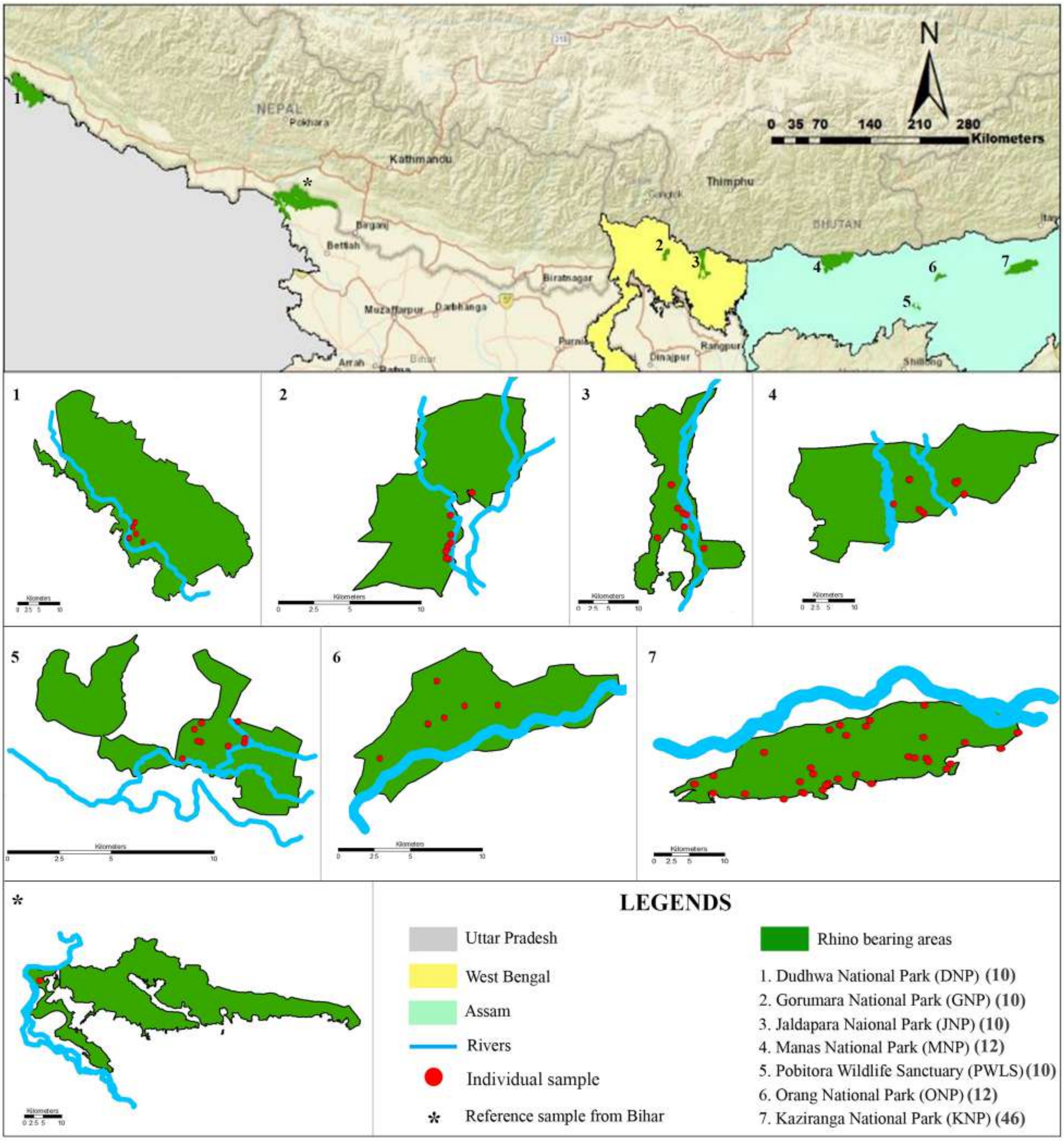

Fig. 4 Map of the study area and distribution of the final samples used in this study (n = 111). The top plate shows the position of the rhino-bearing parks across three Indian states (Uttar Pradesh, West Bengal and Assam). The reference sample of wild rhino received from the state of Bihar (*) is also presented

