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Original Article

Genetic Structure and Diversity Among Historic and Modern Populations of the Sumatran Rhinoceros (*Dicerorhinus sumatrensis*)

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Abstract

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*), once widespread across Southeast Asia, now consists of as few as 30 individuals within Sumatra and Borneo. To aid in conservation planning, we sequenced 218 bp of control region mitochondrial (mt) DNA, identifying 17 distinct mitochondrial haplotypes across modern ($N = 13$) and museum ($N = 26$) samples. Museum specimens from Laos and Myanmar had divergent mtDNA, consistent with the placement of western mainland rhinos into the distinct subspecies *D. s. lasiotis* (presumed extinct). Haplotypes from Bornean rhinos were highly diverse, but dissimilar from those of other regions, supporting the distinctiveness of the subspecies *D. s. harrissoni*. Rhinos from Sumatra and Peninsular Malaysia shared mtDNA haplotypes, consistent with their traditional placement into a single subspecies *D. s sumatrensis*. Modern samples of *D. s. sumatrensis* were genotyped at 18 microsatellite loci. Rhinos within Sumatra formed 2 sub-populations, likely separated by the Barisan Mountains, though with only modest genetic differentiation between them. There are so few remaining Sumatran rhinoceros that separate management strategies for subspecies or subpopulations may not be viable, while each surviving rhino pedigree is likely to retain alleles found in no other individuals. Given the low population size and low reproductive potential of Sumatran rhinos, rapid genetic erosion is inevitable, though an under-appreciated concern is the potential for fixation of harmful genetic variants. Both concerns underscore 2 overriding priorities for the species: 1) translocation of wild rhinos to ex situ facilities, and 2) collection and storage of gametes and cell lines from every surviving captive and wild individual.

Subject areas: Population structure and phylogeography; Conservation genetics and biodiversity
Keywords: mitochondrial DNA, haplotype, microsatellites, museum samples, conservation

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) was once distributed across Southeast Asia from Borneo and Sumatra to the foothills of the Himalayan Mountains. Due to habitat loss coupled with over-hunting, the species (which is the only extant member of its genus) is now close to extinction (Scott et al. 2004; Zafir et al. 2011; Havmøller et al. 2016). In the past 2 decades alone, the total population size of this species has decreased by more than 50% (Pusparini et al. 2015; IRF 2016). The current population is estimated to consist of fewer than 100 individuals (Nardelli 2014; Havmøller et al. 2016), and possibly as few as 30 rhinos (Hance 2017), occupying less than 1% of the former geographic range of the species (Dinerstein 2011) (Figure 1). Three subspecies are recognized (Groves 1965; Groves and Kurt 1972; Rookmaaker 1984); one of them, *D. s. lasiotis*, which is presumed extinct, ranged across Bhutan, Bangladesh, northeast India, Myanmar, northern Thailand, and likely the Indochinese Peninsula and southern China. Subspecies *D. s. harrissoni*, historically found in the island of Borneo, now consists of two individuals from Sabah (Malaysian Borneo) maintained ex situ and a remnant wild population in Kalimantan (Indonesian Borneo), estimated to be 15 in size, although the number in Kalimantan may be much smaller (Groves and Kurt 1972; van Strien et al. 2008; Emslie et al. 2013; Hance 2017). Extensive surveys conducted in Sabah after the last two wild individuals were captured in 2011 and 2014 found no sign of additional rhinos, and the population from Malaysian Borneo is thus considered to be extinct in the wild (Havmoller et al. 2016). The other extant subspecies, *D. s. sumatrensis*, was formerly found in Sumatra, the Malay Peninsula, and southern Thailand (Groves and Kurt 1972). However, this subspecies now consists of fewer than 100 individuals in the Indonesian island of Sumatra in 2 or 3 national parks: Gunung Leuser, Way Kambas, and possibly Bukit Barisan Selatan (where it may now be extirpated) (Pusparini et al. 2015;

