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Genetic differences between the two remaining wild populations of the endangered Indian rhinoceros (*Rhinoceros unicornis*)

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ABSTRACT

The management of rare and endangered species in the wild and in captivity requires an understanding of the characterization of the genetic units within each species and their relationships to each other. The Indian rhinoceros (*Rhinoceros unicornis*) is an endangered species with a current population size of c. 2800 individuals. We analyzed 26 individuals of known origin kept in captivity and 21 wild ranging individuals of the two remnant large wild populations in Assam (India) and Nepal employing mitochondrial and microsatellite markers to determine whether the two geographically isolated populations show distinct patterns of genetic diversity, and whether the genetic diversity of the populations is influenced by past demographic bottlenecks. We identified 10 different mitochondrial D-loop haplotypes, of which 4 were specific to the Assam population (10 sequences examined) and 6 specific to the Nepal population (19 sequences). Similarly, the microsatellite analysis demonstrated a strong genetic differentiation between the Assam and Nepal populations and allowed to assign each individual to its origin with high confidence. Furthermore, our analyses revealed the occurrence of a bottleneck in the Assam population long before the reported bottleneck in 1908, and it revealed that the Nepal population is a recent (probably post-glacial) colonization. In summary, the extent of genetic divergence between the two remnant *R. unicornis* populations suggests separate conservation programs (even for captive individuals) as long as the persistence of the entire species is not severely threatened. The microsatellite markers can also be used to determine the origin of confiscated material such as horns.

1. Introduction

The fourth largest extant terrestrial mammal, the Indian (or greater one-horned) rhinoceros (*Rhinoceros unicornis*), is threatened according to the World Conservation Union (IUCN) red list (Talukdar et al., 2008). Recent estimates revealed a total number of c. 2800 rhinoceros living in isolated populations in India and Nepal (Talukdar, 2009; van Strien and Talukdar, 2007). In historic times (c. 1400 AD), the Indian rhinoceros occurred along the flood plains from north-western Myanmar across the Gangetic plain to the Indus River Valley in northern Pakistan with a minimal total population of more than 450,000 individuals (Blanford, 1891; Dinerstein and McCracken, 1990; Laurie, 1979). Since the 19th century, land alterations significantly reduced and fragmented the habitat suitable for the rhinoceros. Hunting and later poaching further reduced the populations. Today, natural populations of the Indian rhinoceros only occur in the states of Uttar Pradesh, West Bengal and Assam in Eastern India and the Terai of Nepal (Foose and van Strien, 1997).

The population in Assam was estimated to contain less than 20 individuals in the area of today's Kaziranga National Park when hunting was banned in 1908 (Laurie et al., 1983; Ryhiner, 1961; Ullrich, 1972). This population recovered to c. 2300 individuals in 2009 and has expanded into neighboring areas, including the Laokhowa, Pabitora and Orang wildlife sanctuaries (Merenlender et al., 1989; Molur et al., 1995; Ryhiner, 1961; van Strien and Talukdar, 2007). In the Chitwan valley in Nepal, a population of more than 1000 individuals persisted until 1950 when poaching and land alterations began to reduce the number of rhinoceros to 60–80 individuals in 1962 (Laurie, 1979). This population also recovered from its bottleneck, attaining an estimated size of 544 individuals in 2000, with a subsequent decrease to 372 individuals due to renewed poaching (Poudyal et al., 2009) and a slow increase to 408 individuals in 2008 (Talukdar, 2009). A distance of 700 km separates the two main populations in Assam and Nepal and no individuals were translocated between the two populations. Further 180 individuals live in other areas in India and Nepal

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(Talukdar et al., 2008). The captive population comprises 188 Indian rhinoceros kept in 69 zoological institutions in North America, Europe, Asia and Australia in 2010 (von Houwald et al., 2011).

Continued habitat loss and fragmentation result in small, isolated populations which exhibit a greater sensitivity to demographic stochasticity, may have reduced population mean fitness and suffer increased extinction rates because of increased expression of inbreeding depression, decreased levels of genetic diversity and higher probabilities of fixing deleterious mutations relative to pre-fragmentation population structure (Dudash and Fenster, 2000; Frankham, 2010). Genetic diversity in small populations is expected to diminish because of genetic drift and inbreeding. Understanding the extent of phylogenetic distinction between remnant populations is essential to carry out effective conservation programs (Olney et al., 1994).

In the present study, we investigated the genetic variation of the Indian rhinoceros within and between the two main remnant populations in Assam (Kaziranga National Park) and Nepal (Chitwan National Park) employing mitochondrial D-loop analysis and eight microsatellite markers. Since the sampling is difficult for such large animals, our analyses were mainly conducted on samples from wild-born individuals in the captive populations, which we considered as a random sampling of the wild populations. Some mitochondrial analyses were performed on individuals born in captivity with known maternal lineage. We hypothesize that the two geographically isolated and remnant populations of *R. unicornis* show distinct patterns of genetic variability. Protein electrophoresis in 23 individuals from the Nepal population revealed an unexpectedly high heterozygosity in this population (Dinerstein and McCracken, 1990; McCracken and Brennan, 1993), whereas no allozyme variation was found among 3 individuals from the Assam population (Merenlender et al., 1989). However, the genetic variability between and within the two isolated populations has not yet been compared using regions not under selection. We also examined whether the genetic diversity of the two populations was influenced by past demographic fluctuations (bottlenecks). Answers to these questions are essential for improving the future management of this endangered species.

