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RESEARCH ARTICLE

Genetic variation of complete mitochondrial genome sequences of the Sumatran rhinoceros (*Dicerorhinus sumatrensis*)

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Abstract The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is the smallest and one of the most endangered rhinoceros species, with less than 100 individuals estimated to live in the wild. It was originally divided into three subspecies but only two have survived, *D. sumatrensis sumatrensis* (Sumatran subspecies), and *D. s. harrissoni* (Bornean). Questions regarding whether populations of the Sumatran rhinoceros should be treated as different management units to preserve genetic diversity have been raised, particularly in light of its severe decline in the wild and low breeding success in captivity. This work aims to characterize genetic differentiation between Sumatran rhinoceros subspecies using complete mitochondrial genomes, in order to unravel their maternal evolutionary history and evaluate their status as separate management units. We identified three major phylogenetic groups with moderate genetic differentiation: two distinct haplogroups comprising individuals from both the Malay Peninsula and Sumatra, and a third group from Borneo. Estimates of divergence time indicate that the most recent common ancestor of the Sumatran rhinoceros occurred approximately 360,000 years ago. The three mitochondrial haplogroups showed a common divergence time about 80,000 years ago corresponding with a major biogeographic event in the Sundaland region. Patterns of mitochondrial genetic differentiation may suggest considering Sumatran rhinoceros subspecies as different conservation units. However, the management of subspecies as part of a metapopulation may appear as the last resource to save this species from extinction, imposing a conservation dilemma.

Keywords Mitogenomes · Phylogenetics · Genetic structure · Divergence time · Critically endangered species

Introduction

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is the smallest of the five extant rhinoceros species belonging to the family Rhinocerotidae. Also known as the hairy rhinoceros, it is the closest relative of the extinct woolly rhinoceros *Coelodonta antiquitatis* (Orlando et al. 2003; Willerslev et al. 2009). Members of this species once inhabited rainforests, swamps, and cloud forests in Bangladesh, Bhutan, China, India, Indonesia, Laos, Malaysia, Myanmar, and Thailand (van Strien et al. 2008). Currently, fewer than 100 individuals are estimated to remain primarily due to deforestation and illegal poaching in both protected and non-protected areas. The Sumatran rhinoceros is considered critically endangered and thought to be confined to a few remnant upland forest areas on the islands of Sumatra (Barisan Selatan National Park, Gunung Leuser National Park, Gunung Patah, Kerinci-Seblat National Park and Torgamba Forest) and Borneo (Danum Valley Conservation Area, Tabin Wildlife Reserve, and East Kalimantan Province; Miller et al. 2015).

In addition to the critical situation of the Sumatran rhinoceros in the wild, conventional captive breeding has also proven difficult. In the early 1980s, the governments of Indonesia and Malaysia, and international conservation organizations, supported a captive breeding program by transporting

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40 Sumatran rhinoceroses from their native habitats to zoos and reserves across the world. By the late 1990s, not a single rhinoceros had been born in the program, and mortality reached 60% (Foose and van Strien 1997). After years of failed reproductive attempts, the first healthy captive calf was born at the Cincinnati Zoo in September 2001 (Roth 2003). Two other births at the same location followed, in 2004 and 2007 (Christman 2010). The first captive calf in Asia was born in 2012, followed by another birth recently reported in May 2016 in western Sumatra (http://jakartaglobe.id/archive/rare-baby-sumatran-rhinoceros-named-a-gift-from-god/; http://phys.org/news/2016-05-rare-sumatran-rhino-calf-born.htm).

The Sumatran rhinoceros has been divided into three subspecies by Groves (1967) based on measurements of eight morphological characters: *D. sumatrensis sumatrensis* distributed originally on the island of Sumatra and on the Malay Peninsula, *D. s. harrissoni* inhabiting the island of Borneo, and *D. s. lasiotis*, currently extinct, formerly ranging across the rest of Southeast Asia and the Indian subcontinent. Questions regarding whether the populations in the Malay Peninsula, Sumatra and Borneo should be treated as different management units to preserve genetic diversity have been raised (Foose and van Strien 1997; Goossens et al. 2013). Molecular studies using mitochondrial (mt) DNA have led to different interpretations of the conservation status of Sumatran rhinoceros subspecies. For instance, Amato et al. (1995) suggested that an average genetic divergence of 1% between the rhinoceros populations of Sumatra and Borneo was not enough evidence to support more than one conservation unit. Morales et al. (1997) in contrast, analyzing the phylogeographic structure of *D. s. harrissoni* and *D. s. sumatrensis*, identified a maximum of three management units among the remaining populations of the Sumatran rhinoceros: Malay Peninsula–East Sumatra, West Sumatra, and Borneo.

Although genetic studies have played an important role in identifying conservation priorities, the Sumatran rhinoceros crisis requires finding effective strategies to manage the remaining populations in the wild and captivity (Goossens et al. 2013). The consensus among managers and scientists indicates that both subspecies should be managed as a metapopulation, disregarding taxonomic distinction (Nardelli 2014). This means interbreeding populations of the Sumatran rhinoceros to avoid the extinction of the species as a whole. However, concerns about outbreeding depression still exists (Amato et al. 1995; Morales et al. 1997), but this may be outweighed by the risk of extinction due to inbreeding, demographic stochasticity, disease and poaching (Miller et al. 2015).

Since 1986, the San Diego Zoo has banked in its Frozen Zoo® Sumatran rhinoceros genetic material that can be utilized to understand genetic differentiation between living Sumatran rhinoceros subspecies. In this study, we aim to characterize genetic variation in Sumatran rhinoceroses using the complete mt genome, or mitogenome, to unravel their maternal evolutionary history and evaluate the conservation status of subspecies as separate management units. Results from this study contribute to the discussion about the definition of conservation units of critically endangered species, and provide practical applications regarding the management of the Sumatran rhinoceros.

Materials and methods

DNA samples and karyotypes

We examined a total of 14 wild-born and one captive Sumatran rhinoceroses, three individuals from Sabah–Borneo (*D. s. harrissoni*), one individual from Malaysia (*D. s. harrissoni*, likely corresponding to SB26, also caught in Sabah), and 10 from the Malay Peninsula and Sumatra (*D. s. sumatrensis*; Table 1). DNA, tissue, and cell lines were obtained from the San Diego Zoo Global Frozen Zoo®. Utilization of samples was compliant with applicable regulatory procedures for CITES and the US Endangered Species Act. DNA was extracted using the DNeasy tissue and cell line kits (Qiagen, CA, USA) according to the manufacturer's instructions. For karyotyping, chromosomes were derived from lymphocytes and/or fibroblast cell cultures. Cell culturing, harvesting, and chromosome banding followed the techniques described by Houck et al. (1995).

Next generation sequencing data

Whole mitogenomes were amplified in two overlapping amplicons [approximately 10 kilobase pairs (kbp) each] using polymerase chain reaction (PCR) and specific primers designed for this study (Supplementary Table 1). Forward and reverse primers were defined in conserved regions of a multiple-species alignment including all rhinoceros mt sequences obtained from Genbank. PCRs were performed in a 25 µl volume using Eppendorf Mastercycler Gradient thermal cyclers. Each reaction included 2 µl of extracted DNA (60 ng), 2.5 µl of 10× HiFi PCR buffer, 1.25 µl of $MgSO_4$ 50 mM, 2.0 µl of 2.5 mM deoxynucleoside triphosphates, 1.0 µl of 10 µM of each primer, and 0.2 µl of High Fidelity Platinum Taq (Invitrogen Corporation, Carlsbad, CA, USA). The PCR cycling conditions were 94 °C for 2 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing for 30 s, and 68 °C for 10 min, with a final extension at 68 °C for 7 min.

