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Mitochondrial D-loop Genetic Diversity Studies of *Rhinoceros unicornis* in Assam, India

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ABSTRACT

In Assam wild populations of great Indian rhinoceros are found in three protected areas namely Kaziranga National Park, Orange National Park and Pobitora Wildlife Sanctuary. But it remains an area of investigation whether these three populations are homogeneous or not. To resolve this matter present genetic study was performed on mitochondrial DNA control region by using non invasive dung samples collected from the three natural habitats. The study showed a high level of genetic diversity of rhinoceros population in three habitats of Assam with 24 haplotypes from 196 samples and 21 variable sites in 413bp long nucleotide sequences was recorded. The Fst value also showed differences between the groups; and significant values obtained between Kaziranga and Pobitora with Orang groups. AMOVA analyses revealed the total genetic diversity is 91.62% and diversity between populations is only 8.38%. Thus the higher genetic variability found in Indian rhinoceros populations is important for future survivability, management and translocation to new habitat.

Key words: D-loop haplotype, Genetic diversity, Rhinoceros.

INTRODUCTION

Rhinoceros unicornis Linnaeus, 1758 is the largest Perissodactyl characterized by a single large horn found in India and Nepal. In India the largest and growing population is found in the Kaziranga National Park of Assam (Laurie *et al.*, 1983) and the other two habitats are Orang National Park and Pobitora Wildlife Sanctuary. In past (c. 1400 AD), the Indian rhinoceros was found in the flood plains of Brahmaputra river and Gangetic plain, Indus River Valley and Myanmar with more than 450,000 individuals (Blanford, 1891; Laurie, 1978; Dinerstein and Price, 1991). But during early part of 19th century (1908 AD) the rhinoceros population was decreased significantly due to killing, poaching and habitat loss and population become fragmented (Laurie *et al.*, 1983). Now the natural populations of the Indian rhinoceros only available in the states of Assam, Uttar Pradesh and West Bengal in India and the Terai of Nepal (Foose and van Strien, 1997). In Assam, all three rhinoceros populations are thought to be expanded from survived remaining population after 1908 AD without any fitness problems. To know more about genetic diversity at molecular level of the species which is related to fitness and survivability in a changing environments (Freeman and Herron, 1998) the rhinoceros populations from the three habitats were evaluated for genetic diversity based on the D-loop segment of mitochondrial DNA as marker because of its higher mutation rate correlated with evolutionary events (Avise, 1994; Kim *et al.,* 2002).

MATERIALS AND METHODS

In the present study 352 fresh dung samples of rhinos were collected from three wild habitats namely Kaziranga National Park, Orang National Park (ONP) and Pobitora Wildlife Sanctuary (PWLS) out of which only 196 samples gave positive amplification. To obtain genomic DNA, the outermost layer of the dung samples were collected, as this layer contain the cell of intestinal mucosa. Approximately 10g of fresh dung were placed in 50ml polypropylene tube containing 95% ethanol (Merck) and labeled properly and kept at -20°C until DNA isolation.

Genomic DNA extraction was carried out from the alcohol preserved dung samples by QIAmp DNA stool mini kit (QIAGEN Inc.) with slight alternations in the prescribed protocol. During the DNA extraction from dung, in addition to faecal matter approximately 100 µl alcohol was also taken from the bottom of the sample vial. The DNA was evaluated for its quality via agarose gel electrophoresis and quantity was measured using a NanoDrop spectrophotometer.

Primers

To amplify 420 bp (expected) long D-loop control region located in tRNA-Pro and D-loop of mitochondrial genome, a set of region specific generic primers RH-D-F1 and RH-D-R1 (Fernando *et al.*, 2006) were selected. The sequences of the primers are RH-D-F1CATCAACACCCAAAGCTGAAA and RH-D-R1ATGGGCCCGGAGCGAGAACGA.

