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ORIGINAL ARTICLE

Population genetic assessment of extant populations of greater one-horned rhinoceros (*Rhinoceros unicornis*) in India

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Abstract The greater one-horned rhino has a wide range of historical distribution across the northern Indian subcontinent and now being confined to restricted patches in India and Nepal. Development of effective conservation strategy for rhino requires a clear understanding of the extant and spatial distribution of genetic diversity. In the present study, we employed nine microsatellite markers to analyze 238 noninvasively sampled individual rhinos from five protected areas in India, in order to assess genetic diversity and population genetic structure in the wild. We observed a moderate to high level of genetic diversity with allelic richness (Ar) ranging from 2.589 (±0.88) to 3.635 (±0.93) and expected heterozygosity (H_e) ranging from 0.352 (±0.20) to 0.59 (±0.13) in the area. Significant level of genetic differentiation was observed between the Protected Areas of Assam and West Bengal, especially, Gorumara National Park showing a unique genetic signature ($F_{ST} \geq 0.25$; $p < 0.001$, with all other protected areas). Given the degree of population genetic structure observed, prolonged separation of these protected areas is unwanted as this could lead to further loss of genetic diversity, consequently, affecting long-term viability of the species. The results presented here will be crucial in designing in situ conservation and management strategies of the species.

Keywords *Rhinoceros unicornis* · Noninvasive genetics · Microsatellite · Genetic diversity · Population structure

Introduction

The greater one-horned rhinoceros (*Rhinoceros unicornis)*, characterized by its single large nasal horn, is a large free-ranging mammal found in India and Nepal. They were once distributed throughout the Indian subcontinent, with a range extending from the Punjab foothills, Peshawar, Sind and lower Indus to far west to North eastern India and with discrete records of its existence from Bangladesh, China and Burma (Rao 1947, Tun 1956, 1967, Rookmaaker 1980). The population of rhino was reported to have reduced to few hundred individuals in number during early 1900s due to rapid degradation and loss of habitat along with increased poaching activity (Talukdar et al. 2008). However, the rhinos have managed to revive back in recent years and now remnants of the population are distributed in patches of protected areas (PAs) in Assam, West Bengal and Uttar Pradesh of India and parts of Nepal (Laurie et al. 1983). The species is protected under Schedule I of Wildlife (Protection) Act, India 1972 and is listed as Vulnerable in IUCN red list of threatened species (IUCN 2012) and finds its place in Appendix-I of the CITES.

In India, the extant rhino population is restricted to seven PAs, four in the state of Assam, viz., Pabitora Wildlife Sanctuary (PWLS), Kaziranga National Park (KNP), Orang National Park (ONP) and Manas National Park (MNP), two in the state of West Bengal, viz., Gorumara National Park (GNP) and Jaldapara National Park (JNP) and one in the state of Uttar Pradesh, viz., Dudhwa National Park (DNP), respectively. PWLS, KNP and ONP are situated in the flood plains of Brahmaputra river basin characterized by dry and swampy grasslands which provide a good habitat to the rhinos. The

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current population estimates of PWLS, KNP and ONP stands at 93, 2,329 and 100, respectively (2013 census by the Forest Department, unpublished data). All these PAs are connected through series of river islands of the Brahmaputra which are often utilized by the rhinos for movement (Talukdar et al. 2007). GNP and JNP are located in the Terai region of the Himalayan foothills in the state of West Bengal, and are inhabited by 42 and 186 rhinos, respectively (2012 census by the Forest Department, unpublished data). Although GNP and JNP are located at close proximity, they are separated by intense human settlements and agriculture lands, thereby reducing the possibility of rhino movement between the PAs. The West Bengal populations were connected to Assam through Sankosh–Rydak region where rhinos were known to exist till late 1960's, with reports of free movements of individual rhinos from West Bengal to Assam (Bist 1994). Most of the rhino habitats in the region were lost due to clearing of forest for extension of agricultural land, encroachment, erosion etc. and no information on movement of individuals is currently available. The rhino population from MNP, Assam, in the closest proximity to West Bengal was completely wiped out in 1990s due to intense poaching activity during a decade long period of civil unrest. As a part of rhino reintroduction effort in MNP, Indian Rhino Vision 2020, a joint initiative of Government of Assam and other non-governmental agencies, translocated 18 individuals from KNP and PWLS during 2008–2012 and 7 rescued individuals from KNP. The DNP, Uttar Pradesh, is inhabited by around 30 individuals. The DNP population was rescued by seven rhinos (two males and five females) translocated from PWLS and Chitwan National Park, Nepal during 1984–1985 (Sale and Singh 1987).