The construction of genomic libraries involved three steps as described by Parson et al. (2013): enzymatic shearing, ligation of the adapters and size selection. The quantity of amplified DNA was determined with a Nanodrop

Table 1 List of Sumatran rhinoceros samples sequenced for the complete mitochondrial genome

Sample no.	Stud book (SB) no.	Sex	Diploid no.	Status	Birth location	Housing institution at sampling	Mitochondrial haplotype
KB6196	19	F	82	Wild born	Malay Peninsula	Melaka Zoo, Malaysia	H03
KB6197	–	F	82	Wild born	Malay Peninsula	Melaka Zoo, Malaysia	H04
KB6198	1	F	82	Wild born	Malay Peninsula	Melaka Zoo, Malaysia	H03
OR5367	28	M	82	Wild born	Sumatra–Indonesia	San Diego Zoo, USA	H08
OR1266	33	F	82	Wild born	Sumatra–Indonesia	San Diego Zoo, USA	H08
OR1265	24	F	–	Wild born	Sumatra–Indonesia	Cincinnati Zoo, USA	H06
OR1439	25	F	81	Wild born	Sumatra–Indonesia	San Diego Zoo, USA	H05
OR1440	35	M	82	Wild born	Sumatra–Indonesia	San Diego Zoo, USA	H02
OR4280	29	F	82	Wild born	Sumatra–Indonesia	LA Zoo/Cincinnati Zoo, USA	H01
OR3077	27	F	82	Wild born	Sumatra–Indonesia	NY Bronx Zoo, USA	H07
KB20219[a]	43	F	82	Captive born	Cincinnati Zoo, USA	Cincinnati Zoo, USA	H01
OR2142[b]	–	–	–	Wild born	Malaysia	SOS RHINO	H11
KB20276	51	F	82	Wild born	Sabah–Borneo	Tabin Wildlife reserve, Malaysia	H10
KB20277	47	M	82	Wild born	Sabah–Borneo	Tabin Wildlife reserve, Malaysia	H10
KB20278	57	F	82	Wild born	Sabah–Borneo	Tabin Wildlife reserve, Malaysia	H09

[a]Offspring of wild caught Sumatran rhinos SB29 (dam) and SB28 (sire)

[b]This individual likely corresponds to SB26, a wild female caught in Sabah in 1989

spectrophotometer (Nanodrop Products, Wilmington, DE, USA). Both 10 kbp fragments were normalized to a quantity of 300 ng and then pooled. The amplicons were enzymatically sheared into suitable sized fragments using the Ion Xpress Plus Fragment Library Kit (Life Technologies, Foster City, CA, USA) following the manufacturer's recommendations. For the 200 bp sequencing kit, shearing times were reduced to 15 min to yield fragments around 260 bp. Size and quality of fragmented DNA were determined with the Agilent DNA High Sensitivity Kit on the Bioanalyzer (Agilent, Santa Clara, CA, USA) following the manufacturer's recommendations. Specific Ion Torrent compatible adapters were ligated onto the ends of each fragment and linked by nick translation. For the barcoded libraries, the Ion P1 Adapter and the Ion Xpress barcode adapters were applied to allow identifying the source when sequencing multiple samples simultaneously. The fragmented and adapter ligated libraries were size selected using the E-Gel SizeSelect Agarose Gel (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's recommendations.

Targets were then subjected to emulsion PCR using the Ion One Touch (Life Technologies, Foster City, CA, USA) following the manufacturer's recommendations. For clonal amplification, DNA was localized to Ion Sphere particles (Life Technologies, Foster City, CA, USA), which were automatically enriched with the Ion OneTouch ES system (Life Technologies, Foster City, CA, USA). Quality was assessed using the Qubit 2.0 Fluorometer (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's recommendations. Next generation sequencing was performed using the Personal Genome Machine (PGM) and samples were loaded onto PGM 316 chips. All PGM sequences were analyzed with the Ion Torrent Software Suite v4.4 for quality control. The Fastq files were mapped to the reference Sumatran rhinoceros mitogenome (Accession Number NC_012684) using the SeqMan NGen application of the DNAstar Lasergene v11 software (Madison, WI, USA). In SeqMan NGen, a template assembly-special workflow was used selecting the Ion Torrent read technology with default parameters (mer size = 19 nucleotides, maximum percentage match = 90%) and the quality end trim option. Single nucleotide polymorphism (SNP) calculation was performed selecting a haploid Bayesian method with default parameters (minimum SNP percentage = 5, SNP confidence threshold = 10, minimum SNP count = 2, and minimum base quality score = 5). Mapped reads were visualized and consensus sequences exported from the SeqMan Pro application of the DNAstar Lasergene v11 software (Madison, WI, USA). Sumatran rhinoceros mitogenomes were aligned using Sequencher 5.0 (Gene Codes, Ann Arbor, MI, USA) using the woolly rhinoceros (*Coelodonta antiquitatis*) as the outgroup for phylogenetic analyses (Genbank Accession Number NC_012681) for a final alignment consisting of 16,591 bp. All sequences were annotated according to the reference Sumatran rhinoceros mitogenome and deposited in Genbank under the Accession Numbers MF066629 to MF066643.

Phylogenetic analyses

The final mitogenome alignment including the Sumatran and woolly rhinoceroses was used to conduct maximum likelihood analysis and Bayesian inference using PhyML 3.0 (Guindon et al. 2010) and MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), respectively. PhyML was used to compute bootstrap percentages after 1000 replicates. Bayesian analyses were performed considering a single partition with a model of sequence evolution corresponding to TrN + G + I, and six partitions corresponding to: tRNAs, rRNAs, control region (D-loop) and first, second and third codon sites of the protein coding genes. jModelTest 0.1 (Posada 2008) was used to select the model of sequence evolution of six partitions according to the Akaike Information Criterion: GTR + I (first and third codons, and rRNAs), TrN (second codon, tRNAs), and TrN + I (D-loop).

The Bayesian inference consisted of two concurrent runs with four Markov chains (one cold and seven heated chains with a temperature of 0.2), 20 million generations (sampled every 1000 generations), and a 10% burn-in. We verified that potential scale reduction factors were near to 1.0 for all parameters, and that the average standard deviation of split frequencies was below 0.01. We visualized convergence of runs to stationarity using Tracer version 1.4 (Rambaut and Drummond 2007) by verifying no trends in generation versus logL plots. Posterior probabilities were calculated for each node.

Population summary statistics and network

Population summary statistics of mt genes were estimated using DnaSP 5.0 (Librado and Rozas 2009). We looked at the number of polymorphic sites, number of haplotypes, haplotype diversity (H), and nucleotide diversity (π). To compare pair-wise genetic distances between individuals within Sumatran rhinoceros subspecies, Kimura two-parameter genetic distance was estimated using Mega 6.0 (Tamura et al. 2013). To investigate the evolutionary relationships among Sumatran rhinoceros mt lineages, a haplotype network was built using the median-joining approach (Bandelt et al. 1999), as implemented in the software Network 4.5.1.6 (http://www.fluxus-engineering.com/sharenet.htm). We evaluated the extent of population genetic structure estimating pair-wise Φ_{ST} and different hierarchical Analyses of Molecular Variance (AMOVA) in Arlequin 3.5.1.2 (Excoffier and Lischer 2010). Significant deviations from the null hypothesis of no differentiation were assessed with 10,000 permutations.