overlapping primers [57]. For annealing temperature standardisation, gradient PCR was set in 10 µl reactions containing 4 µl of 2× Qiagen PCR buffer mix (QIAGEN Inc., Hilden, Germany), 1 µl of primer (3 µM), 2 µM BSA (4 mg/ml), 1.4 µl of RNase free water and 5 ng of rhino tissue DNA. PCR conditions included an initial denaturation (95 °C for 15 min); 35 cycles of denaturation (95 °C for 30 s), annealing (50–60 °C gradients for 40 s) and extension (72 °C for 40 s); followed by a final extension (72 °C for 10 min). During each set of reactions, PCR and extraction negatives were included to monitor contamination. Amplified products were visualized with 2% agarose gel, cleaned with Exonuclease (Thermo Scientific, Waltham, USA) and Shrimp Alkaline Phosphatase (Amresco, Solon, USA) mixture and sequenced bidirectionally in an ABI 3500XL bioanalyzer (Applied Biosystems). Out of these 23 primers, two did not show amplification in any samples. The remaining sequences (n = 21 from 16 individuals) were aligned with the available one-horned rhino mitogenome (Genbank: X97336, [28]) in Mega v7 [58]. Two primers were designed manually in the flanking conserved regions adjacent to the gaps (Additional file 1: Table S1) and sequences were generated from all the samples (n = 16).

The complete mitochondrial sequences (n = 16) were aligned and manually screened to identify the segregating sites. Further, a total of 15 primers were designed (multiple primers covering the segregating sites) to amplify all the polymorphic sites as < 500 bp fragments to ensure higher success rate from—poor quality dung DNA samples. These primers were standardised following same protocol described above. For all field collected samples (tissue = 56 and dung = 88) individual identification was performed using a panel of 14 microsatellites (described in [55]). After PCR amplification and genotyping of the markers, samples with 12–14 loci data were selected for downstream analysis and genetic recaptures were removed. To ensure removal of closely related individuals in our dataset we selected one sample from adjacent midden sites. Sequence data (2531 bp covering seven genes) was generated for the selected individuals to assess phylogeography patterns.

Complete mitogenome annotation and comparative analysis

All rhino sequences (n = 16) were aligned in Mega v7 to generate a complete mitogenome sequence and manually checked to identify any nucleotide ambiguities. Annotation was done using MITOS2 web with default settings and vertebrate mitochondrial genetic code [59] followed by mitogenome map construction with OGDRAW [60]. The mitogenome annotation was further confirmed with earlier published one-horned rhino mitogenome data (Genbank: X97336, [28]). To ascertain species-wise mitochondrial DNA diversity these sequences were aligned with already published rhino mitogenome sequences from *Diceros bicornis* (n = 2, Genbank: FJ905814, NC012682 [61]), *Ceratotherium simum* (n = 2, Genbank: Y07726, NC001808 [62]), *Dicerorhinus sumatrensis* (n = 15, Genbank: MF066629-MFO66643 [10] and *Rhinoceros sondaicus* (n = 6, Genbank: FJ905815 [61], MK909142, MK909146, MK909148, MK909149, MK909151 [13]). We calculated number of segregating sites (S), nucleotide (π) and haplotype diversity (Hd) using DnaSP v.5 [63] for all genes in the mitogenome.

Genetic diversity in Indian rhinos

Population-wise basic indices of genetic variations (S, π and Hd) were calculated for concatenated sequence data (2531 bp from seven genes) using DnaSP v.5 followed by a median joining [64] haplotype network constructed in PopART v. 1.7 [65]. To ascertain any possible population structure a Bayesian approach implemented in BAPS v.5.3 was used as it considers linked loci data [66]. Pairwise F_{st} and differential hierarchical AMOVA analysis was performed using Arlequin v. 3.0 [67] to confirm the pattern found in BAPS analysis.

Estimation of clade-specific divergence times and demographic history

To identify the clades, Bayesian phylogeny was constructed with MrBayes v. 3.2.7 [68] using 16 Indian rhino mitogenome and Javan rhino sequence (outgroup, as they are the sister clade of one-horned rhinoceros) [69]. Analysis was conducted using GTR + G substitution model determined by jModelTest v2.1.3 [70] (based on Akaike Information Criteria). The MCMC parameters included 2 runs of four chains each of 15 million generations with sampling after 1000 generations till split frequencies were below 0.01. Posterior probabilities were calculated for each node.