Although, the rhino population in India has increased in size, the effects of size contraction (population bottleneck) and habitat fragmentation on it is not clearly known. Prolonged separation of the individuals thriving in patches might have affected the population due to probable loss of genetic diversity. From a conservation point of view, it is important to understand the genetic status of rhino, as isolated populations are often at risk to come under the influence of stochastic factors which may eventually lead to further decline in population size or even extinction (Dyke 2008). Moreover, it is important to define population at spatial scale, as in case of free-ranging animals delineating population boundary is a difficult convention. In this context, use of genetic data for population monitoring can play a crucial role in understanding the status of the rhino population in India.

We undertook a microsatellite-based population genetic assessment of rhino inhabiting PWLS, KNP, and ONP in Assam and GNP and JNP in West Bengal, India. Nuclear microsatellite markers are regarded as excellent tool for population genetic studies involving natural populations (Bruford and Wayne 1993). Due to their highly polymorphic nature, microsatellites have been extensively used in studies related to genetic diversity, effect of bottlenecks and population structure as well as tracing migration patterns (Paetkau et al. 1995, Waits et al. 2000, Harley et al. 2005, Serrano et al. 2009, Vonholdt et al. 2010). We followed a noninvasive sampling approach by collecting dung (faecal) samples which allows collection of biological material without capturing the animal. The methodology has been increasingly applied to a wide variety of species (Taberlet et al. 1997, Goossens et al. 1998, Kohn et al. 1999, Bhagavatula and Singh 2006, Borthakur et al. 2011, Arandjelovic et al. 2011, Karmacharya et al. 2011) particularly in case of rare or elusive free-ranging animals.

In the present study, we investigate the extent of contemporary genetic diversity in the wild populations of rhino in India and estimate the population substructure with the aim to establish population boundaries at a spatial scale. This is the first detailed testimony of genetic diversity and population structure of the remnant populations of rhino in India, providing crucial information for a science based in situ conservation of the species.

Materials and methods

Study area

All the samples were collected from the five wild rhino-bearing PAs of India, viz., PWLS (38.8 km^2, from 26°12′N to 26°15′N latitudes and 91° 57′E to 92°50′E longitudes), KNP (429.93 km^2, from 26°34′N to 26°46′N latitudes and 93°08′E to 93°36′E longitudes) and ONP (78.8 km^2, from 26°29‘ N to 26°40′N latitudes and 92°16′E to 93°27′E longitudes) in the state of Assam, GNP (79.99 km^2, from 26°44′N to 26°75′N latitudes and 88°50′E to 88°60′E longitudes) and JNP (216.51 km^2, 25°58′N and 27°45′N latitudes and 89°08′E and 89°55′E longitudes) in the state of West Bengal, respectively (Fig. 1). MNP and DNP were not considered in the present study as both host reintroduced populations.

Collection of samples and storage

Rhinos defecate on common dung piles across all age and sex classes (Laurie et al. 1983). The dung piles of rhino were located across all the PAs employing multiple field survey teams comprising of four to six persons. Sampling in each protected area was carried out in a single session of 3 to 7 days using vehicle and elephants as mode of transport. About 10 to 15 g of fresh dung samples (not more than 24 h old) were collected in plastic vials containing DMSO EDTA Tris salt saturated (DETs) buffer (Frantzen et al. 1998). To avoid cross contamination in case multiple samples were collected from the same dung pile, samples were collected only from the top