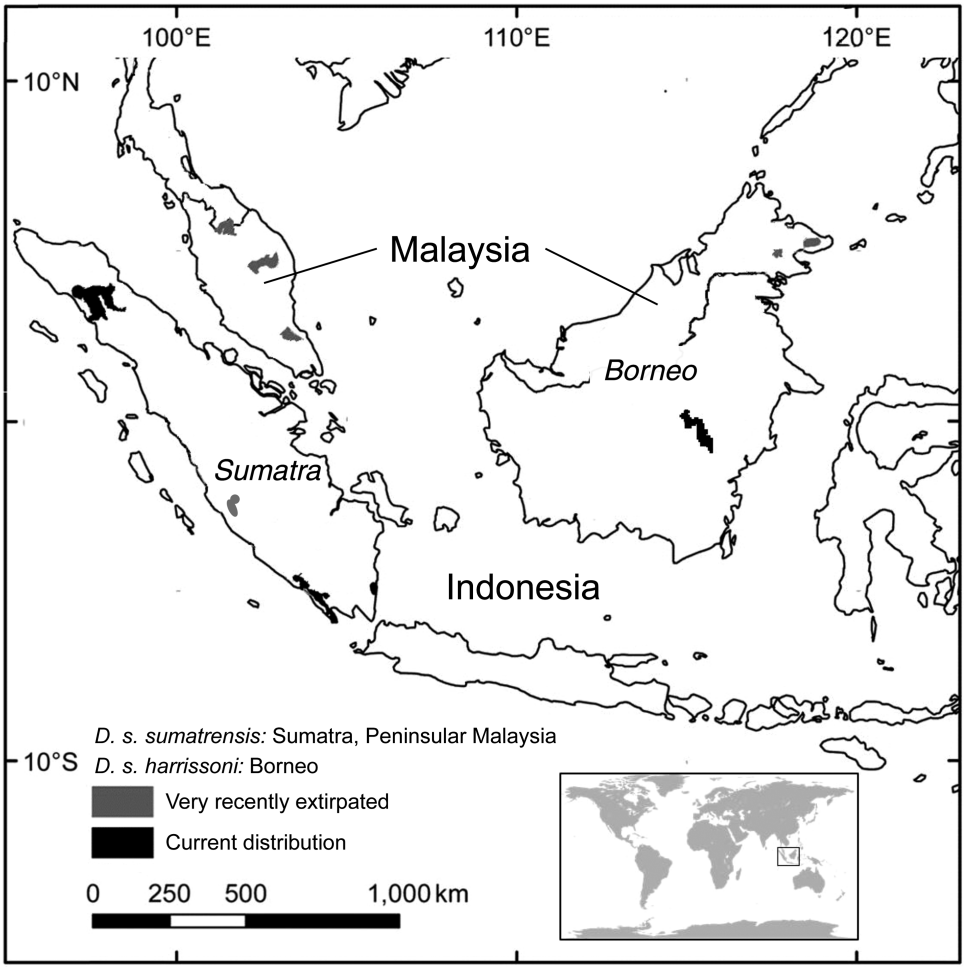

Figure 1. Map showing the current and recent distribution of Sumatran rhinoceros populations. The approximate locations of confirmed surviving Sumatran rhinoceros populations are in black, consisting of three national parks on the Indonesian island of Sumatra, although the population in Bukit Barisan Selatan (southwestern Sumatra) may now be extirpated. An additional small group of Sumatran rhinoceros was recently rediscovered in the West Kutai Regency of the East Kalimantan Province of Indonesian Borneo. Regions in gray are other populations that were very recently extirpated within Sumatra, Malaysian Borneo, and Peninsular Malaysia. Information is based on IUCN (www.iucnredlist.org) and World Wildlife Fund (https://www.worldwildlife.org/species/sumatran-rhino) accounts.

Hance 2017). A further 7 individuals make up the captive breeding program for this subspecies. Overall, the remaining number of Sumatran rhinoceros is unlikely to number more than 90 and may be as low as 30 including the 9 captive individuals (Hance 2017).

In an attempt to conserve the last few remaining Sumatran rhinoceros populations, a series of management strategies was outlined in the Bandar Lampung Declaration (IUCN 2013) and in the Sumatran Rhino Crisis Summit (Havmoller et al. 2016). These affirmed a previous decision to manage the entire remaining Sumatran rhino populations, inclusive of both extant subspecies, as a single population. Although this strategy has not yet been put into action, the national governments of Malaysia and Indonesia are prepared for collaboration. In the face of such a drastic management decision, studies are needed to determine the degree of differentiation within and among subspecies. Earlier studies on the Sumatran rhino utilized mitochondrial DNA (mtDNA) restriction mapping to assess population differentiation and to identify conservation units (Amato et al. 1995; Morales et al. 1997). Amato et al. (1995) suggested that the subspecies are not different enough to represent separate conservation units. Morales et al. (1997) found low genetic divergence between the populations within the island of Sumatra (0.3% haplotype sequence divergence). However, genetic differences detected between rhinoceros on Borneo and those in other populations (1.0% haplotype sequence divergence) were considered large enough to justify management as separate evolutionary lineages.

The Bandar Lampung Declaration indicated that Sumatran rhino populations should be monitored frequently and intensively through collaborative efforts to detect population trends and inform future management decisions. Interbreeding genetically distinct subspecies or populations of Sumatran rhinoceros may result in the loss of genetically unique evolutionary lineages and has the potential to result in outbreeding depression or loss of local adaptations (Allendorf et al. 2001; Edmands 2007). Previous studies did not rely on sequences of the mtDNA; additionally, mtDNA markers sometimes do not reflect the patterns and relationships evident using other genetic markers, so that nuclear genetic markers are helpful in providing a more complete picture of population structure (Toews and Brelsford 2012). In order to further incorporate genetic monitoring of endangered species into management plans, we investigated the genetic diversity and structuring of Sumatran rhinoceros populations using both mtDNA sequences and nuclear microsatellite markers. We wanted to determine how genetic diversity has been reduced across time as the Sumatran rhinoceros population declined, and to establish the genetic structure of current and past Sumatran rhinoceros populations. We found evidence for a reduction in mitochondrial diversity in the species over time, for the distinctiveness of the population on Borneo and for modest genetic differentiation within the island of Sumatra likely due to a geographic impediment to gene flow. Given the small size of each population, every remaining rhino pedigree is likely to carry many unique alleles found in no other individuals, whereas rapid genetic erosion and the fixation of deleterious alleles are likely. There is a strong need both for bringing remaining Sumatran rhinos into breeding centers and especially for the storage of gametes and cell lines from all surviving individuals.

Methods and Materials

Samples

"Modern" samples consisted of Sumatran rhinoceros tissue or blood samples obtained from 15 individuals alive within the past 30 years (Table 1). Whole blood samples were collected from 2 Sumatran rhinoceros at the Cincinnati Zoo during routine veterinary care;

Table 1. Information on the high quality Sumatran rhinoceros samples

Lab ID	Studbook No.	Sex	Name	Capture	Sample	Ancestry	Locality	SDZ No.	Reference
Dsu-28	28	M	Ipuh/Bagus	07/23/90	Blood	Sumatra-W	Bengkulu	OR5367	a
Dsu-33	33	F	Rami	06/12/91	DNA	Sumatra-W	Bengkulu	OR1266	b
Dsu-35	35	M	Tanjung	03/20/92	DNA	Sumatra	—	OR1440	c
Dsu-29	29	F	Emi/Ipak	03/06/91	DNA	Sumatra-W	Bengkulu	OR4280	e
Dsu-63	19	F	Mas Merah	08/26/87	DNA	Peninsular Malaysia	—	KB6196	b, c
Dsu-64	15	F	Minah/Seridelima	05/23/87*	DNA	Peninsular Malaysia	—	—	c
Dsu-66	13	F	Panjang	02/25/87	DNA	Peninsular Malaysia	—	—	c
Ratu	44	F	Ratu	07/01/05	Skin	Sumatra-E	Lampung	—	c, d
TomFoose	Wild	—	—	—	Skin	Sumatra	—	—	—
24	Unknown	—	—	—	Blood	Sumatra	—	—	—
25	12	F	Dusun	09/09/86	Blood	Peninsular Malaysia	—	OR1439	c
126	24	F	Mahato	07/22/88	Muscle	Sumatra-E	Riau	OR1265	b
128	Unknown	—	—	—	Muscle	Sumatra	—	—	—
4273	Wild	—	—	—	Muscle	Sumatra	—	—	—
34965	25	F	Barakas/Kumu	07/24/88	Blood	Sumatra-E	Riau	—	b

M, male; F, female.