To estimate divergence among clades, rate of mutation for Indian rhino was calculated using BEAST v.2.3.6 [32]. Analysis was performed with five extant rhino mitogenome (without D-loop) (n = 11 sequences, India = 7 (haplotypes representing maximum variation in the data), Java = 1, Sumatra = 1, White = 1, Black = 1) along with horse (*Equus caballus*, Genbank: NC001640), donkey (*Equus asinus*, Genbank: NC001788), Asiatic wild ass (*Equus hemionus*, Genbank: NC016061) and zebra (*Equus zebra*, Genbank: NC018780) as outgroups. GTR + G substitution model was selected through jModelTest v2.1.3 for this multi-species data. Birth–death speciation was considered as tree prior [10, 34] along with uncorrelated relaxed log normal clock [10, 33]. During analysis, four established internal node calibration points (based on fossil records) with normal distribution priors were employed: (i) Caballine split (4 ± 0.5 million years ago (Mya)) [71, 72]; (ii) late Oligocene diversification of rhino groups (26 ± 3.5 Mya) [73]; (iii) split of rhinoceros genus (3 ± 0.5 Mya) [1]; (iv) origin of the perissodactyls (55 ± 3 Mya) [74, 75]. The first three calibration points were considered as monophyletic constraint [33] as the last point includes both ingroup and outgroup taxa.

tMRCA (time to Most Recent Common Ancestor) was inferred using the estimated mutation rate with lognormal distribution under strict molecular clock (intra species data, n = 16) [34, 35]. MCMC runs included 100 million generations, sampled at every 10,000 states with 10% burn-in. Data convergence was checked with Tracer v. 1.5 [76] and the final tree (with maximum clade credibility) was estimated with TreeAnnotator [77] and visualised using FigTree v.1.4.2 [78].

To estimate past fluctuations in population size, Bayesian skyline analysis (Bayesian skyline plot or BSP) was conducted using concatenated sequence data with monophyletic constraint to the identified maternal clades ensuring phylogenetic construction. Analysis was conducted with multiple datasets (each clade and combined data, respectively) to ascertain any possible impacts of genetic structure in the data [38]. In all cases coalescent BSP tree prior was used along with strict molecular clock, estimated mutation rate and clade specific divergence date. MCMC parameter settings and data convergence were identical to the tMRCA analysis.

Abbreviations

BSP: Bayesian skyline plot; ESUs: Evolutionary significant units; Kya: Thousand years ago; mtDNA: Mitochondrial DNA; Mya: Million years ago; Ne: Effective population size; NP: National Park; tMRCA: Time to the most common recent ancestor.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12862-022-02045-2.

Additional file 1: Table S1. Details of the primers used in sequencing Indian rhino whole mitogenome and phylogeography data. **Table S2.** Sample (both tissue and dung) of Indian rhinos used in this study. A total of 111 individual rhino samples (72 tissue and 39 dung samples, respectively) were used in this study. Out of the 72 tissue samples, 16 samples representing different parks were used to generate the whole mitogenome data. **Table S3.** Mitogenome organization in *Rhinoceros unicornis*. Codons respective to each tRNA are mentioned in parenthesis. **Table S4.** Comparative analysis results of genetic diversity indices among five extant rhino species. **Table S5.** Details of the variable sites based on concatenated sequence of 2531bp of Indian one horned rhino mtDNA haplotypes.

Additional file 2: Figure S1. Whole mitogenome organisation and annotation of *Rhinoceros unicornis*. **Figure S2.** Gene-wise comparative analyses of polymorphism indices (S, Hd and π) for all extant rhino species. **Figure S3.** Estimation of mitogenome mutation rate for Indian rhino using Caballine (Zebra, Donkey, Asiatic Wild Ass and Horse) as outgroup. Internal node calibration points are presented in italics (bold). Estimated node ages and branch mutation rates with available reference in literature is marked with * [1, 8, 10, 29].

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Author contributions

TG and SM designed the study. AS and SM raised necessary funds and acquisition of permissions to conduct this study. Sample collection was done by TG, SK and PK. TG, SK and KS were involved in data curation and generation, whereas analysis and data visualisation were done by TG and SK. TG and SM wrote the manuscript with inputs from SK, AS, PK and KS. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset generated in this study is available in GenBank with Accession numbers MZ736693–MZ736708 (whole mitogenome sequences) and MZ771364–MZ771458, MZ771459–MZ771553, MZ771554–MZ771648, MZ771649–MZ771743, MZ771744–MZ771838, MZ771839–MZ771933 and MZ771934–MZ772028 (fragmented sequence data for phylogeography). The additional figures and tables are provided as Additional file 1: Tables S1–S4 and Additional file 2: Figs. S1–S3).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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