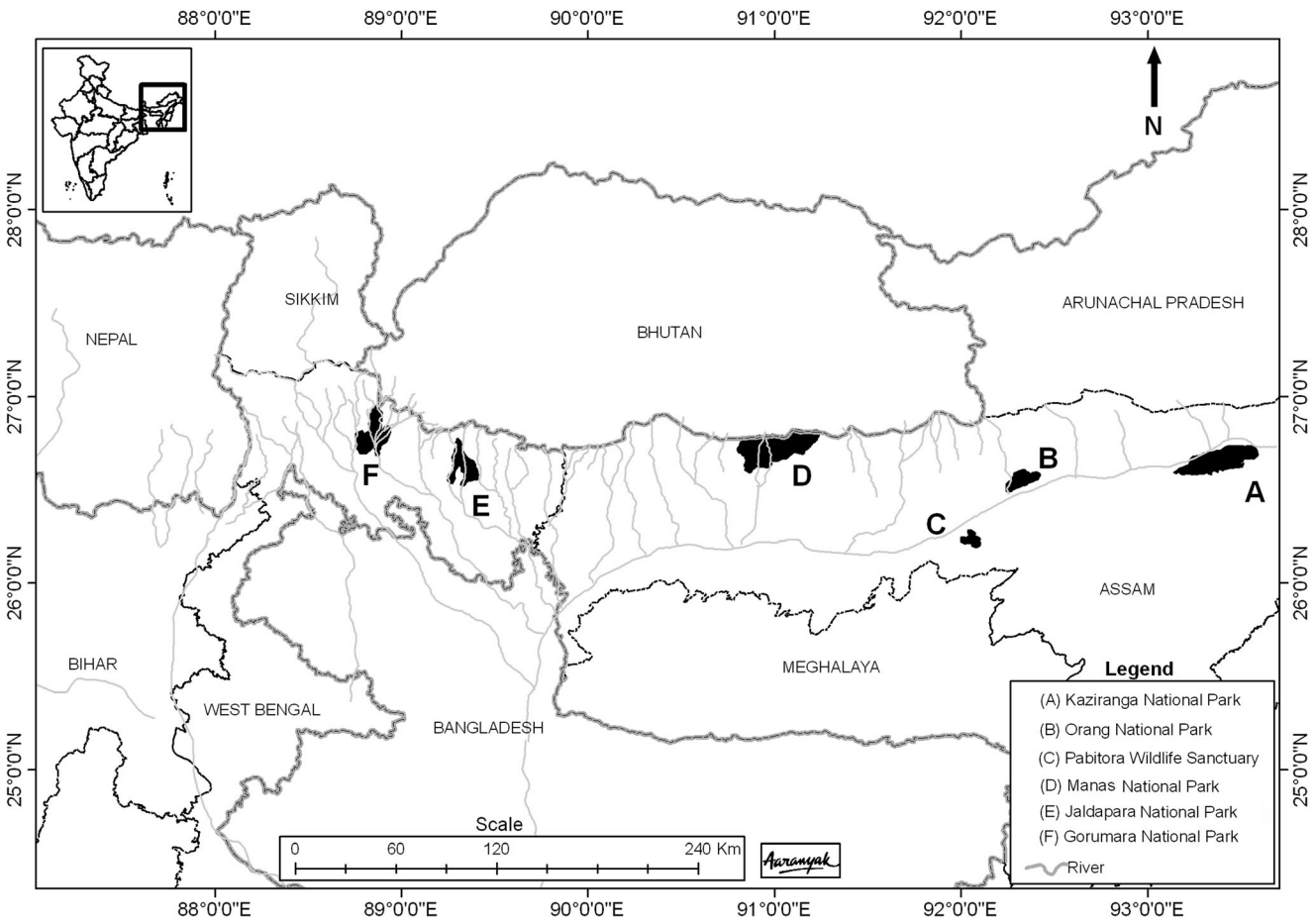

Fig. 1 Rhino-bearing protected areas (PAs) of Assam and West Bengal: **a** Kaziranga National Park (KNP), **b** Orang National Park (ONP), **c** Pabitora Wildlife Sanctuary (PWLS), **d** Manas National Park (MNP), **e** Jaldapara National Park (JNP) and **f** Gorumara National Park (GNP)

most bolus of two visibly different heaps of faeces. Collection of only fresh faecal samples within a short period of time reduces the probability of recapturing same individual; thus, maximize the number of different individuals genotyped. The Global Positioning System (GPS) coordinates readings were recorded for each of the sample location. Opportunistic sampling of tissue samples from carcasses were collected and stored in absolute ethanol. These samples were utilized for initial standardization of microsatellite markers used in the present study. All the samples were stores at −20 °C until DNA was extracted and further analyses conducted.

Extraction of DNA

DNA from dung samples were extracted following two methods: standard commercial kit protocol (QIAamp DNA Stool Kit, QIAGEN Ag., Germany) and the guanidine isothiocyanate-silica based protocol (Boom et al. 1990) with minor modifications as follows: (1) 500 μl of DETs buffer containing faecal sample was added to 1,000 μl of L6 lysis solution in a sterile 1.5-ml microcentrifuge tube (MCT) and incubated overnight at room temperature with intermittent vortexing followed by centrifugation at 8,000 rpm for 1 min; (2) the supernatant was mixed with 100 μl of 10 % polyvinyl polypyrrolidone (PVPP) solution by gentle inversion and the suspension was incubated at room temperature for 30 min and centrifuged at 12,000 rpm for 2 min; (3) the supernatant was mixed with 50 μl of 6 % silica solution in a new 1.5 μl MCT and incubated at room temperature for 30 min; the silica matrix was then pelleted through centrifugation at 12,000 rpm for 1 min; (4) silica pellet was washed twice with 500 μl of L2 solution and 500 μl of ethanol wash buffer followed by once with 500 μl of ice-cold 80 % ethanol (*v*/*v*) and 500 μl with ice-cold acetone; (5) washed pellet was then dried at 55 °C and DNA was eluted at 55 °C with 75 μl of TE buffer. For each extraction, a negative control was included consisting of reagent blank to check cross-contaminations. For reference tissue samples, DNA was extracted using commercially available DNeasy Blood and Tissue Kit (QIAGEN Ag., Germany) following standard kit protocol. All the dung DNA extractions were performed in an isolated facility at Wildlife Genetics

Laboratory (WGL), Aaranyak, dedicated for low quality DNA work.