2. Materials and methods

2.1. Sample collection

Between 1995 and 2004, we collected samples of blood, bone, tissue, saliva and hair from 63 individuals (62 kept in captivity and one from the wild). Fifty individuals were from the Assam population (all kept in captivity, but 12 of them were wild caught), 9 were from the Nepal population (8 wild caught and kept in captivity and one from the wild in the pre-bottleneck period) and four individuals were Assam × Nepal hybrids (all born by dams caught in Nepal and kept in captivity, only used for mtDNA analyses). In addition, DNA samples collected in 1986–1987 from 23 wild ranging individuals living in Chitwan NP (Nepal) were kindly provided by E. Dinerstein and G. McCracken (see Dinerstein and McCracken, 1990). Of these samples totalling 86 *R. unicornis* individuals, 47 samples of unrelated individuals were used in the final analyses (Table 1).

Blood samples from zoos were anticoagulated with EDTA immediately after collection and stored at −80 °C. Since obtaining blood samples usually requires anesthetizing the individuals, several zoos sent us skin scrapings, which are routinely removed from the sole during foot care of Indian rhinoceros. All samples were imported to Switzerland with the necessary CITES export and import permits.

Our *R. unicornis* samples represented 42 maternal lines: 12 from Assam (10 were used in the mtDNA analysis), 29 from Nepal (18 used in the mtDNA analysis), and one animal from the pre-bottleneck period (1924) in Nepal (Table 1).

As outgroup taxa for the mtDNA analysis, we used the complete mitochondrial genomes of the African White rhinoceros (*Ceratotherium simum*; Acc. No. Y07726), the African Black rhinoceros (*Diceros bicornis*; Acc. No. L22010) and the Javan rhinoceros (*Rhinoceros sondaicus*; Acc. No. FJ905815) analyzed by Willerslev et al. (2009), and that of the Garrano horse (*Equus caballus*; haplotype gr6, GenBank Acc. No. AY246231).

2.2. DNA extraction

DNA was extracted following the spin column protocol of QIAGEN (Hombrechtikon; Switzerland) and DNA was isolated from 30 mg dried skin or horn material, or from 100 mg drilled bone powder, or from tissue of 30 hair roots. Tissue digestion of all samples was conducted over night in ATL buffer, with proteinase K digestion at 55 °C. DNA was eluted from the spin columns with 60–80 μl AE buffer (QIAGEN).

2.3. Mitochondrial D-loop sequence analysis

For 29 individuals of *R. unicornis* (only one per maternal lineage), a fragment of the mitochondrial D-loop (c. 428 bp) was amplified with following primers: RhinoForward: 5′-CGTGCATTAAATTGTTTGCC-3′ and RhinoReverse: 5′-ATACCAAATGCATGACACC-3′. PCR was performed in 25 μl volume using PuReTaq™ Ready-To-Go™ PCR Beads (GE HEALTHCARE, Zürich; Switzerland) with 5–50 ng of DNA, 25 pmol of each primer and a final concentration of 1.5 mM of $MgCl_2$. Thermocycling condition was 95 °C (1 min), primer annealing 52 °C (1 min) and polymerase extension 72 °C (1.5 min), repeated in 35 PCR cycles.

PCR products were checked for appropriate size with agarose gel-electrophoresis and amplicons were cleaned and directly sequenced with an automated ABI sequencer (ECOGENICS GmbH, Schlieren; Switzerland). Forward and reverse strands were read from each sample. Sequences are deposited in GenBank (Acc. No. JF825390–JF825418). They were aligned with ClustalX version 2.0 (Larkin et al., 2007) and the alignment (428 bp) was checked in the BioEdit editor (Hall, 2005). Identical haplotypes were detected using Collapse version 1.2 (Posada, 2006). Nucleotide characteristics, nucleotide diversity (P_i), gene flow (G_{ST}; Nei, 1973), and population differentiation (Snn; Hudson, 2000) between Assam and Nepal populations were estimated using DnaSP version 5.00.06 (Librado and Rozas, 2009; Rozas et al., 2003) and significant levels were investigated with permutation tests (1000 replicates), with the nucleotide sequence format set to "mitochondrial".

In the phylogenetic analyses, the sequence set of 34 samples (including outgroup taxa) was aligned with ClustalX and checked with BioEdit. Final length of the alignment was 450 bp due to indels and deletions. Maximum parsimony (MP) analyses were performed (heuristic searches with random stepwise addition for 1000 replicates and TBR branch swapping options) with all indels considered as a new state (5th base) using Paup* version 4.10b (Swofford, 2002). Comparable results were obtained when indels were considered as missing data (data not shown). The combined data set was also analyzed using the maximum likelihood (ML) and neighbor-joining (NJ) methods. Maximum likelihood analyses were conducted with PhyML version 2.4.4 (Guindon and Gascuel, 2003) and NJ with Paup*. For the ML and NJ analyses, a total of 56 substitution models were evaluated using Modeltest version 3.7 (Posada and Crandall, 1998) and Paup*. The best model selected by the Akaike Information Criterion (AIC) was HKY + G (freq $A = 0.3015$; freq $C = 0.2753$; freq $G = 0.1439$; freq $T = 0.2794$;