Divergence time and demography

To estimate the time to the most recent common ancestor (t_{MRCA}) of the Sumatran rhinoceros, we first estimated the rate of evolution of Sumatran rhinoceroses (mean number of substitutions per site per unit of time) versus other perissodactyl species. Complete perissodactyl mitogenomes were obtained from Genbank including the domestic horse, donkey, Malayan and lowland tapirs, and white, black, greater one-horned, and Javan rhinoceroses. These sequences were aligned with the woolly rhinoceros and the most divergent Sumatran rhinoceros haplotypes (including both subspecies) using Sequencher 5.0 (Gene Codes, Ann Arbor, MI, USA). The final alignment was verified by visual inspection. The D-loop was excluded from the final multi-species alignment that consisted in 13 taxa and 14,403 bp.

MrBayes 3.1.2 was used to estimate phylogenetic relationships among perissodactyls considering five partitions as shown in Willerslev et al. (2009), corresponding to tRNAs, rRNAs, and first, second and third codon sites of the protein coding genes. jModelTest 0.1 was used to select the model of sequence evolution of five partitions corresponding to GTR + G + I (all codon positions plus ribosomal RNAs) and HKY + G (tRNAs). The Bayesian inference was performed for 20 million generations (sampled every 1000 generations), and a 10% burn-in. The Bayesian tree supporting the monophyly of all perissodactyl families (Equidae, Rhinocerotidae and Tapiridae) was used as tree model in BEAST 1.6.1 (Drummond and Rambaut 2007) for estimating the mean rate parameter. We assumed a birth–death speciation process for the tree prior and incorporated an uncorrelated lognormal relaxed molecular clock as described in Vilstrup et al. (2013). The birth–death speciation process is a tree prior chosen to model speciation at the species level that describes lineage diversification between samples from different species, assuming that at any point in time every lineage can undergo speciation or go extinct (Drummond and Rambaut 2007).

Three calibration points were considered and normal priors applied, corresponding to: (1) an intermediate age between the early split of caballines about 4 million years ago (mya; Orlando et al. 2013) and corresponding paleontological data supporting their presence in North America two mya (Eisenmann 1992; mean = 3.0; standard deviation (stdev) = 0.5); (2) the split between the Asian and American tapirs 15 mya (stdev = 5; Cozzuol et al. 2013); and (3) the diversification of extant rhinoceroses in the late Oligocene (Steiner and Ryder 2011; mean = 26.0; stdev = 3.5). The age of the root incorporated an exponential prior of 56 mya as initial value (Prothero and Schoch 1989; Benton and Donoghue 2007). The run in BEAST was based on 10^8 MCMC generations, with samples taken every 10^4 steps, and the first 5×10^4 steps removed as burn-in. The program Tracer was

employed to analyze the autocorrelation tree and effective sample size for parameter estimates. The final tree was estimated in TreeAnnotator v1.8.2 (Drummond and Rambaut 2007) and visualized in FigTree v1.4.2 (Drummond and Rambaut 2007; http://tree.bio.ed.ac.uk/).

The second set of BEAST analyses were performed to infer t_{MRCA} of Sumatran rhinoceros mitogenomes, assuming a constant population size as tree prior and strict molecular clock with the species-specific mean rate derived from the first round of analyses. In this analysis, we changed the tree prior to a coalescent mechanistic model, given that we are now trying to model demographic processes occurring between populations of the same species (Drummond and Rambaut 2007). This run was also performed 10^8 MCMC generations, with samples taken every 10^4 steps, and the first 5×10^4 steps removed as burn-in. Finally, we investigated the demographic history of the Sumatran rhinoceros in BEAST by estimating past fluctuations in population size via the Bayesian Skyline Plot approach (Drummond et al. 2005). We assumed a Bayesian skyline tree prior, a strict molecular clock, and the MCMC parameters were the same as in the first set of BEAST analyses.

Results

Next generation sequencing data and karyotypes

Fifteen Sumatran rhinoceroses were sequenced using the PGM platform for a total of 6.3 million reads generated, 5.4 million assembled and 0.8 unassembled reads, and 1.1 billion bp sequenced (Supplementary Table 2). The average number of reads generated per individual was 397,729 and read length about 202 bp. The average contig size was 16,702 bp with contig coverage of 2760X, ranging from 1236X in individual OR1266 to 4548X in OR1265. Six of the rhinoceroses studied already had their karyotypes documented (Houck et al. 1995). Seven additional Sumatran rhinoceroses were also karyotyped with no differences observed in the diploid number (2n = 82) or chromosome morphology between subspecies except for a single female *D. s. sumatrensis* (OR1439) who showed an atypical 2n = 81 (Table 1). This reduction in chromosome number can be accounted for by a Robertsonian translocation (centromere–centromere fusion) mechanism in which two fewer acrocentric chromosomes and one additional metacentric chromosome were observed. This is the first time this type of chromosomal rearrangement has been reported in *Dicerorhinus*. The affected individual had no noted congenital anomalies or morphological differences to our knowledge. However, fission–fusion polymorphisms have been observed in other rhinoceros species, including *Diceros* and *Ceratotherium* (Houck et al. 1994, 1995).

Phylogeny and genetic diversity

Phylogenetic analyses were performed considering 15 Sumatran rhinoceros mitogenomes (16,466 bp), characterized by 131 polymorphic sites and 108 parsimony informative sites. The genetic region showing the highest number of polymorphic sites was the D-loop (32 polymorphic sites with 21 informative; see Supplementary Fig. 1). Three major Sumatran rhinoceros clades were observed, two sister groups corresponding to 11 *D. s. sumatrensis* individuals (Sumatran subspecies), and a third basal clade grouping all four *D. s. harrissoni* rhinoceroses (Bornean subspecies; Fig. 1). Both *D. s. sumatrensis* groups were comprised of individuals from the Malay Peninsula and Sumatra, suggesting the survival of divergent maternal lineages in these separate geographic regions. For instance, clade D in the phylogenetic tree includes individuals from the Malay Peninsula and North-East Sumatra (Riau Province), and clade E comprises individuals from South-West (Bengkulu) and South-East (Kerinci) Sumatra, and the Malay Peninsula. These two terminal nodes (E and D) were highly supported by posterior probability and bootstrap values (>0.97/99). The Bayesian inferences comparing a single model of nucleotide substitution versus six genetic partitions produced the same tree topology, with the woolly rhinoceros as the outgroup. The maximum likelihood approach using PhyML failed to group all *D. s. harrissoni* individuals in a single cluster (tree not shown), and the monophyletic origin of both subspecies was moderately supported (*D. s. sumatrensis* C node = 0.76/63 and *D. s. harrissoni* B node = 0.75/–). A similar result was still observed after excluding the D-loop from the PhyML analysis.

The relationships among haplotypes were also inferred by the median-joining network approach. Eleven haplotypes were identified among the 15 Sumatran rhinoceroses, with haplotype diversity (H) of 0.962 and nucleotide diversity (π) of 0.00292. Three distinct haplotypes were identified for *D. s. harrissoni* (number of variable sites (S) = 15, H = 0.833, $\pi = 0.00046$) versus eight haplotypes in *D. s. sumatrensis* (S = 92, H = 0.945, $\pi = 0.00238$). The number of mutational steps separating the two *D. s. sumatrensis* haplogroups (D and E) was 50, similar to the number of mutations observed separating *D. s. sumatrensis* E haplogroup and *D. s. harrissoni* haplotypes (55 mutational steps; Fig. 2). This suggests the presence of two well-differentiated haplogroups within *D. s. sumatrensis*.