Amplification of microsatellite markers

A total of nine polymorphic microsatellite markers originally developed from *R. unicornis* (Zschokke et al. 2003) were used in the study to generate the multilocus microsatellite genotypes. All the PCRs were performed in multiplex of 10-μl reaction volume; each marker being labelled with one of a set of four different fluorescent tags, viz., 6-FAM, VIC, NED and PET. The microsatellite markers were grouped into three panels, each consisting of three pairs of primers in multiplex reactions (Electronic supplementary material (ESM) Table S1). All the reactions were performed using QIAGEN Multiplex PCR Kit (QIAGEN, Germany) following standard kit protocol for reagent concentration with 0.25 μM of each primer and 2.5 μl of template DNA. For panel I and II, the thermal cycling was performed with 95 °C initial denaturation/ activation of 15 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min with a final extension step at 72 °C for 30 min. For panel III, the annealing temperature was set at 52 °C for 1 min, with rest of the programme remaining same. All the PCR works were performed at WGL of Aaranyak.

The quantity of DNA extracted from noninvasive samples is often low and microsatellite genotyping of such samples generates two types of errors, i.e., allelic dropout (ADO) and false allele (FA) (Taberlet et al. 1999, Fernando et al. 2003). To minimize potential errors, genotyping was carried out in replicates of three or more at each locus for all the samples depending on the genotype discrepancy (Taberlet et al. 1996) and consensus genotypes were created from the repeats. The use of the Multiplex PCR Kit (QIAGEN, Germany) allowed to minimize electropherogram stutter patterns and to prevent ADO. In addition, all the work was carried out in a facility dedicated for low copy number DNA analysis and aerosol-barrier tips were used to prevent sample cross contaminations.

Allele sizing and estimation of error rates

The PCR products were loaded in ABI 3130 Genetic Analyzer (Applied Biosystems, USA) utilizing commercial DNA sequencing facility. Allele sizes for each locus were scored through automated allele calling using the software GENEMAPPER v3.7 (Applied Biosystems, USA) combined with visual inspection of the raw data at WGL of Aaranyak. This combined approach gives the scope to mitigate potential scoring errors like stochastic amplifications within the size range, mistyping of allele due to stuttering, allelic dropout or null alleles (Pompanon et al. 2005, Dewoody et al. 2006). To select the final set of samples for individual identification, quality index value was assigned to each genotype following Miquel et al. (2006). Samples that revealed the same genotype in all three repetitions had a quality index of one, whereas, samples that yielded two same genotypes out of the three amplifications had a quality index of 0.66. For the purpose of selection of samples for final data analysis, quality index of 0.66 was kept as the cut-off value. Estimates of error rates and consensus genotypes were determined using software GIMLET v1.3.3 (Valiere 2002).

Individual identification

The power of the microsatellite markers used in the study to resolve between different individuals was quantified in terms of the probability of identity (P_{ID}) and probability of identity among siblings (P_{ID}-sibs) (Paetkau et al. 1995) which were estimated for 10 known individual rhinos from tissue DNA as well as faecal DNA for respective PAs using software GIMLET v1.3.3 (Valiere 2002).The unique multilocus genotypes, i.e., individual rhinos were identified using the Identity Module of the programme CERVUS (Marshall et al. 1998).

Genetic diversity

Allele frequency and allelic richness (Ar) were estimated using software F_{STAT} v2.9.3 (Goudet 1995). Allelic richness was measured in terms of the number of alleles independent of sample size, so that comparison amongst different sample sizes could be estimated. Observed (H_o) and expected (H_e) heterozygosity as well as inbreeding coefficient (F_{IS}) were calculatedusing software GENETIX v.4.05.2 (Belkhir et al. 1996–2004). Tests for linkage disequilibrium (LD) between all pairs of loci and exact test of Hardy–Weinberg equilibrium were performed with 100 000 steps in Markov–Chain and 10000 dememorization step using the software ARLEQUIN v3.5.1.2 (Excoffier et al. 2005). The p values were adjusted with Bonferroni correction for multiple comparisons (Rice 1989).