Table 1
List of individuals used for the genetic analyses. The first letter of the ID denotes geographical origin of the individual or of its wild born ancestors (A = Assam, H = 'hybrid' Assam × Nepal, N = Nepal), the remainder of the ID identifies the individual. Individuals with an **S** as the second letter of their ID are or were kept in a zoo. In these individuals, the remainder of the ID denotes the studbook number in the International Studbook (von Houwald et al., 2010). Individuals with **D** as the second character are those sampled by Eric Dinerstein during his study on the genetic diversity of free ranging *R. unicornis* in Nepal (Dinerstein and McCracken, 1990; Dinerstein, 2003). Haplotype: an entry in this column indicates the mtDNA haplotype. The source indicates the zoo (or if preceded by an M) the Museum which provided us with the sample; Rhino TAG = Rhinoceros taxon advisory group of the species survival program.

ID	Name	Sex	Origin	Haplotype	Sample type	Source
AS005	Gadadhar	M	Assam		Bone	M Bern
AS007	Joymothi	F	Assam		Bone	Basel
AS018	Arjun	M	Assam		Bone	M Karlsruhe
AS029	Jaypuri	F	Assam		Blood	Gulf Breeze
AS035	Herman	M	Assam	H2	Blood	Los Angeles
AS051	Roopa	F	Assam		Skin	Whipsnade
AS066	Pinky	F	Assam	H3	Blood	NY Bronx
AS067	Radha	F	Assam	H3	Liver	Rhino TAG
AS069	Patrick	M	Assam	H1	Saliva & hair	Toronto
AS079	Indira	F	Assam	H4	Dried blood	Toronto
AS093	Numa	F	Assam	H3	Tissue	Dvur Kralove
AS097	Gomati	F	Assam	H4	Hair	Singapore
AS106	Rabha	M	Assam	H4	Skin	Fort Worth
AS145	Ropen	M	Assam	H4	Blood	Dvur Kralove
AS197	Stillborn	M	Assam	H4	Liver	Rhino TAG
HS238	Chitwan	F	Hybrid	H7	Blood	Cumberland OH
HS239	Himal	M	Hybrid	H7	Blood	Cumberland OH
HS324	Stillborn	F	Hybrid	H7	DNA	Miami
NS049	Mohan	M	Nepal	H7	Blood	NZP-Washington
NS050	Shanti	F	Nepal		Blood	Miami
NS190	Arun	M	Nepal	H7	Blood	SDWAP
NS191	Arati	F	Nepal	H10	Blood	Fort Worth
NS193	Rapti	F	Nepal	H5	Blood	Munich
NS204	Sani	F	Nepal	H9	Horn	Stuttgart
NS256	Beluki	F	Nepal	H7	Skin	Whipsnade
NS257	Behan	F	Nepal	H7	Skin	Whipsnade
ND01	Kame	M	Nepal		DNA	E. Dinerstein
ND02	Conan	M	Nepal		DNA	E. Dinerstein
ND03	Kankantuna	M	Nepal	H7	DNA	E. Dinerstein
ND04	Bange		Nepal		DNA	E. Dinerstein
ND05	Kalilo	F	Nepal		DNA	E. Dinerstein
ND06	Haune	F	Nepal	H8	DNA	E. Dinerstein
ND07	Yadav	M	Nepal		DNA	E. Dinerstein
ND08	Hero		Nepal	H7	DNA	E. Dinerstein
ND09	Tikoli calf		Nepal		DNA	E. Dinerstein
ND10	Kothar		Nepal		DNA	E. Dinerstein
ND11	Mudflap	F	Nepal		DNA	E. Dinerstein
ND13	Thuliputhi	F	Nepal	H7	DNA	E. Dinerstein
ND15	Yuali colt	M	Nepal	H6	DNA	E. Dinerstein
ND16	Puchar		Nepal		DNA	E. Dinerstein
ND17	Jawanik	M	Nepal		DNA	E. Dinerstein
ND18	son of Abire	M	Nepal		DNA	E. Dinerstein
ND19	son of Gumour	M	Nepal	H7	DNA	E. Dinerstein
ND20	Gumour	F	Nepal		DNA	E. Dinerstein
ND22	son of Laxmi	M	Nepal	H7	DNA	E. Dinerstein
ND23	Bhayaadhila	F	Nepal	H7	DNA	E. Dinerstein
N1924	#1953.50.1		Nepal	H10	Bone	M Edinburgh

Gamma distribution shape parameter = 0.1899) and this model was consequently used for the NJ and ML analyses. The robustness of the branching pattern of the trees was tested by 1000 bootstrap replicates for MP, ML and NJ analyses. In addition, Bayesian analyses were carried out using the Monte Carlo Markov Chain (MCMC) method implemented in MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Four chains were run for 1,000,000 iterations (sampling every 50th generations) and the first 10% were discarded as burn in.

While NJ, MP and ML show difficulties in resolving the relationships among closely related haplotypes, statistical parsimony allows a display of genealogical relationships among sequences with a limited number of mutations. The results of statistical parsimony among haplotypes were represented graphically using a network calculated with TCS version 1.21 software (Clement et al., 2000).