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PCR amplification

PCR amplification was performed in 25µl reaction mix using 2µl genomic DNA (10-30ng) , 2µl 100µg/ µL BSA, 2.5 µl 1.5Xtaq buffer A, 0.5 µl (1.5mM) $MgCl_2$, 2.5 µl (0.25mM) dNTP separate (SIGMA), each primer forward and reverse of 0.5 µl (10 µM), 0.1 µl AmpliTaq Gold DNA polymerase and 14.4 µl water. Amplification of specific product was carried out in Master cycler employing initial denaturation at 95°C for 4 min followed by 50 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min and primer extension at 72°C for 1.5 min. On completion of the cycles, the reaction mixture was incubated further at 72°C for 5 min and 4°C forever. The amplified PCR products (approximately 1.5 µl) were checked for appropriate size with 2% agarose gel (Fig 1) with suitable molecular marker. After electrophoresis, the PCR products were cleaned up by adding 3µl of Exo-SAP mixture (Shrimp Alkaline Phosphatase) per 20µl reaction. For purification of a few samples gel extraction method was also followed. The purified products were then sequenced in both forward and reverse direction in ABI automated sequencer (Applied Biosystems).

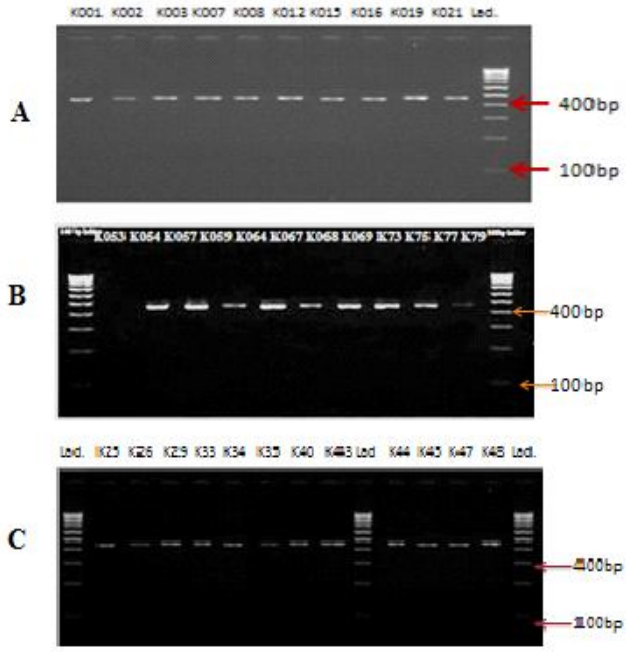

Fig 1: PCR products of 420bp size A to C with 100 bp ladder.

Data analysis

Amplified 413bp of D loop fragment of *R. unicornis* were aligned using CLUSTALW program (Thompson *et al.,* 1994), inbuilt in the genetic analysis package MEGA 7.0 (Kumar *et al*., 2016). Sequences were then checked with Finch TV1.4 (Geospiza. com) and then visually refined. A total of 24 haplotypes were obtained from 196 D-loop sequences.The haplotype sequenced data were then analyzed with the help of BLAST for homology search. In addition to the samples collected in this study, two GenBank data (Acc. No.X97336.1 and NC 001779.1) were used to compare and draw phylogenetic tree. Identical haplotypes, polymorphic sites, haplotype diversity and variance of haplotype diversity within the population were detected in DnaSP 5.0 (Rozas *et al*., 2003). Nucleotide composition of all haplotype sequences, type of substitutions, haplotype distance matrix, pairwise Fst Matrix (Weir and Cockerham, 1984) and pairwise differences (Nei's standard genetic distances) of three Indian rhino population and for all samples of *R. unicornis* were determined by Arlequin 3.0 (Excoffier *et al.,* 2005). Hierarchical genetic structure was inferred using analysis of molecular variance (AMOVA) for all haplotype data were also calculated in Arlequin 3.0. Different phylogenetic trees were constructed for all haplotypes with the *Diceros bicornis* sequence (Acc. No. L22010) as an out_group based on the p-distance and Kimura's 2 parameter model using MEGA 7.0. Bootstrap analysis (1000 data sets) was used to assess confidence in the branching order into the dendrogram. Genetic distances among different haplotypes were also calculated by Kimura 2 parameter method using MEGA7.0. A parsimony network linking all haplotypes was developed using NETWORK 4.6.1.1 (Bandelt *et al*., 1999) for a visualization of the relationship among the haplotypes separately.