Population differentiation and genetic structuring

Pairwise F_{ST} among the five PAs and estimates of molecular variance (AMOVA) among and within PAs were performed using software ARLEQUIN (Excoffier et al. 2005). To investigate the pattern of genetic structure in the rhino population, two approaches were taken into consideration. First, a Bayesian clustering method as implemented in software STRUCTURE v2.3.1 (Pritchard et al. 2000) which does not require any prior information on population. Second, a Bayesian approach following Rannala and Mountain (1997) with 'leave one out' option (Cornuet et al. 1999) using software GeneClass v2.0.h. (Piry et al. 2004) which requires prior population information. Under admixure model with correlated allele

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

Results

A total of 292 dung samples (58 from PWLS, 84 from KNP, 54 from ONP, 60 from GNP and 36 from JNP) were collected and used for microsatellite genotyping. Based on the quality index criteria a total of 249 out of 292 samples were selected. ADO and FA were found to be in the range of ≤7 % (Table 1). The estimates of cumulative P_{ID} and P_{ID}-sibs were observed to be 2.66×10^{-7} and 4.17×10^{-3}, respectively, in case of the 10 reference tissue samples. The estimates of cumulative P_{ID} and P_{ID}-sibs for faecal samples were observed to be 7.406×10^{-6} and 6.050×10^{-3} in PWLS, 8.145×10^{-7} and 2.310×10^{-3} in KNP, 1.793×10^{-6} and 2.990×10^{-3} in ONP, 4.961X 10^{-4} and 2.907×10^{-2} in GNP and 7.148×10^{-5} and 1.439×10^{-2} in JNP, respectively. The individual identity analysis yielded 238 (45 from PWLS, 72 from KNP, 44 from ONP, 43 from GNP and 34 from JNP, respectively) unique multilocus genotypes which were used for further genetic analysis.

Genetic diversity

The number of alleles observed per locus varied in all PAs from 2 to 6 with an average of 4.6 (±1.33) alleles per locus across all the samples with exception of Locus Rh1 in GNP where only one allele was observed. Estimates of the allelic richness, observed and expected heterozygosity and inbreeding coefficient are represented in the Table 2. The allelic richness across all loci varied from 2.589±0.88 (GNP) to 3.635± 0.93 (ONP). The level of heterozygosity was observed to be highest in KNP while lowest in GNP. The mean expected heterozygosity (H_e) ranged from 0.352 (±0.2) to 0.59 (±0.13). Observed heterozygosity (H_o) was slightly higher ranging from 0.409 (±0.27) to 0.67 (±0.15) generating an overall excess of heterozygosity across all the PAs with mean F_{IS} ranging from −0.027 (ONP) to −0.158 (GNP). Although no significant LD was observed between pairs of loci, three loci (Rh11 in PWLS, KNP, ONP and GNP; Rh10 in KNP and

Table 1 Estimates of number of allele (NA), allele dropout (ADO), false allele (FA) and percentage of PCR success across loci in rhino-bearing PAs of India

Loci	PWLS				KNP				ONP				GNP				JNP			
	NA	ADO	FA	% PCR success	NA	ADO	FA	% PCR success	NA	ADO	FA	% PCR success	NA	ADO	FA	% PCR success	NA	ADO	FA	% PCR success
Rh1	3	0.043	0.042	77	3	0.038	0.029	84	3	0.022	0.024	81	1	0.000	0.000	85	3	0.083	0.045	91
Rh3	3	0.024	0.038	99	4	0.039	0.014	82	3	0.042	0.031	90	4	0.025	0.050	78	3	0.000	0.000	81
Rh4	5	0.022	0.042	86	6	0.031	0.019	80	5	0.025	0.021	81	2	0.000	0.000	90	4	0.063	0.038	91
Rh5	3	0.028	0.034	95	4	0.022	0.034	82	5	0.038	0.000	66	3	0.045	0.000	76	3	0.053	0.000	91
Rh6	2	0.045	0.028	89	2	0.044	0.038	91	2	0.033	0.034	95	2	0.000	0.000	87	2	0.000	0.000	87
Rh7	3	0.043	0.042	76	2	0.017	0.022	79	4	0.042	0.031	69	3	0.037	0.000	77	2	0.000	0.019	90
Rh9	3	0.063	0.000	89	3	0.043	0.029	79	3	0.050	0.029	72	2	0.000	0.000	81	2	0.029	0.029	90
Rh10	5	0.045	0.020	98	5	0.043	0.019	80	4	0.025	0.000	82	3	0.036	0.069	76	6	0.045	0.043	65
Rh11	5	0.036	0.026	77	5	0.033	0.017	78	5	0.019	0.000	64	4	0.036	0.045	72	3	0.029	0.029	91

Table 2 Comparison of allelic richness (Ar), observed (H_o) and expected (H_e) heterozygosity, estimates of inbreeding coefficient (F_{IS}) and deviation from HWE in Indian PAs hosting *Rhinoceros unicornis*

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

Ar is based on minimum of 27 diploid individuals

[a] Significantly deviated from Hardy–Weinberg equilibrium after bonferroni correction

Rh5 in ONP) significantly deviated from Hardy–Weinberg equilibrium after Bonferroni corrections (Table 2).