We evaluated the possible evidence for historic population expansion from a low-diversity population (Rogers and Harpending, 1992). Pairwise mismatch distributions for Assam and Nepal groups separately and for all samples of *R. unicornis* were plotted and tested for goodness-of-fit distribution using parametric bootstrapping (1000 replicates) with Arlequin version 3.0 (Excoffier et al., 2005). Moreover, Fu's F_s test (Fu, 1997) was calculated to detect any potential excess of rare alleles, which would indicate a recent population expansion (significance levels were evaluated using 1000 simulations).

2.4. Microsatellite analysis

Fingerprinting of the eight microsatellite loci (Rh1, Rh3, Rh4, Rh5, Rh6, Rh9, Rh10, and Rh11; Zschokke et al., 2003; Table A1) followed the PCR protocol and Elchrom® electrophoretic techniques described in Zschokke et al. (2003). Microsatellite results were checked for null alleles and mis-scoring using Micro-Checker version 2.2.3 (Van Oosterhout et al., 2004) for each population separately. We estimated allele frequency, allelic richness (Ar;

corresponds to the number of alleles independent of sample size), observed and expected heterozygosity (H_O, H_E) using FSTAT version 2.9.3 (Goudet, 1995). Differences in genetic diversity and covariance components between Assam and Nepal populations were examined using ANOVA (conducted with SPSS 18.0; SPSS Inc., Chicago, US) and AMOVA (using ARLEQUIN), respectively. Heterozygote deficiencies within populations (F_{IS}) was estimated and divergence from Hardy–Weinberg (HW) equilibrium was tested using FSTAT with 1000 permutations. In addition, genetic differentiation (F_{ST}) was calculated between populations using FSTAT. Because the use of F_{ST} has been criticized recently due to its relationship to the within population diversity, we have additionally calculated G''_{ST}, the corrected G'_{ST} when the number of populations is limited, as well as θ, which is based on the effective number of alleles, using SMOGD version 1.2.5 (Crawford, 2010; see Meirmans and Hedrick, 2011 for a general discussion on the differences between the methods).

The potential occurrence of bottlenecks was examined using two different approaches. First, the software BOTTLENECK version 1.2.02 (Piry et al., 1999) was applied to test for heterozygosity excess, which can be interpreted as a signature of a bottleneck occurring in a temporal range of 2–4 N_e generations (Piry et al., 1999). This approach is based on a faster allelic diversity reduction compared to the heterozygosity decrease, and a past bottleneck can consequently be detected when the observed heterozygosity is larger that the heterozygosity expected based on the observed allele number (Piry et al., 1999). We used a two-phase model (TPM; di Rienzo et al., 1994) with parameters set to 95% of single-step mutation (SSM; Ohta and Kimura, 1973) and a variance among multiple steps equal to 12, as suggested by Piry et al. (1999). To assess the robustness of these tests, additional simulations with different parameter values (80–99% of SSM and a variance of 2–24 among multiple steps) were performed. In addition, the Bayesian modeling approach implemented in MSVAR version 0.4.1b (Beaumont, 1999) was used to detect declines and/or expansions using multi-locus microsatellite genotypes. This program performs coalescent simulations using a Markov Chain Monte Carlo (MCMC) algorithm to estimate posterior distributions of demographic parameters, such as the rate of population change $r = N_0/N_1$ (N_0 = current effective number of gene copies; N_1 = number of gene copies at the time of population decline or expansion), the time (expressed in generations) when the population started to decline or expand ($t_f = t_a/N_0$; t_a = number of generations since the beginning of the decline or expansion and the genetic parameter $\theta = 2N_0\mu$, where μ = mutation rate of the locus). A result of $\log_{10}(r)$ smaller than 1 indicates a declining population. To check for stability of the simulations, we ran six independent computations for each population using different parameter configuration and starting values. Each MCMC run lasted 2×10^8 iterations, with 20,000 samplings completed every 10,000 steps and the first 10% of the samples for each run were discarded as burn-in.

A very recent bottleneck is expected to lead to a strong reduction of the present effective population size (Piry et al., 1999). Thus, another indirect approach to detect a recent severe bottleneck is to evaluate the present effective population size based on genetic data (Frankham et al., 2002). In our study, the effective population size (N_e) could not be assessed with methods based on temporal changes in allele frequency. We therefore used three different methods developed for a single temporal sample. The first method we used is based on gametic disequilibrium (Hill, 1981), including the bias correction developed by Waples (2006) as implemented in the program LDNE version 1.31 (Waples and Do, 2008). This method assumes selective neutrality of unlinked markers and an isolation of the populations. It has been shown that this method produces reliable results in populations with a skewed sex ratio or non-random variance in reproductive success (Waples, 2006). Confidence intervals (95% CI) were calculated using a jackknife approach, assuming random mating and a level of excluded alleles $\leqslant 0.05$ (as suggested by Waples and Do, 2008). The second method infers effective population size from one- and two-locus genetic data (Vitalis and Couvet, 2001b) as implemented in ESTIM version 1.2 (Vitalis and Couvet, 2001a). This method has been shown to perform well in estimating N_e for a variety of sampling conditions, especially with a limited number of loci in small populations (Vitalis and Couvet, 2001a,b). The third method estimates N_e based on summary statistics and Bayesian computations as implemented in ONeSAMP version 1.2 (Tallmon et al., 2004). This approach assumes unlinked and neutral loci, and a closed population. Values of 3 and 1000 were used as lower and upper bounds on the priors for N_e (Tallmon et al., 2004). Similar results were obtained with simulations with other priors (data not shown).