— indicates information is unavailable.

*Date of birth in captivity

SDZ No. is the institution number for samples from the San Diego Zoo

References are for locality information: (a) Maynard 1993; (b) Morales et al. 1997; (c) Christman 2010; (d) personal communication, Dr. John Payne; (e) personal communication, Dr. Zainal Zahari.

samples were collected in EDTA tubes to prevent clotting and kept frozen or refrigerated until DNA isolation (<1 week from time of collection). Other samples of whole blood or tissue were kept frozen at –20 °C after collection until the time of extraction (Table 1). From museums in North America and Europe, 28 Sumatran rhino bone samples collected between ca. 1860 to 1941 were obtained (Table 2). DNA from 4 museum samples was extracted prior to importation; DNA from all other museum samples was isolated (see below) after arrival at the University of Illinois at Urbana-Champaign (UIUC). Specimens were imported from international collaborators under CITES/ESA Permit 14US84465A/9 and CITES COSE Permit 12US757718/9. Endorsement for the proposed rhinoceros research was obtained from the Association of Zoos and Aquariums, and all work was conducted with UIUC IACUC approval (protocol 15053).

Sample Preparation and DNA Extraction

DNA was isolated from whole blood or tissue samples using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's recommended protocol. Sample preparation and DNA extraction for museum specimens were conducted in a designated ancient DNA laboratory facility at the Carl. R. Woese Institute for Genomic Biology, UIUC, with the following protocol designed for ancient DNA (Cui et al. 2013). Bones were surface decontaminated by submersion in undiluted bleach for 5 min, followed by 3 rinses in DNA-free ddH_2O, and a final rinse in isopropanol. Samples were placed in a UV-crosslinker for a minimum of 10 minutes or until completely dry. Approximately 0.2 g of each bone was crushed into small pieces or a fine powder using a mortar and pestle in a designated drilling hood and collected in a sterile 15-mL centrifuge tube. All surfaces in the drilling hood and equipment were sterilized between samples with 10% bleach and/or DNA-Off (Clonetech Laboratories Inc., Mountainview, CA) followed by at least 10 min of exposure to UV light. Crushed samples were incubated for 24–48 h in 4 mL of extraction buffer (0.5 M EDTA, 33.3 mg/mL Proteinase K, 10% N-lauryl sarcosine) at 37 °C. Seven or fewer samples were run at one time, and a negative extraction control was included with each set of samples. The extraction solution containing digested sample was concentrated to approximately 250 µL using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (UFC903024; Millipore, Burlington, MA). Remaining undigested bone fragments were kept at 4 °C for future extractions. Concentrated digest was put through the QIAquick PCR Purification kit (Qiagen Inc.) 2 times and eluted in a final volume of 60 µL.

PCR Amplification

An approximately 450 base pair fragment of the mitochondrial control region was amplified by PCR in the modern samples using previously published primers mt15996L (TCCACCATCAGCACCCAAAGC) (Campbell et al. 1995) and mt16502H (TTTGATGGCCCTGAAGTAAGAACC) (Campbell et al. 1995). Samples from 2 modern individuals were excluded from analysis to prevent spurious results as the maternal sample was present in the data set. Reaction mixtures of 10 µL included the following final concentrations: 0.4 µM of each forward and reverse primer, 0.2 mM of each dNTP (Applied Biosystems Inc. [ABI], Foster City, CA), 1× PCR buffer, 1.5–2 mM $MgCl_2$, and 0.4 units of AmpliTaq Gold DNA polymerase (ABI, Foster City, CA). Given

Table 2. Sample information for archival Sumatran rhinos representing the historical population

Sample number	Tissue type	Institution	Collection location	Collection year
AMNH4-54763	DNA	American Museum of Natural History	Myanmar	1924
AMNH5-81892	DNA	American Museum of Natural History	Malaysia	1933
AMNH6-173576	DNA	American Museum of Natural History	Sumatra	Unknown
AMNH7-54764	DNA	American Museum of Natural History	Myanmar	1924
USNM198854	Bone	National Museum of Natural History—Smithsonian	Borneo	1914
USNM199551	Bone	National Museum of Natural History—Smithsonian	Borneo	1912
USNM102076	Bone	National Museum of Natural History—Smithsonian	Unknown	1900
1880-1233	Tissue	National Museum of Natural History (Paris)	Unknown	Unknown
1902-308	Tissue	National Museum of Natural History (Paris)	Unknown	Unknown
1903-329	Bone	National Museum of Natural History (Paris)	Unknown	Unknown
539	Bone	National Museum of Natural History of the Netherlands	Borneo	1896
4947	Bone	National Museum of Natural History of the Netherlands	Sumatra	1941
19594	Bone	National Museum of Natural History of the Netherlands	Sumatra	1860
19595	Bone	National Museum of Natural History of the Netherlands	Sumatra	1883
19596	Bone	National Museum of Natural History of the Netherlands	Sumatra	1880
56616	Bone	Natural History Museum of Bern	Sumatra	Unknown
56618	Bone	Natural History Museum of Bern	Sumatra	Unknown
1500	Bone	Natural History Museum Vienna	Unknown	1884
3082	Bone	Natural History Museum Vienna	Unknown	1910
4294	Bone	Natural History Museum Vienna	Unknown	1873
7529	Bone	Natural History Museum Vienna	Unknown	1920
8173	Bone	Natural History Museum Vienna	Laos	1904
29566	Bone	Natural History Museum Vienna	Sumatra	Unknown
29567	Bone	Natural History Museum Vienna	Sumatra	Unknown
29568	Bone	Natural History Museum Vienna	Unknown	Unknown
19-0311	Bone	Palaeontological Museum Munich	Borneo	1903
1908/571	Bone	Palaeontological Museum Munich	Borneo	1908
190312	Bone	Palaeontological Museum Munich	Borneo	1903

the fragmented nature of DNA in museum specimens, novel primers (DisuCR-F: TGATTTGACTTGGATGGGGTA and DisuCR-R: TTGAGATACACCCCGCTATG) were designed to amplify a 218 bp region of the Sumatran rhino mitochondrial control region (inclusive of primer lengths) that was also part of the region amplified in the modern samples. Reaction mixtures of 20 µL included the following final concentrations: 0.3 µM of each forward and reverse primer, 0.4 mM of each dNTP (New England Biolabs [NEB], Ipswich, MA), 1× PCR buffer, 5 mM $MgCl_2$, and 0.75 units of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Negative controls containing only water were included with each PCR. The step down PCR algorithm for all (modern or museum) mitochondrial control region amplifications was: initial denaturation at 95 °C for 9:45 min; 20 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C for 3 cycles; 20 s at 94 °C, 30 s at 58 °C, 56 °C, 54 °C, and 52 °C (5 cycles at each temperature), 30 s at 72 °C; followed by 22 additional cycles with 50 °C annealing, with a final extension at 72 °C for 7 min. For all samples (modern or museum) that amplified, only a single fragment of the expected size was evident.