Estimates of genetic distance (Kimura two-parameter) revealed that *D. s. harrissoni* and *D. s. sumatrensis* differ by 0.39% (stdev = 0.03), which is similar to the value observed between the two clades of *D. s. sumatrensis* (mean = 0.41% ± 0.01; Supplementary Table 3). Among *D. s. harrissoni* individuals, the average genetic distance was 0.05% (stdev = 0.04), and 0.24% among *D. s. sumatrensis*

Fig. 1 Bayesian inference of relationships among 15 Sumatran rhinoceros mitogenomes. The phylogenetic tree was rooted using the woolly rhinoceros as the outgroup. Nodes are indicated by *letters*, and posterior probability/bootstrap values are shown in *boldface*. Average genetic distances (Kimura two-parameter) and the standard deviations are indicated in *italics* for major internal nodes. Branches in the tree in *light gray* represent individuals from Borneo–Malaysia, in *dark gray* from the Malay Peninsula and *black* from Sumatra. Lack of phylogenetic support in nodes is indicated by *continuous line*

(stdev = 0.18). Other species and subspecies of rhinoceroses were also compared using their mitogenomes (excluding the D-loop). The average genetic distance observed between *D. s. harrissoni* and *D. s. sumatrensis* (0.30%) was less than the observed distance between the southern and northern white rhinoceroses (*Cerathotherium simum simum* and *C. s. cottoni*; 0.76%), suggesting that <1% genetic distance may be expected between rhinoceros subspecies for whole mitogenome comparisons (Table 2). Between sister species such as white versus black rhinoceroses (*Diceros bicornis*), Javan (*Rhinoceros sondaicus*) and greater one-horned (*Rhinoceros unicornis*), and Sumatran versus the extinct woolly rhinoceros, genetic distance ranged between 6.55 and 9.46%. More distantly related rhinoceros taxa displayed genetic distance up to 12%.

The pair-wise Φ_{ST} between the two Sumatran rhinoceros subspecies was 0.098, but this estimate was not significantly different from zero ($P = 0.05762 \pm 0.0077$) likely due to the limited number of samples analyzed in this study. Similarly, between *D. s. sumatrensis* haplogroups (D and E), and haplogroups B (*D. s. harrissoni*) and E (*D. s. sumatrensis*), Φ_{ST} values corresponded to 0.080 in both cases but were not significantly different from zero ($P > 0.05$). Given that Φ_{ST} estimates may be driven by the high intra-specific haplotype diversity and limited sampling size, we additionally performed AMOVA analysis splitting the haplotype data into the two subspecies and three clades according to the phylogenetic analysis. The AMOVA analysis showed that most of the genetic variation occurred within populations (87.6%; Table 3),

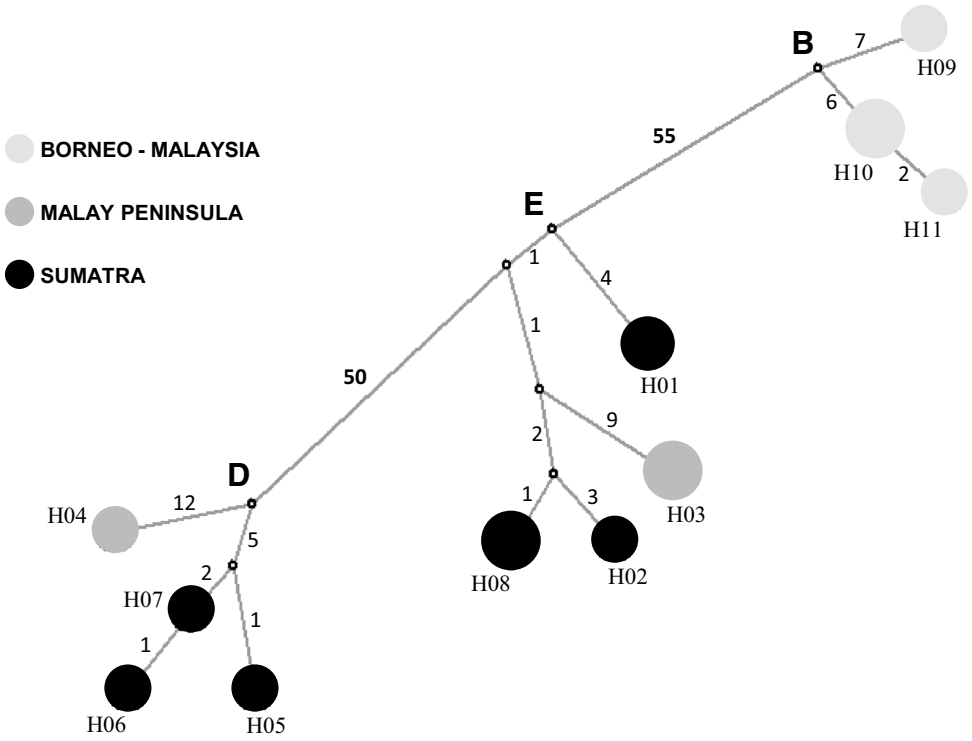

Fig. 2 Median-joining network representing Sumatran rhinoceros haplotypes. The area of each *circle* is proportional to the haplotype frequency. The *light gray circles* correspond to *D. s. harrissoni* (clade B). *D. s. sumatrensis* is represented by two haplogroups (E and D) with *dark gray circles* corresponding to the Malay Peninsula haplotypes H04 (individual KB6197) and H03 (KB6196, KB6198). *Black circles* indicate the Sumatran Island haplotypes H01 (OR4280, KB20219), H02 (OR1440), H08 (OR5367, OR1266), H07 (OR3077), H06 (OR1265), and H05 (OR1439)

Table 2 Pair-wise genetic distance (Kimura two-parameter) estimates between rhinoceros species/subspecies using mitogenomes (excluding the D-loop)

Rhinoceros sp./ssp.	1	2	3	4	5	6	7	8
Black rhinoceros	–							
Southern white rhinoceros	7.43	–						
Northern white rhinoceros	7.45	0.76	–					
Javan rhinoceros	12.68	12.54	12.57	–				
Greater one-horned rhinoceros	12.34	11.96	12.03	6.55	–			
Woolly rhinoceros	12.39	12.54	12.50	12.82	12.65	–		
Sumatran rhinoceros (Sumatran)	12.62	12.45	12.32	12.69	12.68	9.46	–	
Sumatran rhinoceros (Bornean)	12.48	12.31	12.16	12.61	12.64	9.35	0.30	–

Numbers stand for: (1) Black rhinoceros, (2) Southern white rhinoceros, (3) Northern white rhinoceros, (4) Javan rhinoceros, (5) Greater one-horned rhinoceros, (6) Woolly rhinoceros, (7) Sumatran rhinoceros (Sumatran), and (8) Sumatran rhinoceros (Bornean)

Table 3 AMOVA analysis of Sumatran rhinoceros haplotypes partitioned by mitochondrial phylogenetic clades (populations) and subspecies (groups)

Source of variation	d.f.	Sum of squares	Components of variance	Percentage of variation	Fixation indices[a]	P value
Among groups	1	0.756	0.02122	4.19	$\Phi_{CT} = 0.04190$	0.33624
Among populations within groups	1	0.656	0.04172	8.24	$\Phi_{SC} = 0.08599$	0.11158
Within populations	12	5.321	0.44345	87.57	$\Phi_{ST} = 0.12429$	0.00980**

**$P < 0.01$

[a]Φ_{CT} = variation among groups divided by total variation, Φ_{SC} = variation among sub-groups divided by the sum of variation among sub-groups within groups and variation within sub-groups, Φ_{ST} = the sum of variation groups divided by total variation

suggesting that populations are constituted by genetically distinct haplotypes.