Individual genetic differentiations were evaluated using Cavalli-Sforza and Edwards Dc evaluation (Cavalli-Sforza and Edwards, 1967) and graphical representation was conducted with POPULATIONS (Langella, 1999). To determine whether the two populations of *R. unicornis* differ sufficiently to allow assigning an individual of unknown origin to the proper population, we also performed a population assignment for all *R. unicornis* individuals following the approach by Waser and Strobeck (1998) with ARLEQUIN version 3.0 (Excoffier et al., 2005).

3. Results

3.1. Mitochondrial DNA

Sixteen variable nucleotide positions were found in the 29 sequences of *R. unicornis* (plus two indel positions), i.e. in the 428 bp long alignments approximately 4.2% of the nucleotide positions showed variation. Ten different haplotypes were found (including those with indels). The overall nucleotide diversity P_i was 0.069 per site. In the Assam population ($n = 10$ sequences; Table 1), 4 haplotypes were detected and the average number of nucleotide differences was 3.87. In the Nepal population ($n = 19$), 6 haplotypes were detected and the average number of nucleotide differences was 1.20. All haplotypes consisted of individuals from a single population. Maternal lineages from the Assam population had a higher nucleotide diversity ($P_i = 0.0091$) than those from the Nepal population ($P_i = 0.0028$). The genetic differentiation in the population structure was pronounced, as indicated by the G_{ST} and Snn values of 0.295 and 1.000, respectively ($p < 0.001$ for both).

The phylogenetic reconstruction revealed a low differentiation between the different haplotypes of *R. unicornis*. All groups had bootstrap values lower than 70, except the clade grouping the haplotypes H1 and H2 belonging to the Assam population (see Fig. 1 and Table 1). A clade with a limited bootstrap support (<50 for NJ, between 60 and 70 for the other methods used) regrouped all individuals analyzed from the Nepal population (H5–H9) except H10. In addition, haplotypes from the Assam population seem to have a basal position compared to individuals from the Nepal population.

In contrast, the network analysis conducted with TCS evaluated H7 (from the Nepal population) as the historical haplotype. The high number of H7 haplotypes did not influence this assessment. The Nepal samples (except for the haplotype H10) form a star-like structure, whereas the haplotypes in the Assam population do not resemble any particular structure (see Fig. 1).

The mismatch-distribution analyses revealed no significant signal of recent expansion neither in the Assam ($F_s = 3.46$, $p = 0.57$; test of goodness-of-fit, $p = 0.007$) nor in the Nepal population ($F_s = -1.33$, $p = 0.17$; test of goodness-of-fit, $p = 0.57$) nor in the combined data ($F_s = 2.35$, $p = 0.89$; test of goodness-of-fit, $p = 0.34$).

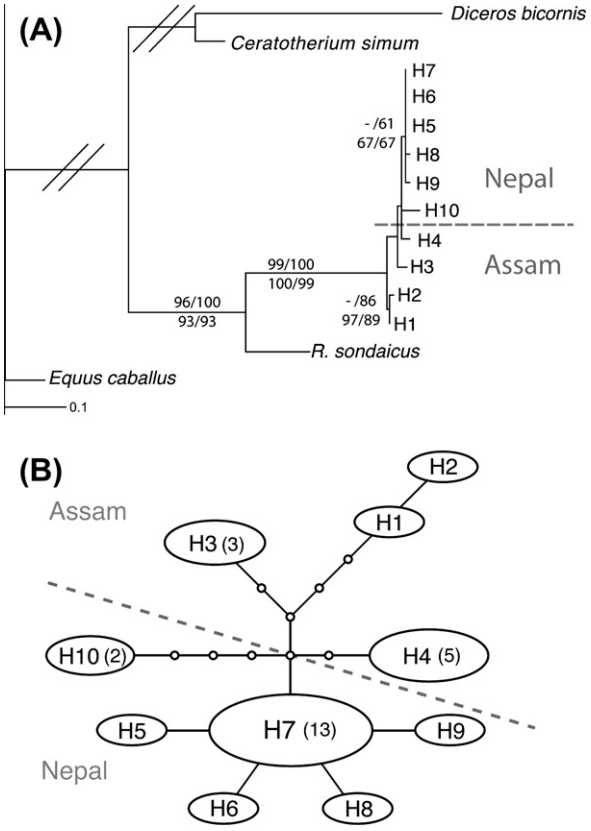

Fig. 1. Genetic structure of all *R. unicornis* individuals based on mtDNA sequence data. Haplotype ID corresponds to the ID in Table 1. (A) Maximum-likelihood tree with bootstrap support values for nodes found in more than 50% of 1000 trees for Neighbor Joining (top left), Maximum Parsimony (top right), Maximum Likelihood (bottom left) and Bayesian analyses (bottom right); (B) Parsimony network of all *R. unicornis* obtained with TCS version 1.21 (Clement et al., 2000). Haplotype size is proportional to its frequency and the number (N) of individuals sharing the same haplotype is written in brackets when $N > 1$. Small circles in the network represent haplotypes not detected in our study.