In addition, 18 microsatellite loci developed in Sumatran rhinos (*Disu542*, *Disu501*, *Disu556*, *Disu863*, *Disu448*, *Disu201*, *Disu847*, *Disu393*, *Disu733*, *Disu149*, *Disu783*, *Disu50*, *Disu748*, *Disu476*, *Disu151*, *Disu127*, *Disu89*, and *Disu582*) were amplified in the modern samples (Brandt 2016). As described by Ishida et al. 2012, PCR products were fluorescently labeled using M13-tailed forward primers (TGTAAAACGACGGCCAGT). A primer mix consisting of 8.5 µM reverse primer, 0.6 µM of M13-tailed forward primer, and 8.5 µM of fluorescently labeled M13 forward primer was used for PCR. Reaction mixtures of 10 µL included the following final concentrations: 2 mM $MgCl_2$, 200 µM of each dNTP (ABI, Foster City, CA), 1× PCR buffer, and 0.4 units of AmpliTaq Gold DNA Polymerase (ABI, Foster City, CA). Negative controls containing water were included with each PCR. A step down PCR algorithm was used with an initial 95 °C for 10 min; cycles of 15 s at 95 °C; followed by 30 s at 60 °C, 58 °C, 56 °C, 54 °C, 52 °C, (2 cycles at each temperature) or 50 °C (last 30 cycles); and 45 s at 72 °C; and a final extension of 30 min at 72 °C. Previous studies have found that historic samples often do not contain DNA suitable for microsatellite amplification (Arandjelovic and Thalmann 2012; Ishida et al. 2012); therefore, museum samples were not genotyped.

Mitochondrial Control Region Sequencing and Analysis

Mitochondrial PCR products with a discrete single amplicon of the expected size on an ethidium bromide stained agarose gel were enzymatically purified (Hanke and Wink 1994) using Exonuclease I and shrimp alkaline phosphatase (ExoSAP). Purified PCR products were Sanger sequenced in both directions using the BigDye Terminator System (ABI, Foster City, CA), and resolved on an ABI 3730XL capillary sequencer at the UIUC Core Sequencing Facility. Resulting chromatograms were trimmed, concatenated, and edited using the software SEQUENCHER (Gene Codes Corporation, Ann Arbor, MI). Control region sequences from both modern and museum samples were trimmed to be the same length for analysis. Samples were grouped as modern or museum for initial analyses; further categorization into geographic region of origin was done within the museum sample set for additional analyses. DNAsp v5 (Librado and Rozas 2009) was used to estimate basic diversity indices, haplotype (h) and nucleotide (π) diversity. Due to the unequal sample size between the museum and modern sample sets, rarefaction was conducted using HP-RARE v1.0 (Kalinowski 2005). Control region sequences were used to generate a median-joining network using the software NETWORK version 4.6.1 (Bandelt et al. 1999).

Microsatellite Genotyping and Analysis

PCR amplification success for microsatellite loci was examined on a 1% agarose gel stained with ethidium bromide. Samples that successfully amplified were genotyped on an ABI 3730XL Genetic Analyzer and scored using GENEMAPPER v3.7 software (ABI, Foster City, CA). All samples were verified as distinct individuals (had different genotypes). Microsatellite variability was assessed using the following parameters calculated by FSTAT, v2.9.3.2 (Goudet 1995), GENEPOP, v4.0 (Raymond and Rousset 1995), and GenAlEx, v6.1 (Peakall and Smouse 2006; Peakall and Smouse 2012): number of alleles per locus, expected heterozygosity, and observed heterozygosity. Exact tests (Guo and Thompson 1992) were performed in GENEPOP, v.4.0 to determine whether each microsatellite locus across and within all populations was in Hardy–Weinberg equilibrium (HWE). GENEPOP, v.4.0 was used to estimate, for all microsatellites the F_{IS} values and the reduction of heterozygosity due to non-random mating. FSTAT was used to calculate linkage disequilibrium between pairs of loci using a log-likelihood ratio statistic.

STRUCTURE v2.3.3 (Pritchard et al. 2000) was used to assess patterns of genetic partitioning among Sumatran rhinos. Four models with varying assumptions regarding individual ancestry and relatedness among populations were implemented. The 4 models considered were: 1) admixture with correlated allele frequencies; 2) admixture with independent allele frequencies; 3) no admixture with correlated allele frequencies; and 4) no admixture with independent allele frequencies. Each model was run 3 times for values of K = 1 through K = 6 with 1 000 000 Markov chain Monte Carlo steps and a burn in of 100 000 steps. The most likely number of population clusters (K) was evaluated by 2 ad hoc methods in STRUCTURE HARVESTER (Earl and vonHoldt 2012): ΔK (Evanno et al. 2005) and log probability of data, lnP(D) (Pritchard et al. 2000). A factorial correspondence analysis (FCA) was conducted in GENETIX, v4.02.2 (Belkhir et al. 2004) to assess the genetic similarities across individuals in the population.

Results

Mitochondrial Control Region

A total of 26 (93%) of the 28 museum specimens yielded DNA of sufficient quality for PCR amplification and sequencing, while 13 high-quality samples from tissue or cell lines were also sequenced. From the 15 modern samples, 13 were included in control region analysis. After alignment and trimming of priming sequences, 177 bp of mitochondrial control region was used for analysis. Among all samples combined a total of 17 distinct haplotypes (designated as Ds1–Ds17) were identified, with 36 mutations detected. Haplotype diversity was 0.90, and nucleotide diversity was 0.040 (Figure 2; Table 3).