Divergence time and demography

Bayesian estimates within the Perissodactyla estimated mean number of substitutions per site per million years to be 6.1×10^{-3} (1.2% per million years) in the Sumatran rhinoceros (Supplementary Fig. 2), which is similar to the mutation rate reported for tapirs (Ruiz-García et al. 2012). With this mean rate, species tree inference was used to estimate t_{MRCA} of Sumatran rhinoceroses around 360,000 years ago (kya), with a 95% highest posterior density (HPD) interval of about 435–291 kya (Fig. 3). For *D. s. harrissoni* and *D. s. sumatrensis*, the age of the most recent common ancestor was 75 kya (95% HPD: 113–40 kya) and 332 kya (95% HPD: 403–263 kya), respectively, suggesting an older coalescent time for *D. s. sumatrensis* that approaches the divergence time of the species. Interestingly, all three mitochondrial haplogroups had a similar coalescent time about 80 kya. In terms of the demographic trajectory of the species, the Bayesian skyline plot analysis did not detect any change in effective population size of the Sumatran rhinoceros for the past 300 kya, except by a relatively recent population decline that started about 25,000 years ago and continued until the present time (Fig. 4).

Discussion

Genetic diversity

The rapid decline of the Sumatran rhinoceros in the wild due to poaching and habitat destruction, and the difficulties of breeding this species in captivity, requires urgent agreement between geneticists and conservationists about future management strategies. The use of complete mt DNA sequences seems to better inform levels of genetic diversity and haplotype structure in the Sumatran rhinoceros than previous studies using single mt gene approaches (Amato et al. 1995; Morales et al. 1997). This study showed the presence of three distinct Sumatran rhinoceros haplogroups, two corresponding to *D. s. sumatrensis* with eight haplotypes, and a third clade representing *D. s. harrissoni* with three haplotypes. These haplotypes were not shared between subspecies, suggesting the management of *D. s. sumatrensis* as two separate conservation units based on the ancestry of mt lineages, and a third management unit for the Borneo–Malaysia population. Three management units in the Sumatran rhinoceros have previously been proposed by Morales et al. (1997) by examining the D-loop, but they corresponded to the geographic regions of Malay Peninsula–East Sumatra, West Sumatra, and Borneo.

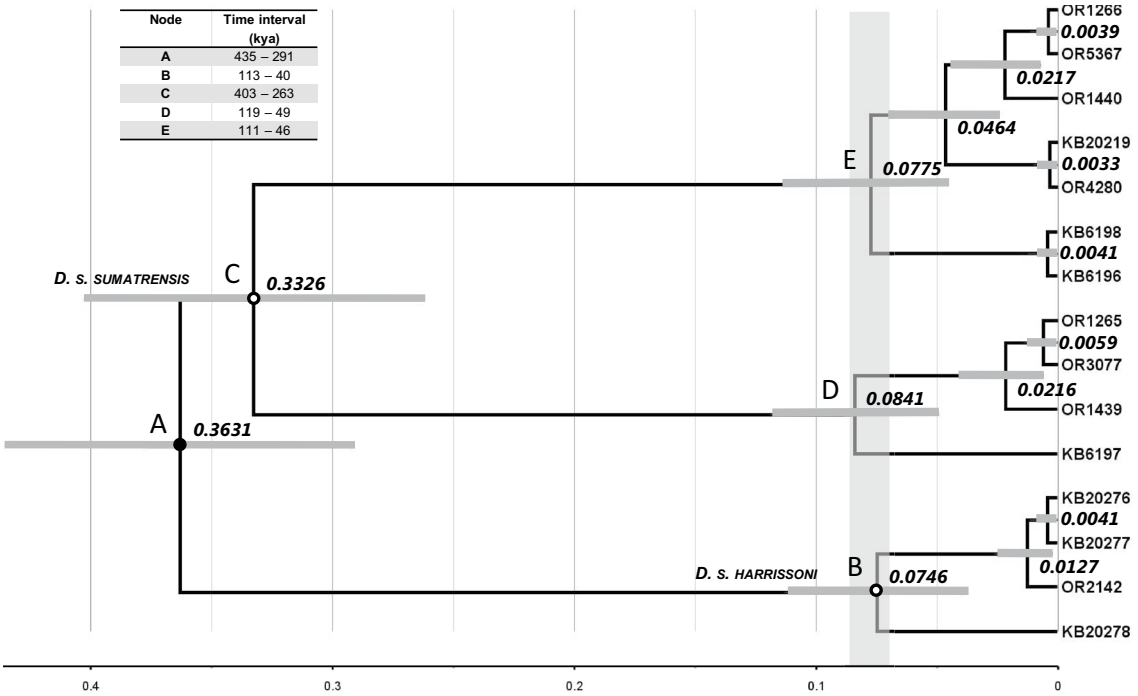

Fig. 3 Divergence time estimates for the Sumatran rhinoceros using complete mitochondrial sequences. Age is indicated for each node with the 95% highest posterior density bar. The most recent common ancestor of the Sumatran rhinoceros is shown by a *black dot*, while the ancestors of *D. s. sumatrensis* and *D. s. harrissoni* are indicated by *open circles* in the topology. The time of the Toba mega-eruption event about 71 kya is represented by the *vertical shaded* region