3.2. Genetic diversity and genetic differentiation based on microsatellite data

No putative null alleles were detected with Micro-Checker. The observed genetic diversity was slightly but not significantly higher in the Assam than in the Nepal population (Ar: $F = 1.21$; $p = 0.29$; H_O: $F = 1.25$; $p = 0.28$; H_E: $F = 1.44$; $p = 0.25$; Table 2). No significant deviations from Hardy–Weinberg equilibrium were detected within either population of *R. unicornis*. The AMOVA revealed that 75.6% of the variation was observed within individuals and 19.2% among populations.

Table 2
Comparison of allelic richness (Ar), observed (H_O) and expected (H_E) heterozygosities, heterozygote deficit (F_{IS}) and mitochondrial diversity (P_i) between the two *R. unicornis* populations in Assam and Nepal. Calculations were based on 8 microsatellite markers developed by (Zschokke et al., 2003) and conducted using Fstat version 2.9.3 (Goudet, 1995) and Arlequin version 3.0 (Excoffier et al., 2005). Allelic richness is based on 12 diploid individuals in both popupations. Mean values ± 1 SD are shown. ANOVAs were conducted to test for significant differences between populations (Assam vs. Nepal).

	Assam ($n = 12$)	Nepal ($n = 26$)	ANOVA
Ar	3.75 ± 1.49	2.95 ± 1.42	$F_{1,14} = 1.21$; $p = 0.29$
H_O	0.57 ± 0.23	0.43 ± 0.29	$F_{1,14} = 1.25$; $p = 0.28$
H_E	0.60 ± 0.20	0.45 ± 0.30	$F_{1,14} = 1.44$; $p = 0.25$
F_{IS}	0.05	−0.24	
P_i	0.009 ($n = 10$)	0.003 ($n = 19$)	

The genetic differentiation based on Fstat indicated a strong isolation between the Assam and Nepal populations ($F_{ST} = 0.202$; $p < 0.001$). This pronounced differentiation was also supported by the population assignment test, where all individuals were unambiguously assigned to their original population (Fig. 3). This strong genetic differentiation was also supported by the additional evaluation of F_{ST} analogs methods ($G''_{ST} = 0.393$; $D = 0.641$).

3.3. Demographic history

Evidence of heterozygosity excess (as defined by Piry et al., 1999) was detected only in the Assam population based on the simulations conducted with Bottleneck (probability for heterozygosity excess: $p = 0.010$). Modifications of parameter values did not change this result. The simulations conducted with MsVar strongly suggested marked declines in both the Assam ($\log_{10}(r) = -3.76$; 95% HDP: −5.59 to −1.93) and the Nepal population ($\log_{10}(r) = -2.87$; 95% HDP: −3.79 to −1.97). These simulations indicated that the present population sizes are c. 0.02% (range from 0.00026% to 1.17%) of the historical (see below) population size in the Assam region and c. 0.13% (range from 0.016% to 1.07%) of the historical population size in the Nepal region. The average mode of $\log_{10}(t_f)$ was estimated to 0.458 (95% HPD: 0.227–0.688) for the Assam population and 0.608 (95% HPD: 0.373–0.833) for the Nepal population. Assuming a present effective population size N_e ranging from 12 to 60 in the Assam population and from 20 to 100 individuals in the Nepal population (see below), and an average generation time of 12 years (Dinerstein and McCracken, 1990), the decline in the Assam population was estimated to have occurred 800–4200 years ago and that in the Nepal population 2000–10,000 years ago. Six independent simulations conducted with different priors yielded similar results.

3.4. Effective population size and individual genetic differentiation

The present effective population size N_e was estimated for both populations using three different methods (Table 3). The Bayesian method, estimated a smaller N_e (17.5) for the Assam population than for the Nepal population (25.9). The other two methods revealed an estimate of N_e only for the Nepal population and 95% confidence intervals including infinity (Table 3).

Both pairwise genetic distance (Fig. 2) and population assignment (Fig. 3) showed a well defined split between Assam and Nepal populations based on nuclear regions. No putative hybrids were detected in the analyzed samples.

4. Discussion

4.1. Comparison between the Assam and the Nepal population

The reduction of population size and the fragmentation of the distribution area in isolated populations are of major concern for most endangered species. Particularly in these situations, genetic information is often critical for evaluation of the existing animals and for future management. In the present study, a strong genetic separation between the Assam and Nepal populations of *R. unicornis* was demonstrated by the microsatellite analysis ($F_{ST} = 0.202$). Similarly, specific mitochondrial alleles were found in each population with no overlap between the two populations. This suggests a strong genetic separation, even if no absolute genetic separation based on the phylogenetic analysis of the D-loop could be found, because in some analyses the haplotype H4 was regrouped with the Nepal clade, depending on the phylogenetic method used. Using microsatellite data, all individuals from our study could be assigned unambiguously to the proper population of origin (see

Table 3
Estimates of the present effective population size (N_e) of *R. unicornis* in the Assam and Nepal populations. Results of three different methods (all based on microsatellite data) are presented (see Section 2 for details).