A median joining network was generated to assess the relationships across the control region haplotypes. Haplotypes grouped by geographic region of origin and also formed clusters consistent with traditional subspecies designations, with at least 5 mutations separating the haplotypes for subspecies *D. s. harrissoni* from those of *D. s. sumatrensis* (Figure 2). Individuals of known origin carrying haplotypes Ds1–Ds10 originated from populations of the subspecies *D. s. sumatrensis* in Sumatra and Peninsular Malaysia. Additionally, all samples of unknown origin were identified as having haplotypes Ds1–Ds10; thus, they fell within the identified variation of *D. s. sumatrensis*. Haplotypes Ds11 and

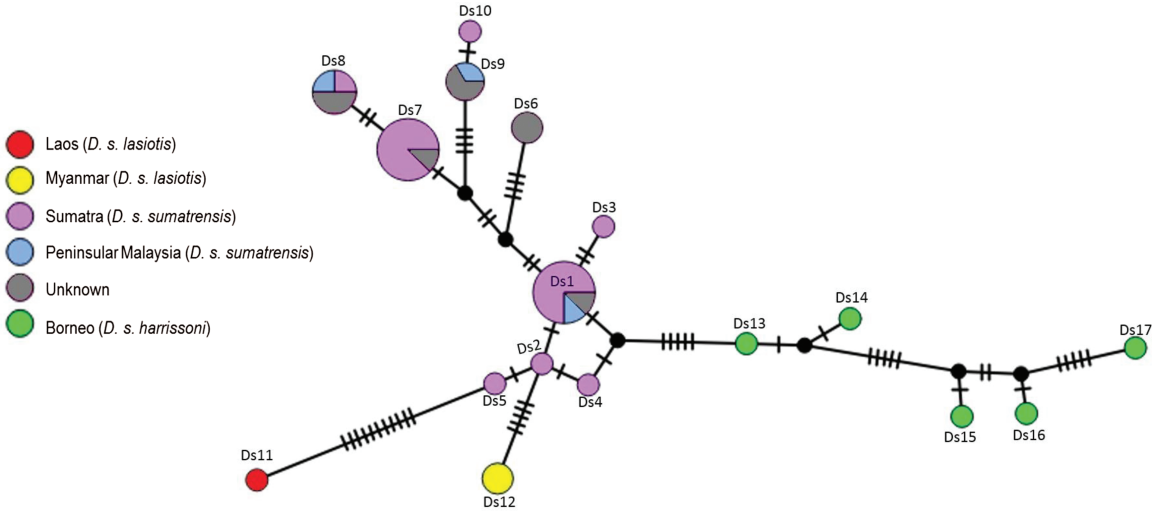

Figure 2. Median joining network of Sumatran rhinoceros mitochondrial control region haplotypes. Each circle in the network represents one of the 17 distinct mitochondrial control region haplotypes detected using both museum samples and cell lines collected more recently. Hash marks indicate the number of mutations separating haplotypes. Circle sizes are proportional to the number of rhinos carrying each haplotype and are color coded by sampling locality. Individuals of known origin carrying haplotypes Ds1–Ds10 were from populations in Sumatra (purple) and Peninsular Malaysia (blue), and are members of the subspecies *D. s. sumatrensis*. All samples of unknown origin (gray) carried haplotypes within the Ds1–Ds10 group; thus, they fell within known variation of *D. s. sumatrensis* individuals. Haplotypes Ds11 and Ds12 were sequenced in samples from Myanmar (yellow) and Laos (red), respectively, representing the subspecies *D. s. lasiotis*. Bornean individuals (green) from the subspecies *D. s. harrissoni* carried haplotypes Ds13–Ds17.

Table 3. Sumatran rhinoceros mitochondrial control region genetic diversity

Sample set	*N*	*H*	*h*	π
Recent *D. s. sumatrensis*	13	5	0.74	0.02
Museum *D. s. sumatrensis*	17 (13)	8 (7.2)	0.90	0.03
All museum	26	15	0.95	0.04
All	39	17	0.90	0.04

N is the number of samples; *H* is the number of observed haplotypes; *h* is haplotype diversity; π is nucleotide diversity.

Rarefied values, in parentheses, allow for different samples sizes to be compared (first 2 listed sets).

Recent individuals were collected beginning ca. 1986 as high quality samples.

Ds12 were found in samples from Myanmar and Laos, respectively, representing the subspecies *D. s. lasiotis*, which is presumed extinct. These haplotypes were unique to *D. s. lasiotis* and divergent by 5 (Ds12) and 11 (Ds11) mutations from the closest haplotypes identified in *D. s. sumatrensis*. All Bornean individuals from the subspecies *D. s. harrissoni* had haplotypes Ds13–Ds17. One sample, incorrectly identified in museum records as originating from Borneo, had a haplotype clustering within the *D. s. sumatrensis* clade; however, historical records show that this sample was unlikely to have originated from Borneo (Supplementary material). Therefore, there was no evidence of haplotypes being shared among subspecies.

The recent samples were all from the subspecies *D. s. sumatrensis* and carried a total of 5 distinct haplotypes ($h = 0.74$; $\pi = 0.022$) (Table 3). Their diversity was compared to that of the historic haplotype diversity in museum specimens, excluding archival samples from the Bornean subspecies (*D. s. harrissoni*) and the western mainland subspecies (*D. s. lasiotis*). The remaining museum dataset ($N = 17$) contained a total of 8 distinct haplotypes ($h = 0.90$; $\pi = 0.032$) (Table 3). Three haplotypes (Ds1, Ds8, and Ds9) were found in both the modern and museum samples sets. Two haplotypes (Ds4 and Ds5) were found only in the modern sample set and 5 haplotypes (Ds2, Ds3, Ds6, Ds8, and Ds10) were restricted to the museum samples (Figure 3). To account for unequal sample size between modern and museum datasets, rarefaction analysis was used (Kalinowski 2005). After rarefaction of the museum dataset to 13 samples, the number of haplotypes was 7.2, higher than the 5 haplotypes among the more recently collected modern samples. Likewise, a total of 12 mutations separated haplotypes in modern samples while 19 mutations separated haplotypes in the museum samples. It should be noted that the modern sampling includes populations that have been extirpated since the samples were collected (including Peninsular Malaysia), so that the decline in mtDNA diversity in the currently surviving populations of *D. s. sumatrensis* is likely to be more extreme.