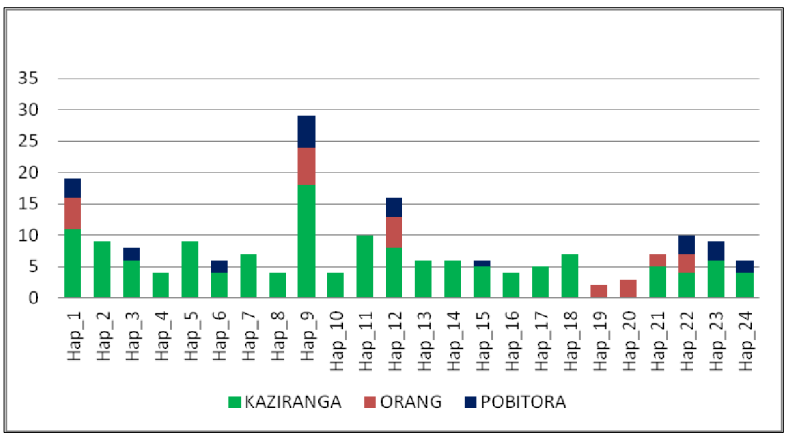

Fig 2: D-loop haplotypes of *R. unicornis* in three habitats.

RESULTS AND DISCUSSION

The Nucleotide positions of the sequenced segment of DNA were assigned according to the complete rhinoceros mtDNA reference sequence (15412-15824 nts) GenBank Accession No. X97336. 21 variable sites were found in total 24 haplotypes obtained from 196 D-loop sequences from three different rhino habitats. The same haplotype obtained from different habitat given same ID code placing first letter of the habitat (H1 from Kaziranga as KH1). Out of 21 polymorphic sites are 8 Singleton variable sites and remaining 13 are parsimony informative sites. In the rhino group of Kaziranga National Park (KNP) 22 different haplotypes were obtained, 9 haplotypes were detected in Pobitora WLS (PWLS) and 7 haplotypes were found in Orang National Park (RONP). In Fig 2 the distribution and frequencies of different haplotypes of *R. unicornis* in three protected areas of Assam are presented. The mean haplotype diversity of the three rhino groups is (Hd) 0.97571±0.011. The 21 Polymorphic sites within 24 haplotypes of *R. unicornis*_obtained from three areas of Assam and two GenBank sequences was given in Fig 3 with reference to that sequence acc, no. X97336. The variable positions of nucleotides are found commonly in particular locus. Highest genetic diversity (0.99567) was observed among the rhinos of Kaziranga National Park. No statistical significance for Fu's Fs or Tajima's D values was observed for whole population or population for each habitat ($P > 0.10$). The average nucleotide composition of all haplotype sequences were 34.18%A, 29.34%T, 12.76% G and 23.72% C and the average nucleotide content of A + T (63.52%) was obviously higher than that of G + C (36.48%). The nucleotide compositions of D-loop sequence from three rhino groups are nearly similar. The phylogenetic tree (Fig 4) revealed a low differentiation between the different haplotypes of *R. unicornis* of three different habitats. Bootstrap analysis showed bootstrap values lower than 70, except in the few haplotypes such as H21, H22 and H23.

The Medium-joining network (Haplotype network) of all mtDNA haplotypes of *R. unicornis* (Fig 5) indicated that there could have more D-loop haplotypes in the wild populations. The network analysis (conducted with NETWORK 4.6.1.1) depicted that H9 as a historical haplotype. The other haplotypes have link with H9, on the other hand H1 is another stock in whom a large number of haplotypes have connections.