Method	Software	Assam population N_e (95% CI)	Nepal population N_e (95% CI)
Bayesian	ONeSAMP Tallmon et al. (2004)	17.5 (13.0–30.2)	25.9 (18.3–52.0)
Gametic disequilibrium	LDNE Waples and Do (2008)	– (54.1–∞)	56.8 (12.9–∞)
One- and two-locus data	ESTIM Vitalis and Couvet (2001a)	∞ (7.7–∞)	96.0 (22.2–∞)

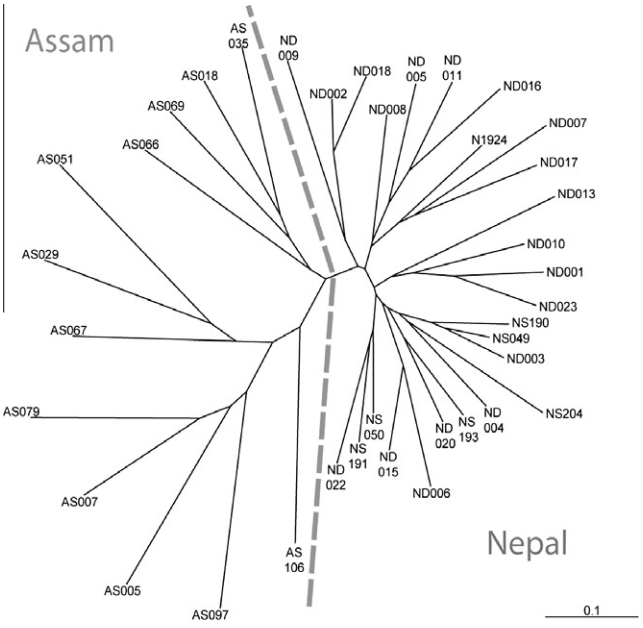

Fig. 2. UPGMA tree based on Cavalli-Sforza and Edwards (1967) genetic distances (microsatellite data) between *R. unicornis* individuals. Codes indicate the individuals (see Table 1).

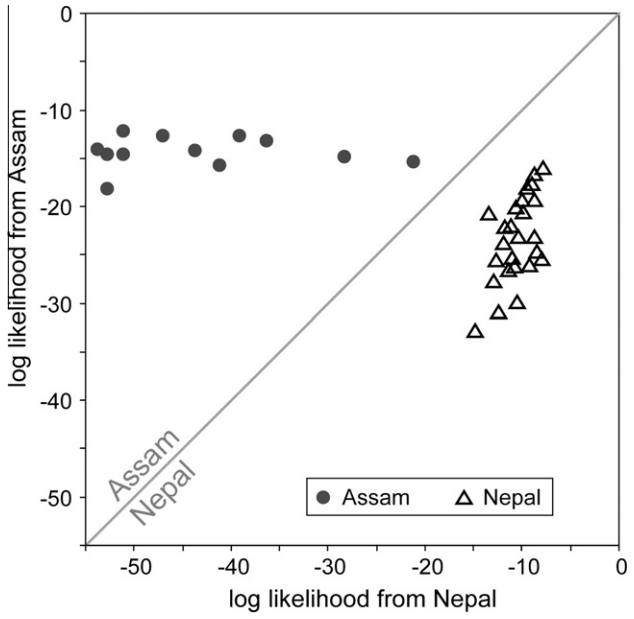

Fig. 3. Population assignment of all *R. unicornis* individuals based on microsatellite data. All individuals were assigned to their population of origin.

Fig. 3). Consequently, we are confident that any assignment to the origin group and detection of putative hybrids can be conducted based on the eight microsatellite loci used in the present study.

The higher genetic diversity, as well as the position of the Assam haplotypes in the phylogenetic reconstruction (Fig. 1), suggest a historical occurrence of the species in the Assam region. The position of the Nepal haplotypes in the phylogenetic tree and the lower genetic diversity in this population suggest a secondary colonization of this region by individuals from Assam. We hypothesize that the detected bottleneck in the Nepal population 2000–10,000 years ago may be the result of a post-glacial recolonization.

4.2. Assam population

It has been believed that the Assam population went through a very severe bottleneck consisting of fewer than 20 individuals at the beginning of the 20th century (Laurie et al., 1983; Ryhiner, 1961; Ullrich, 1972). Then the population recovered and increased during the past 100 years (c. 8 generations). However, we found a high genetic diversity in this population, both in mitochondrial (4 different haplotypes in 10 analyzed samples) and in nuclear DNA (Ar: 15% higher in the Assam than in the Nepal population; H_O: 25% higher in the Assam than in the Nepal population). Different lines of evidence indicate that the current high genetic diversity is unlikely to be the result of a very recent expansion of a very small population with fewer than 20 individuals. Firstly, our genetic analyses indicated an effective population size larger than 15 individuals. Assuming a rather high N_e/N ratio of 0.3, then the smallest population size 100 years ago should have been at least 2.5 times larger than so far assumed. Secondly, a population size reduction to 20 individuals would have impacted the present genetic diversity both on the nuclear and mitochondrial level in a more pronounced way, as observed e.g. in the lions (*Panthera leo*) of the Ngorongoro crater (Packer et al., 1991), the North American elephant seals (*Mirounga angustirostrus*; Hoelzel, 1999), the saddlebacks (*Philesturnus carunculatus*; Taylor et al., 2007) or the Dice snake in Swiss lakes (*Natrix tessellata*; Gautschi et al., 2002). We therefore conclude that the bottleneck in the Assam population in 1908 must have been less severe than previously thought. Taking into account that even with modern techniques an accurate estimate of the number of rhinoceros living in the wild is difficult (Lahan and Sonowal, 1973), it is understandable that it was not possible to get a reliable estimate of the population size of rhinoceros more than 100 years ago. In addition, overlooked individuals from neighboring areas may have immigrated into the region. It is, however, also possible that a low population size was reported intentionally with the purpose to persuade the decision makers to protect the species, as has been done in the White rhinoceros around 1900 (Rookmaaker, 2000; Skinner and Smithers, 1990).