Microsatellite Analysis

Multilocus genotypes for 18 microsatellite loci were generated from 13 individuals (all corresponding to *D. s. sumatrensis*) for which high quality samples were available. No linkage disequilibrium at microsatellite loci was detected after correction for multiple comparisons ($P < 0.0003$). Two-tailed tests for departure from Hardy–Weinberg equilibrium indicated significant deviation at 6 loci ($P < 0.05$), possibly due to population structure (see below). The average number of alleles per locus was 2.8 and ranged from 2 to 5. Overall mean observed heterozygosity was low ($H_O = 0.28$) compared to expected heterozygosity ($H_E = 0.50$), and fixation index values were high overall ($F_{IS} = 0.44$) (Table 4). Values based on deviations from HWE would be consistent with population structure.

Nuclear genetic partitioning among the high quality Sumatran rhino individuals was examined with STRUCTURE. Ad hoc

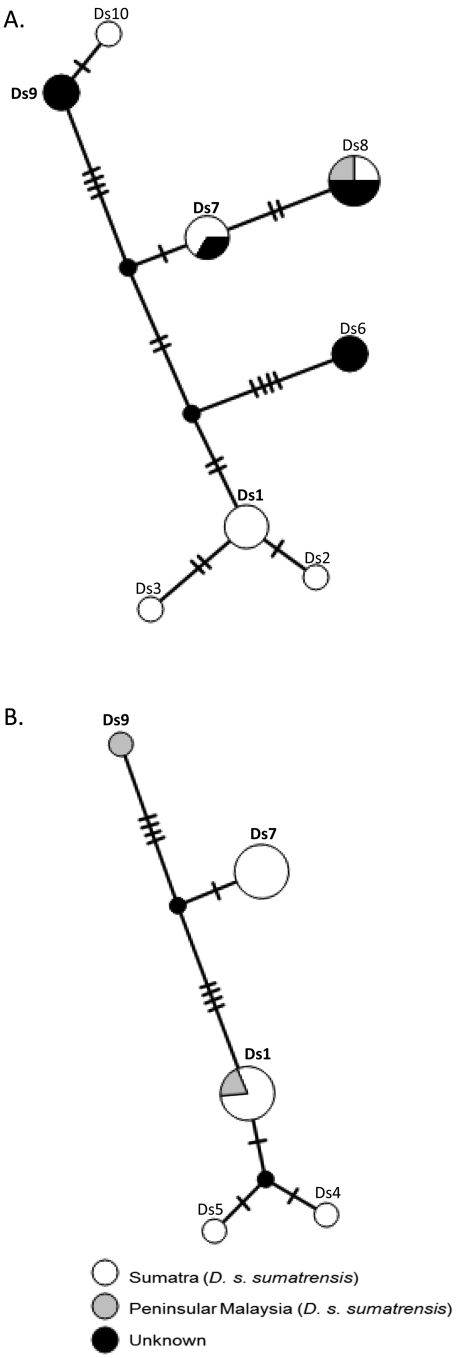

Figure 3. Median joining networks of mitochondrial control region haplotypes for archival and modern samples within the subspecies *D. s. sumatrensis*. Panel A shows only museum samples, while panel B shows recently surviving populations sampled in the 1980s or later (Table 1). Each circle represents a distinct mitochondrial control region haplotype; each hash mark indicates one mutation. Circles are proportional to the number of rhinos carrying each haplotype and shaded by sampling location as indicated. Three haplotypes labeled in boldface, Ds1, Ds7, and Ds9, were detected in both modern and museum samples. Haplotypes Ds2, Ds3, Ds6, Ds8, and Ds10 were detected only in museum samples (A), while Ds4 and Ds5 were only identified in modern samples (B). There were a total of 12 mutations separating haplotypes in modern samples while 19 mutations separate haplotypes in museum samples. Within-subspecies diversity in the modern samples was also reduced compared to museum samples after rarefaction to account for sample size differences (Table 4). Among currently surviving populations, a further reduction of mtDNA diversity is likely given that populations have been extirpated since the 1980s (Figure 1), including all in Peninsular Malaysia.

Table 4 Genetic diversity of the recently sampled Sumatran rhinoceros at 18 microsatellite loci

Locus	A	F_{IS}	H_E	H_O
Disu542	2	−0.091	0.212	0.231
Disu501	2	−0.063	0.508	0.538
Disu556	2	0.529	0.518	0.25
Disu863	3	0.040	0.48	0.462
Disu448	2	0.520	0.471	0.231
Disu201	2	0.842*	0.471	0.077
Disu847	4	0.445	0.545	0.308
Disu393	2	−0.200	0.323	0.385
Disu733	3	1.000*	0.537	0.000
Disu149	4	0.048	0.726	0.692
Disu783	3	0.318	0.668	0.462
Disu050	3	0.865*	0.551	0.077
Disu748	3	0.104	0.428	0.385
Disu476	3	0.286	0.532	0.385
Disu151	2	0.442	0.271	0.154
Disu127	3	0.514*	0.465	0.231
Disu098	5	0.665*	0.725	0.25
Disu582	3	1.000*	0.542	0.000
Overall	2.83	0.440	0.499	0.284

A is the mean number of alleles per locus; F_{IS} is the average deviation from Hardy–Weinberg proportions; H_E is the mean expected heterozygosity; H_O is observed heterozygosity.

The samples listed were collected after ca. 1980.

*statistically significant, $P < 0.05$.

methods to determine the number of partitions provided inconsistent estimates for the value of *K*, perhaps in part due to the limited available samples. Using the Δ*K* method provided support for $K = 2$, regardless of model assumptions. When estimating the most likely number of genetic partitions based on LnP(D) values, $K = 3$ was found for models assuming independent allele frequencies, and $K = 4$ was best supported in models assuming correlated allele frequencies. Given the inconsistent estimates, the guideline put forth by Pritchard and colleagues (2000) was followed that a priori information, notably regarding the biogeography of the study area, should be taken into consideration when assessing the potential number of genetic clusters identified by STRUCTURE (Pritchard et al. 2000). Important differences in clustering patterns were identified when the value of *K* was raised from $K = 2$ to $K = 3$, corresponding to the biogeography of regions inhabited by Sumatran rhinos. However, when the *K* value was further increased to $K = 4$ no additional groupings were apparent. At $K = 3$, genetic distinctiveness between rhinos from the island of Sumatra and the Malay Peninsula was evident (Figure 4), in addition a clear separation was observed between 2 groups of individuals from Sumatra. A factor correspondence analysis conducted using the software GENETIX supported the genetic partitions shown by STRUCTURE at $K = 3$ (Figure 5). The collection locality within Sumatra was not known for all of the rhinos used in our study, but by relying on individuals of known provenance, the division was consistent with separation into populations east and west of the Barisan Mountains, in accordance with previous reports (Morales et al. 1997; Steiner et al. 2017). If the individuals within each genetic partition are considered to derive from separate populations (tentatively, in the case of the 2 partitions in Sumatra), F_{ST} values are only modest between pairs of populations within *D. s. sumatrensis* (0.087 between the 2 Sumatran groups, 0.076 between the Malay Peninsula and Sumatra-East, 0.131 between the Malay Peninsula and Sumatra-West).