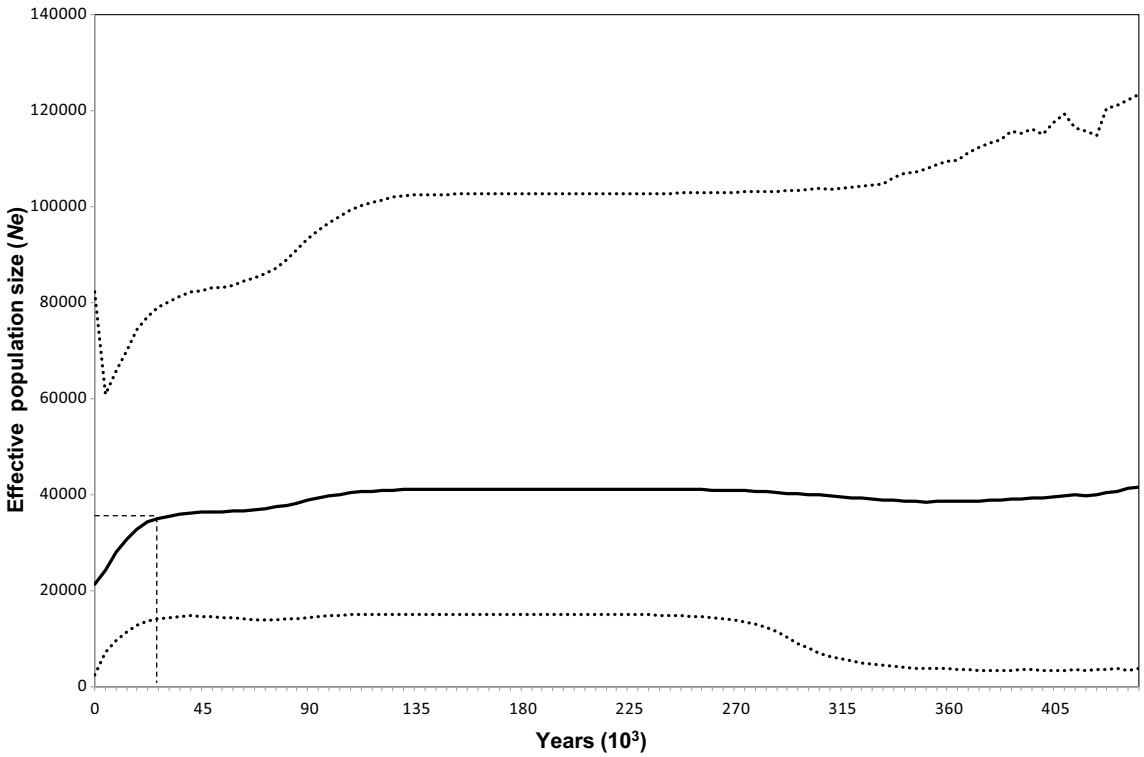

Fig. 4 Bayesian skyline plot derived from Sumatran rhinoceros mitogenomes. The *horizontal middle line* refers to mean effective population size (*y*-axis), with lower and upper confidence interval. The *dashed lines* on the *x* and *y*-axes represent the time period of population decline beginning about 25 kya. The *x*-axis depicts time on a scale of thousands of years

Despite the distinct Sumatran rhinoceros haplogroups, we observed moderate levels of genetic differentiation between Sumatran rhinoceros populations, e.g., <1%. If comparable data for *D. s. lasiotis* were available, an improved understanding of the relative divergence of *D. s. sumatrensis* and *D. s. harrisoni* could be obtained. However, studies comparing other rhinoceros species seem to indicate that the Sumatran rhinoceros is one of the species with the lowest level of intraspecific genetic variation. Fernando et al. (2006), using the D-loop, estimated 4.8–5.1% genetic divergence between geographically isolated populations of Javan rhinoceroses (*R. sondaicus*) in Java (Ujung Kulon) and Vietnam (Cat Tien), compared to 7.2% between white rhinoceros subspecies (*C. s. simum* and *C. s. cottoni*), 3.4–4.3% among black rhinoceroses (*D. b. minor* and *D. b. michaeli*), and no genetic differentiation between the two Sumatran subspecies. These authors recommended the management of the two Javan rhinoceros populations as separate conservation units based on their levels of genetic differentiation.

Additional studies have also shown relatively high levels of genetic differentiation in the D-loop of the black rhinoceros, corresponding to 2–4% (Brown and Houlden 2000; Anderson-Lederer et al. 2012), and this species is managed according to the three subspecies: *D. b. bicornis, D. b. michaeli* and *D. b. minor*. In white rhinoceroses, 1.4% nucleotide divergence has been documented between *C. s. simum* and *C. s. cottoni* using restriction enzymes in the mt DNA (George et al. 1993). White rhinoceros populations have even been suggested as distinct species based on morphological measurements (Groves et al. 2010). Greater one-horned rhinoceros (*R. unicornis*) populations from Assam (India) and Nepal have also been considered as two separate management units due to pronounced genetic differentiation in the D-loop, the presence of non-overlapping mt haplotypes and high nuclear variation (Zschokke et al. 2011; Das et al. 2015).

Our results also showed that the Sumatran rhinoceros subspecies have a common ancestor around 363 kya. This divergence time seems to be relatively more recent than the one estimated for other rhinoceros subspecies. For instance, Brown and Houlden (2000) suggested a divergence time for the two black rhinoceros subspecies between 1.30 and 0.93 mya, and 7 mya divergence between white and black rhinoceroses. A divergence time of 1.84 mya has also been suggested between white rhinoceros subspecies (Groves et al. 2010). However, comparing divergence times among genetic studies has to be done with caution given that data sets are neither comparable in size (single mt gene vs. mitogenome) nor in rate of nucleotide substitution (2% vs. 1.2%).

For the three Sumatran rhinoceros haplogroups, their coalescence time seems to correspond roughly with the Toba super-eruption event that occurred in Sumatra around 71 kya (Zielinski et al. 1996). This eruption was a major biogeographic event that dispersed huge volumes of ash across much of the Indian Ocean, Indian Peninsula and South China Sea, likely modifying atmospheric conditions and forcing a climatic cooling, although no volcanic winter has been confirmed (Lane et al. 2013). The same catastrophic event has been linked in humans to severe demographic changes during the Late Pleistocene (Ambrose 1998). Our results may suggest that the Toba super-eruption event likely impacted the genetic structure of the Sumatran rhinoceros in the Malay Peninsula and the island of Sumatra. As indicated by Louys (2007), mammalian species may have survived in refugia immediately following the eruption, and repopulated shortly after in vast areas of the devastated environment. This contraction and further population expansion may have contributed to the genetic structure of the Sumatran rhinoceros. As a cautionary tale, results obtained from this study are only based on mt DNA sequences. The incorporation of nuclear genetic data may also contribute to better understanding patterns of genetic variation, geographic structure, and divergence time between *D. s. sumatrensis* and *D. s. harrissoni*, as well as the incorporation of historical samples from the declared extinct *D. s. lasiotis*.

Demography

The Bayesian skyline approach can reveal insights about the factors driving past population dynamics and the correlation between demographic and paleoclimatic events (Ho and Shapiro 2011). In the Sumatran rhinoceros, the population decline observed about 25 kya may correspond to the Last Glacial Maximum about 26,500 years ago (Clark et al. 2009), during which the rainforest cover was greatly diminished in Southeast Asia with few refugia surrounded by tropical grasslands, except on the east and west extremities of the Sundaland shelf (Woodruff 2010). This may have produced the fragmentation of Sumatran rhinoceros populations and reduction of population size. A further continuous population decline of the Sumatran rhinoceros may also be associated with the maximum sea level rise about 7000 years ago (Bird et al. 2005) and the human population expansion and dispersion in Southeast Asia. Between 15,000 and 7000 years ago, around half of the land area of the continent of Sundaland was lost to the sea; coastally-located human populations may have expanded and dispersed to accommodate the intense competition for optimal resources (Soares et al. 2008). Furthermore, prehistoric hunter-gatherer societies in the Holocene about 4–5 kya may have impacted local animal populations by hunting and clearing of land for agriculture (Sharma et al. 2012). However, results from the skyline approach also need to be interpreted with caution. The skyline plot method assumes that the populations are panmictic and receive no gene flow from other populations (Ho and Shapiro 2011). Additionally, population structure and migration between subspecies can produce false signals of population size changes, including population decline or expansions (Heller et al. 2013). Consequently, by studying these mitogenomes alone, it can be difficult to distinguish between the genetic signature of a structured population and a panmictic population that has changed in size.