Population differentiation and genetic structuring

Estimates of pairwise F_{ST} were observed to be significantly high ($p<0.001$) between PAs of West Bengal, viz., GNP and JNP to PAs of Assam, i.e., PWLS, KNP and ONP (Table 3). The highest estimate of pairwise F_{ST} was observed between GNP and PWLS (F_{ST}, 0.382; $p<0.001$). Although, GNP and JNP are at the closest proximity, pairwise F_{ST} between the two PAs was 0.312 ($p<0.001$). Within the PAs of Assam, the pairwise F_{ST} were observed to be relatively low ($F_{ST}\leq$ 0.062; $p<0.001$, between all pairs). The AMOVA results showed 16.9 % variation amongst the populations in comparison to 83.1 % variations within populations.

STUCTURE analysis identified two modes (Fig. 2), one at $K=2$ ($\Delta K=1232.32$) and the other at $K=5$ ($\Delta K=58.28$). At $K=2$, the observed average proportion membership (q) for each cluster were 0.9706 and 0.9707. At this mode samples from PWLS, KNP, ONP and JNP were assigned to one cluster while the samples from GNP were assigned to a separate cluster (Fig. 3a). At $K=5$ a greater level of admixture among individuals from different clusters was observed. The samples from GNP clustered together with average membership proportion $q=0.911$. Samples from JNP, although clustering together with the samples from PWLS, KNP and ONP at $K=2$, formed a separate cluster at $K=5$ with average membership proportion $q=0.826$. The rest of the clusters showed admixture with average membership proportions of $q=0.673$, $q=0.668$ and $q=0.675$, respectively (Fig. 3b). Keeping this in view, another set of simulation was run with STRUCTURE to find any fine scale sub-structuring among the samples assigned to the cluster 1, at $K=2$, i.e., PWLS, KNP, ONP and JNP. The ΔK value was found to be highest at $K=4$ (ESM Fig. S2). The JNP samples were grouped into a unique cluster with average membership proportion of $q=0.786$ together with seven samples from KNP and three samples from ONP (Fig. 4). Rest of the three clusters showed admixure with average membership proportion $q=0.621$, $q=0.661$ and $q=0.640$, respectively. Assignment test (Rannala and Mountain) also produced similar results to that of STRUCTURE, showing considerable amount of admixure among samples from PAs of Assam. Only 25.5, 20 and 9 % of the samples from these PAs were correctly assigned to its respective sampling location, i.e., PWLS, KNP and ONP, respectively. Rest of the samples were assigned to more than one population. However, 97 % of the samples from GNP and 70 % of samples from JNP were correctly assigned to their respective sampling location. Although JNP depicted some degree of admixure with the PAs of Assam, specific signature of genetic differentiation was observed. Therefore, to confirm this, pairwise F_{ST} values among JNP, all the PAs Assam combined as one population and GNP were estimated. It was observed that JNP significantly differes from Assam and GNP with pairwise F_{ST} values of 0.104 and 0.312 ($p<0.001$), respectively (ESM Table S2). The pairwise F_{ST} between GNP and Assam was also observed to be high ($F_{ST}=0.255$, $p<0.001$).

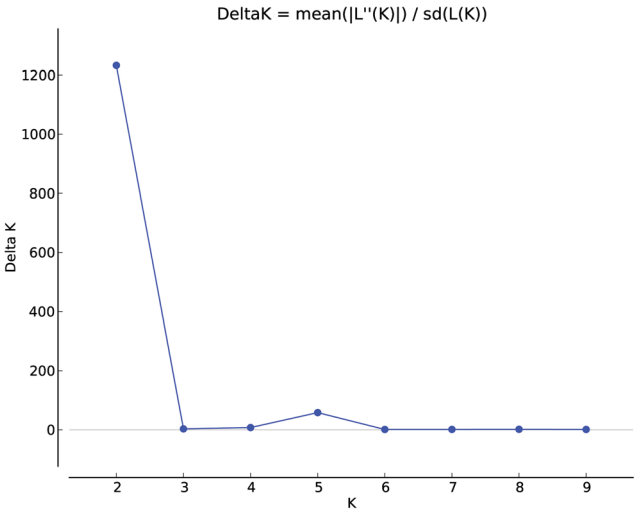

Fig. 2 ΔK (adhoc) values corresponding to K from STRUCTURE simulations under admixture model with correlated allele frequency for rhino samples from all the PAs. Two modes: one at $K=2$ and other at $K=5$ were observed