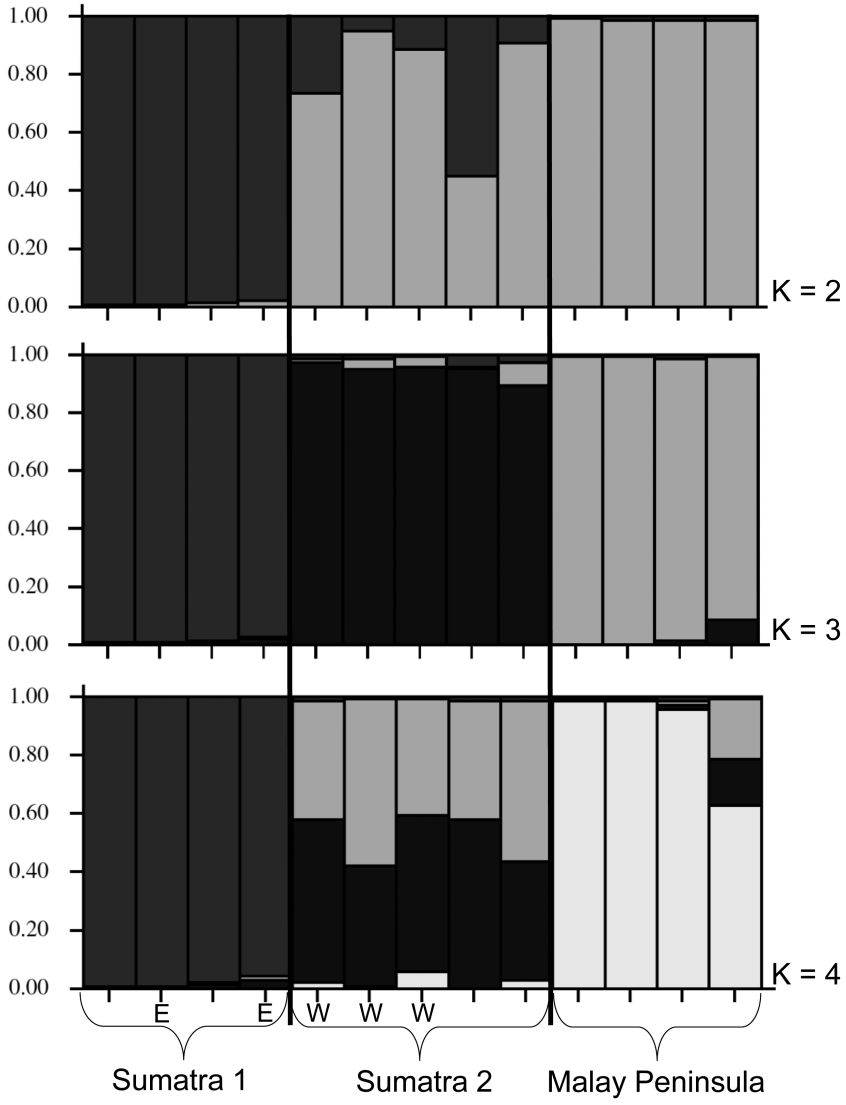

Figure 4. Nuclear genetic partitioning among Sumatran rhinoceros populations. STRUCTURE analyses of multilocus genotypes of 18 microsatellites using 13 high quality Sumatran rhinoceros samples. At $K = 3$, genetic distinctiveness was evident among rhinoceros from the Malay Peninsula (light gray), while those from within the island of Sumatra fell into 2 distinct populations (medium and dark gray). Software settings assumed admixture between populations and correlated allele frequencies. *Ad hoc* methods to determine the number of partitions provided support for a varying number of clusters, with a minimum of 2 and a maximum of 4. Given the biology and biogeography of Sumatran rhinos, clustering patterns at $K = 3$ were informative, while $K = 4$ did not provide any additional information. Individuals known to have originated from Sumatra east of the Barisan Mountains are labeled "E" and those known to have originated from west of the Barisan Mountains are labeled "W."

Discussion

This study is novel in investigating genetic diversity across historical and modern Sumatran rhino populations, vital for the assessment of management units and identification of unique evolutionary lineages within species (Crandall 2009; Schwartz 2009; Oliver et al. 2014). High amplification and sequencing success rate (93%) for a 218 bp portion of the mitochondrial control region was achieved in the museum bone specimens collected between 1860 and 1941. Using archival Sumatran rhinoceros specimens allowed for the evaluation of range-wide historic genetic diversity and the comparison to modern levels of diversity in a species that has experienced severe decline in range and number. Museum specimens can thus be a valuable source of information on the genetic diversity of historic rhino populations, as has been established for other taxa (Leonard et al. 2005; Tsangaras et al. 2012). Some species that currently exhibit low genetic diversity have historic populations with similarly low diversity, for example, koalas (Tsangaras et al. 2012) and Tasmanian devils (Miller et al. 2011). However, this was not the case for the Sumatran rhinoceros (Table 3; Figure 3). Even when sampling was restricted to specimens that were collected from Sumatra or the Malay Peninsula or clustered with known *D. s. sumatrensis* individuals in a haplotype network, diversity in the museum specimens was high ($H = 8$, $h = 0.90$, $\pi = 0.03$) in comparison to the modern samples of *D. s. sumatrensis* ($H = 5$, $h = 0.74$, $\pi = 0.02$), even after rarefaction to adjust for differing sample sizes ($H = 7.2$). The value calculated for haplotype diversity is high, especially in the museum samples, likely due to the modest number of available samples, the broad geographic span, and the isolation of populations and subspecies from each other.