Management

The divergence time estimates and the mt genetic structure of the two Sumatran rhinoceros subspecies may suggest their management as separate conservation units, in the wild and captivity. Nonetheless, due to the magnitude of the Sumatran rhino crisis, with severe population decline in the wild >80% over the past 50 years, genetic and geographical arguments may be overshadowed in deciding the most urgently needed conservation interventions (Goossens et al. 2013). The lack of a sustainable captive population and the risk of extinction of remnant populations in Sumatra and Borneo may indicate that the best chance for this species to survive is by interbreeding populations (Nardelli 2014). As suggested by Ryder (1986), the interbreeding of threatened populations may be justified when the extinction of the species as a whole is jeopardized. However, outbreeding depression could represent a risk for these rhinoceros populations, as shown in other species including humans (Sankararaman et al. 2014). Hybridization may confer the risk for diseases and even male hybrid infertility, but also may help to adapt the Sumatran rhinoceros to new environmental changes by overcoming the effects of reduced genetic variation. Simulation analysis has suggested that managing captive Sumatran rhinoceroses as a single population would greatly increase the probability of population persistence, when compared to scenarios in which captive animals are managed on a regional scale with reduced transfers among regional subpopulations (Ellis et al. 2011). The resolution of this conservation dilemma may reside in concentrating on the urgent measures for saving the Sumatran rhinoceros from extinction, such as managing the captive population on a global basis and adding additional wild-caught individuals (Ellis et al. 2011), implementing intensive management zones in the wild (Havmøller et al. 2016), and developing advanced reproductive technologies for increasing breeding success in captivity, as proposed for other rhinoceros species (Saragusty et al. 2016).

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References

Amato G, Wharton D, Zainuddin ZZ, Powell JR (1995) Assessment of conservation units for the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). Zoo Biol 14:395–402. doi:10.1002/zoo.1430140502

Ambrose SH (1998) Late Pleistocene human population bottlenecks, volcanic winter, and differentiation of modern humans. J Hum Evol 34:623–651. doi:10.1006/jhev.1998.0219

Anderson-Lederer RM, Linklater WL, Ritchie PA (2012) Limited mitochondrial DNA variation within South Africa's black rhino (*Diceros bicornis minor*) population and implications for management. Afr J Ecol 50:404–413. doi:10.1111/j.1365-2028.2012.01333.x

Bandelt HJ, Forster P, Ruhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48. doi:10.1093/oxfordjournals.molbev.a026036

Benton MJ, Donoghue PCJ (2007) Paleontological evidence to date the tree of life. Mol Biol Evol 24:26–53. doi:10.1093/molbev/msl150

Bird MI, Taylor D, Hunt C (2005) Palaeoenvironments of insular Southeast Asia during the Last Glacial Period: a savanna corridor in Sundaland? Quat Sci Rev 24:2228–2242. doi:10.1016/j.quascirev.2005.04.004

Brown SM, Houlden BA (2000) Conservation genetics of the black rhinoceros (*Diceros bicornis*). Conserv Genet 1:365–370. doi:10.1023/A:1011579807460

Christman J (2010) The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) international studbook. Disney's Animal Kingdom, Orlando

Clark PU, Dyke AS, Shakun JD, Carlson AE, Clark J, Wohlfarth B, Mitrovica JX, Hostetler SW, McCabe AM (2009) The last glacial maximum. Science 325:710–714. doi:10.1126/science.1172873

Cozzuol MA, Clozato CL, Holanda EC, Rodrigues FHG, Nienow S, de Thoisy B, Redondo RAF, Santos FR (2013) A new species of tapir from the Amazon. J Mamm 94:1331–1345. doi:10.1644/12-MAMM-A-169.1

Das PK, Borthakur U, Sarma HK, Talukdar BK (2015) Population genetic assessment of extant populations of greater one-horned rhinoceros (*Rhinoceros unicornis*) in India. Eur J Wildl Res 61:841–851. doi:10.1007/s10344-015-0960-2

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:e214. doi:10.1186/1471-2148-7-214

Drummond AJ, Rambaut A, Shapiro B, Pybus OG (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. Mol Biol Evol 22:1185–1192. doi:10.1093/molbev/msi103

Eisenmann V (1992) Origins, dispersals, and migrations of *Equus* (Mammalia, Perissodactyla). Cour Forsch Inst Senckenberg 153:161–170

Ellis S, Ivy J, Ramono WS (2011) Future directions towards the persistence of the captive Sumatran rhino population. White paper accessed 24 Jan 2011. http://www.rhinoresourcecenter.com/index.php?s=1&act=pdfviewer&id=1404633462&folder=140

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetic analyses under Linux and Windows. Mol Ecol Resour 10:564–567. doi:10.1111/j.1755-0998.2010.02847.x

Fernando P, Polet G, Foead N, Ng LS, Pastorini J, Melnick DJ (2006) Genetic diversity, phylogeny and conservation of the Javan rhinoceros (*Rhinoceros sondaicus*). Conserv Genet 7:439–448. doi:10.1007/s10592-006-9139-4

Foose TJ, van Strien N (1997) Asian rhinos—status survey and conservation action plan. IUCN, Gland

George M, Chemnick LG, Cisova D, Gabrisova E, Straril A, Ryder OA (1993) Genetic differentiation of white rhinoceros subspecies: diagnostic differences in mitochondrial DNA and serum proteins. In: Ryder OA (ed) Rhinoceros biology and conservation. Zoological Society of San Diego, San Diego, pp 105–113

Goossens B, Salgado-Lynn M, Rovie-Ryan JJ, Ahmad AH, Payne J, Zainuddin ZZ, Nathan SKSS, Ambu LN (2013) Genetics and the last stand of the Sumatran rhinoceros *Dicerorhinus sumatrensis*. Oryx 47:340–344. doi:10.1017/S0030605313000045

Groves CP (1967) The rhinoceroses of Southeast Asia. Säugetierkd Mitt 15:221–237

Groves CP, Fernando P, Robovsky J (2010) The sixth rhino: a taxonomic re-assessment of the critically endangered northern white rhinoceros. PLoS ONE 5(4):e9703. doi:10.1371/journal.pone.0009703

Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321. doi:10.1093/sysbio/syq010

Havmøller RG, Payne J, Ramono W, Ellis S, Yoganand K, Long B, Dinerstein E, Williams AC, Putra RH, Gawi J, Talukdar BK, Burgess N (2016) Will current conservation responses save the critically endangered Sumatran rhinoceros *Dicerorhinus sumatrensis*? Oryx 50:355–359. doi:10.1017/S0030605315000472

Heller R, Chikhi L, Siegismund HR (2013) The confounding effect of population structure on Bayesian skyline plot inferences of demographic history. PLoS ONE 8(5):e62992. doi:10.1371/journal.pone.0062992

Ho SYW, Shapiro B (2011) Skyline-plot methods for estimating demographic history from nucleotide sequences. Mol Ecol Resour 11:423–434. doi:10.1111/j.1755-0998.2011.02988.x

Houck ML, Ryder OA, Vahala J, Kock RA, Oosterhuis JE (1994) Diploid chromosome number and chromosomal variation in the white rhinoceros (*Ceratotherium simum*). J Hered 85:30–34. doi:10.1093/oxfordjournals.jhered.a111387

Houck ML, Ryder OA, Kumamoto AT, Benirschke K (1995) Cytogenetics of the Rhinocerotidae. Verh Berl Erkrank Zootiere 37:25–32

Lane CS, Chorn BT, Johnson TC (2013) Ash from the Toba supereruption in Lake Malawi shows no volcanic winter in East Africa at 75 ka. Proc Natl Acad Sci USA 110:8025–8029. doi:10.1073/pnas.1301474110

Librado P, Rozas J (2009) DnaSP ver. 5: a software for comprehensive analysis of DNA polymorphism data. Bioinform Appl Note 25:1451–1452. doi:10.1093/bioinformatics/btp187