Table 3 Estimates of pairwise F_{ST} between rhino-bearing PAs of India

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

***$p<0.001$)

Discussion

Genetic diversity

Genetic diversity is indispensable for evolutionary adaptation which is key to the long-term survival of any species (Schemske et al. 1994). It is often associated to fitness of a population, long-term adaptability and ability of a population to retort novel challenges (Lacy 1997, Amos and Balmford 2001).

The current study reveals that the wild rhinos restricted to the different PAs of India have moderate to high levels of heterozygosity (H_e, 0.352 to 0.59). Similar observations were also made by Dinerstein and McCracken (1990) and

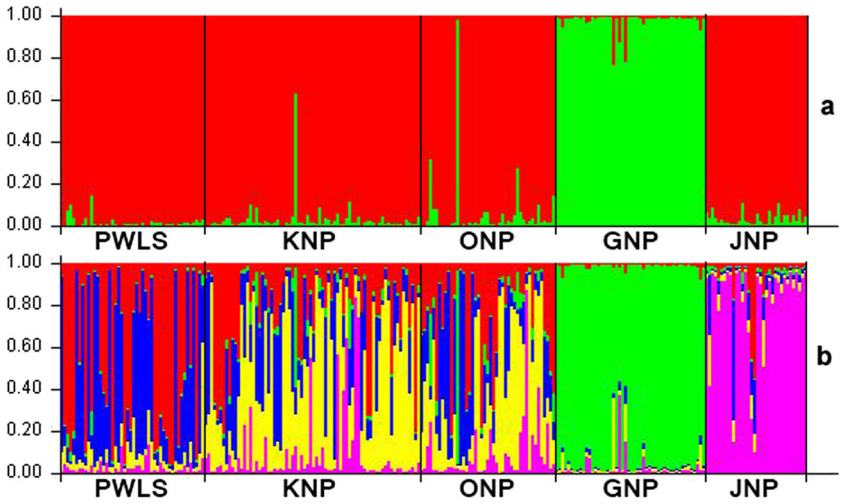

Fig. 3 *Bar diagram* showing clusters inferred by STRUCTURE simulations under admixture model with correlated allele frequencies (**a**) at K=2 and (**b**) at K=5 for rhino samples from all the PAs

Zschokke et al. (2011). Based on allozyme allele diversity, Dinerstein and McCracken (1990) reported higher levels of heterozygosity in rhino population of Royal Chitwan National Park, Nepal. However, heterozygosity in our present work and that obtained through allozyme analysis are not directly comparable. Zschokke et al. (2011) also reported higher levels of heterozygosity (H_e, 0.60±0.20) in captive Assam population in comparison to Nepal (H_e, 0.45±0.30).

The PAs of Assam, i.e., PWLS, KNP and ONP, were observed to retain higher levels of heterozygosity (H_e, 0.502, 0.590 and 0.571, respectively) in comparison to the PAs of West Bengal, viz., GNP and JNP (H_e, 0.352 and 0.428, respectively). The present rhino-bearing areas of Assam are connected through a chain of river islands of Brahmaputra. Migration of rhinos through such islands from one PA to the other (Talukdar et al. 2007) and their subsequent contribution to the gene pool could have contributed to the retained heterozygosity in these PAs. Moreover, the long generation time (average generation time being 15 years; Wirz-Hlavacek et al. 1998) and lifespan of the rhino could have allowed populations to retain the observed levels of diversity. These factors might have contributed together in retaining higher levels of heterozygosity in rhino population of Assam. Although, the number of individual rhinos in GNP and JNP has increased in last few decades, they have a rather fluctuating trend of population size in the past century and have been under constant biotic as well as anthropogenic stress (Bist 1994). Zschokke et al. (2011) were of the view that the higher levels of heterozygosity observed in the Assam population were not due to the recent expansion of the rhino population. According to them the actual population size of rhino in early 1900s was larger than what were reported and individuals from neighbouring areas might have also immigrated into the region in subsequent time.