The Standardized variance in allele frequencies (Fst) calculated by Arlequin based on Kimura 2 parameters for three groups rhinos suggested that Kaziranga rhinoceros are more homogenous to Orang rhino group. Whereas according to Kimura 2 parameter model, there is a difference between Kaziranga and Orang rhino groups (Fst = 0.0393) and Pobitora and Orang (Fst=0.0220) which signifies little difference (Fst value 0.0 to 0.05; Wright, 1965). When analysis was done by taking Kaziranga and Pobitora as one group and Orang as other group, the Fst based on F-Statistics was found 0.09259 which is a significant.

Three different AMOVA analyses were done to explain genetic variation (within and among the groups) among rhinos revealed that 91.62% of the total genetic diversity existed within populations and only 8.38% accounted for between populations. The average number of nucleotide

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

Fig 3: Polymorphic sites within 24 D-loop haplotypes of *R. unicornis* two GenBank sequences (Acc. No.X97336.1 and NC 001779.1).

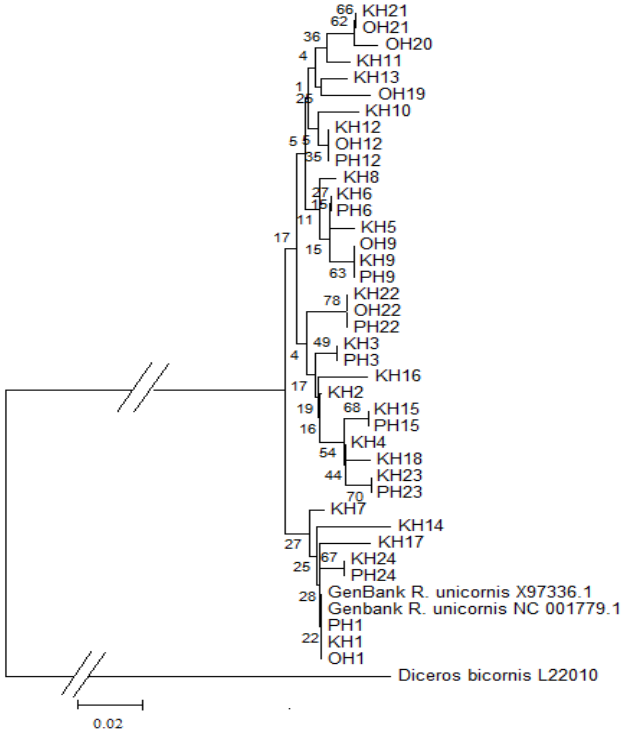

Fig 4: The Neighbour-joining tree of *R. unicornis* haplotypes with two GenBank sequence (Acc. No.X97336 and NC 001779) of *R. unicornis* and one out-group, *Diceros bicornis* (Acc. No. L22010).

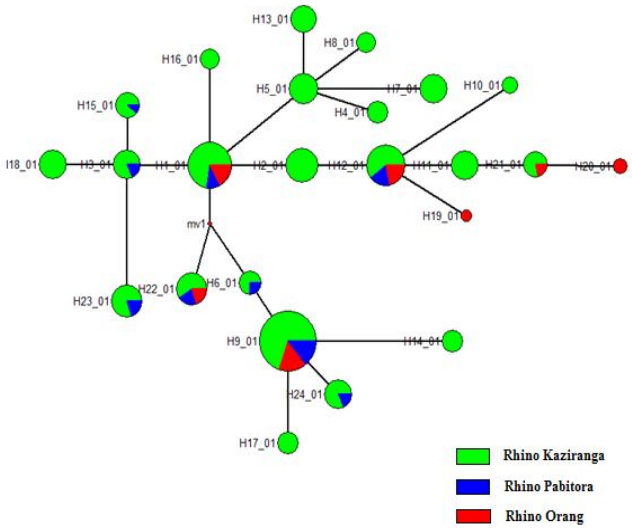

Fig 5: Medium-joining networks (Haplotype network) of mtDNA haplotypes. Each circle represents a haplotype and its size is proportional to the haplotype frequency in different habitats. The partially or completely coloured filled circles illustrate the relative frequency of a haplotype in the three habitats. Small black circles is median vector (mv) unsampled, hypothetical sequences which have not been found in this study or extinct ancestral sequences.