The Assam population of *R. unicornis* is one of the few known examples in captive breeding, in which inbreeding does not lead to an increased juvenile mortality (Krummenacher, 2006; Zschokke and Baur, 2002). Until now, the absence of inbreeding depression in this population has been suggested to be the result of purging of deleterious alleles during the reported bottleneck in 1908 (Zschokke and Baur, 2002). However, our study indicates that the bottleneck in the Assam population was not as severe as

previously thought. It is therefore unclear, why this population does not show any signs of inbreeding depression.

The simulations conducted with MsVar and Bottleneck suggested a bottleneck in the Assam population, which was dated by MsVar to a period between 800 and 4200 years before present. As this is long before the historically known bottleneck 100 years ago, it appears that there might have been two separate bottleneck events in the Assam population.

4.3. Conservation implications

Rhinoceros unicornis is a charismatic species (flagship species) for conservation programs. The total protection of the species decided in 1910 led to an increase in the Assam population up to the present size estimated to exceed 2300 individuals (Talukdar, 2009). Furthermore, captive breeding programs are successfully conducted in several zoos, with large numbers of newborns in San Diego wild animal park and Basel Zoo. As a result, the worldwide captive population is largely sustainable and does no longer depend on newly imported individuals from the wild (von Houwald et al., 2010).

The results of our study demonstrated that the two populations from Assam and Nepal are genetically distinct and that the origin of each individual can be assigned with a high confidence level. Even if the Nepal population is probably a postglacial colonization by individuals from Assam, both populations are presently completely isolated and cannot naturally interbreed. The genetic differentiation and present isolation suggest to treat both populations as separate Management Units (Moritz, 1994, 2002), which implies that mating between *R. unicornis* from the two populations should be avoided, even in captivity. Consequently, a strict breeding program should be followed to avoid crossing between individuals stemming from different populations. This has become feasible with the recent progress in artificial insemination in *R. unicornis* (Stoops et al., 2007, 2010). Unfortunately, some Assam × Nepal matings have already been conducted, both in captive breeding (Zschokke and Baur, 2002) and in the reintroduction programs to Dudhwa National Park, India, where *R. unicornis* both from Nepal and Assam were released (Sinha and Sawarkar, 1993). We suggest that no hybrid animals should be used in further captive breeding and that individuals born in Dudhwa National Park, which are potential hybrids, should not be exported.

It has not been entirely resolved, whether or not there exist any negative effects of outbreeding between the two populations. Zschokke and Baur (2002) found a reduced survival of Assam x Nepal offspring in the captive zoo population, whereas a later study concluded that the observed higher mortality in the outbred offspring was due to a high proportion of primiparous offspring, which are known to have a higher mortality (Pluháček et al., 2007). The study of Pluháček et al. (2007) was based on a larger sample size, but comprised biased assumptions, like the assignment of one individual ('Raju', SB#157) to the Assam population, even though it was caught in the Champaran Forest (State of Bihar, India, across the border from Chitwan NP, Nepal; Rookmaaker, 1998) and should consequently have been grouped with the Nepal population (Rookmaaker, 2002).

As shown in the present study, individuals can be reliably assigned to the Assam or Nepal population using microsatellite markers. This method is rapid, inexpensive (between 10 and 20 € for one individual or sample) and can be conducted using different kinds of DNA samples such as hairs or potentially even feces (not tested here) without the need of capturing individuals. Furthermore, since DNA can be extracted from parts or powder of horn, this method can also be used to determine the origin of confiscated rhinoceros horns.

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Appendix A. Appendix 1

(See Table A1).

Table A1
Summary of the characteristics of all microsatellite loci used in the analysis. The second header line indicates the origin of the sampled individuals: A: Assam ($n = 12$ individuals), N: Nepal ($n = 26$), N_{rar}: Nepal (rarefied to 12 individuals), U: all *R. unicornis* ($n = 38$). When the sample size of the Nepal population was rarefied to that of the Assam population, the number of alleles found in the Assam population was significantly lower than that in the Nepal population (paired t-test; mean difference = 0.826, df = 7, $t = 3.588$, $p = 0.009$). Without rarefaction the difference was not significant (mean difference = 0.500, df = 7, $t = 1.871$, $p = 0.104$).

Locus	# alleles				Allelic size range		
	A	N	N_{rar}	U	A	N	U
Rh1	3	2	1.46	3	4	2	4
Rh3	3	3	2.97	3	32	32	32
Rh4	5	4	3.72	7	26	26	26
Rh5	5	6	4.78	9	10	14	14
Rh6	2	2	2.00	2	2	2	2
Rh9	2	1	1.00	2	22	0	22
Rh10	6	5	4.46	8	12	14	16
Rh11	4	3	3.00	4	12	12	12
Mean	3.75	3.25	2.92	4.75	15.00	12.75	13.50
SD	1.39	1.56	1.28	2.63	9.95	10.86	10.38

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