Population differentiation and structuring in rhinoceros populations of India

The F_{ST} and the AMOVA results suggest that there is considerable amount of genetic differentiation amongst rhino-bearing PAs of India. The pairwise F_{ST} estimates between GNP and all other PAs were observed to be significantly high (F_{ST}>0.25; p<0.001). It was interesting to observe that although GNP and JNP are in close geographical proximity, the pairwise F_{ST} was significantly high (F_{ST}, 0.31; p<0.001) between the two PAs. STRUCTURE and other Bayesian based assignment tests assigned samples from GNP to a separate cluster which is also suggestive of strong genetic differentiation between GNP and rest of the rhino-bearing PAs. Grouping of JNP with PAs of Assam, i.e., PWLS, KNP and ONP in the first STRUCTURE simulation (at K=2) might be due to the

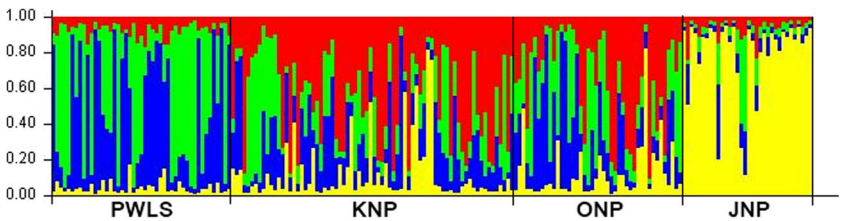

Fig. 4 Bar diagram showing clusters inferred by STRUCTURE simulations under admixture model with correlated allele frequencies at K=4 for rhino samples from PWLS, KNP, ONP and JNP

fact that it was connected to the later through Sankosh–Rydak–Manas landscape. Rhinos from Sankosh–Rydak region became locally extinct during late 1960s, but there are isolated reports of individuals straying from JNP to this region (Bist 1994). Such reports suggest possible movement of individual rhinoceros from JNP via Sankosh–Rydak region to Manas. Therefore, a potential genetic exchange between the Assam and JNP cannot be rejected till the rhinos from Manas were locally extinct due to intense poaching during the civil unrest in the region in late 1990s. This hypothesis could not be ascertained due to unavailability of samples representing the true Manas population. STRUCTURE simulation excluding GNP samples and the pairwise F_{ST} value observed between JNP and combined Assam population (F_{ST}=0.10; p<0.001) suggest JNP also hold a unique genetic signature. These results clearly indicate that both the PAs of West Bengal, i.e., GNP and JNP are genetically differentiated from Assam as a whole. The observed results are important as testify the effect of genetic drift in rhino populations of India that have been restricted to isolated patches only in recent times primarily due to anthropogenic factors. Similar magnitude of population structure within a short time scale was also observed in case of other species (Mondol et al. 2013) in India.

Zschokke et al. (2011) observed a strong genetic differentiation between Assam and Nepal rhino populations based on a study involving microsatellite as well as mitochondrial markers. They reported a high pairwise F_{ST} estimate (F_{ST}= 0.202; p<0.001) between Assam and Nepal populations along with population specific mitochondrial alleles with no overlap between them, but failed to detect any putative hybrids. However, Zschokke et al. (2011) did not include the populations of West Bengal in their study. Therefore, the positioning of the PAs of West Bengal, with respect to metapopulation genetic structure of rhino in the region will be important and necessitates further study examining genetic relationship between Nepal and West Bengal populations. Given the present level of habitat fragmentation and continuous anthropogenic stress imposed on the rhinos in India, it is evident that the remnants of the populations will remain in isolation. Prolonged genetic and demographic separation between the rhino-bearing PAs is unwanted which could lead to further loss of genetic diversity through inbreeding and genetic drift. Immigration and gene flow within the metapopulation will, therefore, crucial in maintaining the genetic variability, thus, long-term viability of the species.

Zschokke et al. (2011) recommended treating rhino populations of Nepal and Assam as separate management units owing to the possible negative effects of outbreeding (Zschokke and Baur 2002) given the level of differentiation observed. On the contrary, in case of Sumatran rhino (*Dicerorhinus sumatrensis*), Goossens et al. (2013) recommended breeding programme for mixing of populations considered as different subspecies, in order to avoid deleterious effects of inbreeding. Pluháček et al. (2007) diverged from the observations of Zschokke and Baur (2002) in terms of possible negative effect of outbreeding in captive Greater One-horned Rhinos. Considering the relatively low genetic diversity in GNP and JNP rhinos and their apparent loss of natural connectivity with other source populations in recent time, we recommend treating Assam and West Bengal populations as one single management unit. We recommend undertaking restocking of West Bengal populations by translocating individuals from Assam, in addition to the current efforts to reintroduce rhinos within Assam. We further recommend that the government should initiate inter-change of rhinos between JNP and GNP besides exploring the possibilities of translocating individuals from Assam.

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