differences between Kaziranga and Orang rhino group is 4.481 and the same differences between Kaziranga and Pobitora rhino group is 4.020. The average number of nucletide substitution per site between populations (Dxy) Kaziranga and Orang rhino group is 0.01085 while this Fig is 0.00973 between Kaziranga and Pobitora rhino group.

The present study recorded 24 haplotypes from 196 D loop sequences of rhinoceros where as earlier studies (Das and Goswami, 2012a) reported only 3 haplotypes (Hap03, Hap04 and Hap05) from 14 samples in KNP might be due to small sample size. Significant haplotype diversity (0.99567) is found in the present study indicated a rich genetic diversity of rhinoceros population suggesting multiple ancestral lineages. Haplotype 9 (hap 09) was found to be more prominent and abundant in three rhino habitats.

Hap01 was found to be the second most available haplotype. The variations of the D-loop sequences are very less which was found from polymorphic site analysis indicating rapid evolving nature of D-loop and its high mutation rate (Saccone *et al.*, 1991). Such variation of allele is caused due to genetic drift, selection, gene flow and local adaptation imposed by environment and humans (Hartl and Clark, 1997). The genetic diversity is probably related to adaptation to changing environmental conditions (Hirayama *et al.,* 2010). The genetic diversity has been always found to have positive implications to a population. The phylogenetic analyses based on different criterion showed that though the rhino population has genetic diversity but their differentiations do not reach to a level to categorize as a sub population. They cannot be considered as separate clade until more analysis on different nuclear DNA is done. The presence of same haplotypes in the three habitats might be due to gene flow between the groups and movement across habitats as the species is highly mobile and good swimmers. The patterns of demography and hierarchical genetic structure of species with limited geographic range are important elements in determining the population structure, in the development of an effective and sustainable management plan (McCracken *et al.*, 2001). The genetic diversity database of rhinoceros population will help in various conservation efforts such as translocation of individuals and population viability assessments (Florescu *et al.,* 2003; Harley *et al.,* 2005). Large sample size in Kaziranga national park has possibility to obtain more D-loop haplotypes. The genetic diversity in rhinos found in this study did not correspond to reports of Zschokke and Baur (2002); who reported monomorphic pattern. However, recent studies by Zschokke *et al.* (2011) on captive rhino population from different zoos have demonstrated that *R. unicornis* population is genetically diverged. In another explanation it can be inferred that as there were a large number (24) of D-loop sequence haplotypes for the mtDNA was found in the rhino population in Assam there may be recent population expansion. One possible explanation for the high diversity of *R. unicornis* found in Assam particularly the KNP have harbor the remaining population in the late 19th and early 20th century that had to moved into the Park from nearby forest areas after losing habitat in the historic range of Brahmaputra River basins.

Appropriate information on the genetic diversity of wild animals is now essential for wildlife conservation and management and initiatives (Soltis and Gitzendanner, 1999). The greater the genetic diversity within a population, the better it is for the survival of the species (Kierstein *et al.*, 2004). Protection of suitable rhino habitat seems to be important aspect of rhino conservation (Das and Goswami, 2012b).

The high level of genetic variation found in Indian rhinoceros populations has ample scope for evolution to occur and helps in the management and translocation of the rhino population to new habitat. The ongoing translocation programme of rhino from Kaziranga and Pobitora and reintroduction it to the former habitat in Manas National Park of Assam should also follow the genetic guideline for better success in the project.

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