Louys J (2007) Limited effect of the Quaternary's largest super-eruption (Toba) on land mammals from Southeast Asia. Quat Sci Rev 26:3108–3117. doi:10.1016/j.quascirev.2007.09.008

Miller PS, Lees C, Ramono W, Purwoto A, Rubianto A, Sectionov Talukdar B, Ellis S (2015) Population viability analysis for the Sumatran rhino in Indonesia. IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, MN

Morales JC, Andau PM, Supriatna J, Zainuddin Z-Z, Melnick DJ (1997) Mitochondrial DNA variability and conservation genetics of the Sumatran rhinoceros. Conserv Biol 11:539–543. doi:10.1046/j.1523-1739.1997.96171.x

Nardelli F (2014) The last chance for the Sumatran rhinoceros? Pachyderm 55:43–53

Orlando L, Leonard JA, Thenot A, Laudet V, Guerin C, Hänni C (2003) Ancient DNA analysis reveals woolly rhino evolutionary relationships. Mol Phylogenet Evol 28:485–499. doi:10.1016/S1055-7903(03)00023-X

Orlando L, Ginolhac A, Zhang G, Froese D, Albrechtsen A, Stiller M, Schubert M, Cappellini E, Petersen B, Moltke I, Johnson PLF, Fumagalli M, Vilstrup JT, Raghavan M, Korneliussen T, Malaspinas A-S, Vogt J, Szklarczyk D, Kelstrup CD, Vinther J, Dolocan A, Stenderup J, Velazquez AMV, Cahill J, Rasmussen M, Wang X, Min J, Zazula GD, Seguin-Orlando A, Mortensen C, Magnussen K, Thompson JF, Weinstock J, Gregersen K, Røed KH, Eisenmann V, Rubin CJ, Miller DC, Antczak DF, Bertelsen MF, Brunak S, Al-Rasheid KAS, Ryder O, Andersson L, Mundy J, Krogh A, Gilbert MTP, Kjær K, Sicheritz-Ponten T, Jensen LJ, Olsen JV, Hofreiter M, Nielsen R, Shapiro B, Wang J, Willerslev E (2013) Recalibrating *Equus* evolution using the genome sequence of an early Middle Pleistocene horse. Nature 499:74–78. doi:10.1038/nature12323

Parson W, Strobl C, Huber G, Zimmermann B, Gomes SM, Souto L, Fendt L, Delport R, Langit R, Wootton S, Lagace R, Irwin J (2013) Evaluation of next generation mtGenome sequencing using the Ion Torrent Personal Genome Machine (PGM). Forensic Sci Int Genet 7:543–549. doi:10.1016/j.fsigen.2013.06.003

Posada D (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol 25:1253–1256. doi:10.1093/molbev/msn083

Prothero D, Schoch R (1989) The evolution of perissodactyls. Oxford University Press, New York

Rambaut A, Drummond AJ (2007) Tracer [computer program]. http://beast.bio.ed.ac.uk/tracer

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574. doi:10.1093/bioinformatics/btg180

Roth TL (2003) Breeding the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) in captivity: behavioral challenges, hormonal solutions. Horm Behav 44:31. doi:10.1016/S0018-506X(03)00068-0

Ruiz-García M, Vásquez C, Pinedo-Castro MO, Sandoval S, Castellanos A, Kaston F, Thoisy B, Shostell J (2012) Phylogeography of the Mountain Tapir (*Tapirus pinchaque*) and the Central American Tapir (*Tapirus bairdii*) and the origins of the three Latin-American tapirs by means of mtCyt-B sequences. In: Anamthawat-Jónsson K (ed) Current topics in phylogenetics and phylogeography of terrestrial and aquatic systems. InTech, Rijeka, pp 83–116

Ryder OA (1986) Species conservation and systematics: the dilemma of subspecies. Trends Ecol Evol 1:9–10. doi:10.1016/0169-5347(86)90059-5

Sankararaman S, Mallick S, Dannemann M, Prüfer K, Kelso J, Pääbo S, Patterson N, Reich D (2014) The genomic landscape of Neanderthal ancestry in present-day humans. Nature 507:354–357. doi:10.1038/nature12961

Saragusty J, Diecke S, Drukker M, Durrant B, Friedrich Ben-Nun I, Galli C, Göritz F, Hayashi K, Hermes R, Holtze S, Johnson S, Lazzari G, Loi P, Loring JF, Okita K, Renfree MB, Seet S, Voracek T, Stejskal J, Ryder OA, Hildebrandt TB (2016) Rewinding the process of mammalian extinction. Zoo Biol 35:280–292. doi:10.1002/zoo.21284

Sharma R, Arora N, Goossens B, Nater A, Morf N, Salmona J, Bruford MW, Van Schaik CP, Krützen M, Chikhi L (2012) Effective population size dynamics and the demographic collapse of Bornean orangutans. PLoS ONE 7(11):e49429. doi:10.1371/journal.pone.0049429

Soares P, Trejaut JA, Loo J-H, Hill C, Mormina M, Lee C-L, Chen Y-M, Hudjashov G, Forster P, Macaulay V, Bulbeck D, Oppenheimer S, Lin M, Richards MB (2008) Climate change and postglacial human dispersals in Southeast Asia. Mol Biol Evol 25:1209–1218. doi:10.1093/molbev/msn068

Steiner CC, Ryder OA (2011) Molecular phylogeny and evolution of the Perissodactyla. Zool J Linnean Soc 163:1289–1303. doi:10.1111/j.1096-3642.2011.00752.x

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) Mega6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. doi:10.1093/molbev/mst197

van Strien NJ, Manullang B, Sectionov IW, Khan MKM, Sumardja E, Ellis S, Han KH, Boeadi Payne J, Bradley ME (2008) *Dicerorhinus sumatrensis*. The IUCN Red List of Threatened Species 2008: e.T6553A12787457

Vilstrup JT, Seguin-Orlando A, Stiller M, Ginolhac A, Raghavan M, Nielsen SCA, Weinstock J, Froese D, Vasiliev SK, Ovodov ND, Clary J, Helgen KM, Fleischer RC, Cooper A, Shapiro B, Orlando L (2013) Mitochondrial phylogenomics of modern and ancient equids. PLoS ONE 8(2):e55950. doi:10.1371/journal.pone.0055950

Willerslev E, Gilbert MTP, Binladen J, Ho SYW, Campos PF, Ratan A, Tomsho LP, da Fonseca RR, Sher A, Kuznetsova TV, Nowak-Kemp M, Roth TL, Miller W, Schuster SC (2009) Analysis of complete mitochondrial genomes from extinct and extant rhinoceroses reveals lack of phylogenetic resolution. BMC Evol Biol 20099:95. doi:10.1186/1471-2148-9-95

Woodruff DS (2010) Biogeography and conservation in Southeast Asia: how 2.7 million years of repeated environmental fluctuations affect today's patterns and the future of the remaining refugial-phase biodiversity. Biodivers Conserv 19:919–941. doi:10.1007/s10531-010-9783-3

Zielinski GA, Mayewski PA, Meeker LD, Whitlow S, Twickler MS, Taylor K (1996) Potential atmospheric impact of the Toba mega-eruption ~71,000 years ago. Geophys Res Lett 23:837–840. doi:10.1029/96GL00706

Zschokke S, Armbruster GFJ, Ursenbacher S, Baur B (2011) Genetic differences between the two remaining wild populations of the endangered Indian rhinoceros (*Rhinoceros unicornis*). Biol Conserv 144:2702–2709. doi:10.1016/j.biocon.2011.07.031