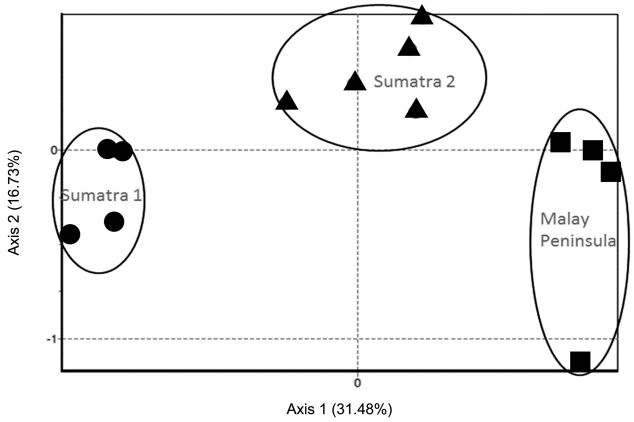

Figure 5. Genetic clustering of Sumatran rhinoceros. Multivariate factorial correspondence analysis (FCoA) was implemented in the program GENETIX using 18 microsatellite loci genotyped in 13 high quality samples of Sumatran rhinoceros. Each icon represents the multilocus genotype of a single individual. The icon shapes were based on the STRUCTURE partitions to which each rhino was primarily assigned: Sumatra 1 (circle), Sumatra 2 (triangle), and Malay Peninsula (square). Axis 1 of the FCoA separates the rhinos into the three groups identified by Structure: from left to right, Sumatra 1, Sumatra 2, and Malay Peninsula. Axis 2 further differentiates the group designated "Sumatra 2" from the other groups. Variation explained along Axes 1 and 2 was 31.48% and 16.73%, respectively.

Within the subspecies *D. s. sumatrensis*, mitochondrial haplotypes were shared between rhinos sampled in the Malay Peninsula and those on the island of Sumatra (Figure 2), suggesting that gene flow was occurring between these regions when they had been connected by land in the Late Pleistocene (see below for further discussion). When genetic clustering techniques were applied to microsatellite genotyping data, 3 partitions were inferred within *D. s. sumatrensis* that correspond to geographic regions (Figures 4 and 5). One partition corresponded to the population in the Malay Peninsula. Sumatra and the Malay Peninsula are currently separated by the Malacca Strait, but were connected by land during the Pleistocene (Heaney 1991; Morales et al. 1997; Leonard et al. 2015). The nuclear genetic differentiation likely reflects genetic drift following vicariant separation of populations by the rise in sea level after the end of the last glacial period. Incongruence between mtDNA and nuclear genetic patterns are common in many species (Toews and Brelsford 2012); in this case, even though insufficient time passed for lineage sorting of mtDNA to produce monophyletic clades (Figure 2), it appears that modest population nuclear genetic differentiation occurred due to drift following the geographic separation of Sumatra and the Malay Peninsula (Figure 4). The rhinoceros population of the Malay Peninsula is believed to have been recently extirpated.

There is also evidence of modest (F_{ST} = 0.087) genetic differentiation of rhinos within the island of Sumatra (Figures 4 and 5). Although the exact provenance of rhinos within Sumatra was only known for some of the samples, the partitions are consistent with the Barisan Mountains forming a barrier to gene flow. The Barisan Mountains are a volcanic arc, active for millions of years, that spans Sumatra from northwest to southeast (Morales et al. 1997). They are likely to have acted as a persistent if incomplete impediment to gene flow between rhinos on either side of the mountain range. It appears that west of the Barisan Mountains, no rhinos have been detected in Bukit Barisan Selatan National Park since 2014, when camera traps identified 1 or 2 (Hance 2017). Some rhinos may survive in Gunung Leuser west of the Barisan Mountains, but they may be unlikely to survive in sufficient numbers to be managed as a separate conservation unit. Since only modest differentiation exists between rhinos west and east of the Barisan Mountains, there would be no major concern in combining the two groups within Sumatra into a single management unit.

The current proposed strategy for managing Sumatran rhinoceros populations aims to combine the two surviving subspecies (in Sumatra and Borneo) into a single conservation management unit (Havmoller et al. 2016). Although our sampling did not allow the generation of microsatellite genotypes for *D. s. harrissoni*, mtDNA haplotypes for the two subspecies fell into different groups, and there is no overlap detected in mtDNA haplotypes. This is in line with previous studies that found mitochondrial genetic differentiation between the 2 Sumatran rhino subspecies (Amato et al. 1995; Morales et al. 1997; Steiner et al. 2017). However, our study included a larger number of individuals and haplotypes, thus further validating this separation (Figure 2). During the last glacial maximum when sea levels were low, there was land connecting the Malay Peninsula, Sumatra, and Borneo (Heaney 1991; Morales et al. 1997; Leonard et al. 2015). Yet despite this potential connectivity across the Sunda Shelf, a semiarid corridor and river basins may have hindered the migration of terrestrial species between Borneo and the other regions (Morales et al. 1997; Morley and Flenley 1987). This may account for the mtDNA distinctiveness of the extant subspecies *D. s. harrissoni* and *D. s. sumatrensis*. Previous research estimated that the two extant subspecies split ca. 363 000 years ago (95% highest posterior density interval of 291–435 kya) (Steiner et al. 2017). This degree of separation could be corroborated by comparing the full nuclear genome now available for *D. s. sumatrensis* (Mays et al. 2018) with that of *D. s. harrissoni*. Yet given their depth of separation and the lack of evidence for subsequent gene flow between them, the 2 subspecies would under normal circumstances be strongly recommended for conservation as distinct units. However, other factors must also be considered, such as whether sufficient individuals are present within each of the genetically distinct subspecies for management practices to maintain them as separate units and to permit breeding opportunities. The population of *D. s. harrissoni* in Sabah consists of only 2 individuals kept ex situ, while in Kalimantan strong evidence only exists for the survival of two rhinoceros individuals (Hance 2017). Thus, even though combining these populations would remove their genetic differences and merge distinctive evolutionary lineages (Allendorf et al. 2001; Edmands 2007), the extremely low numbers for both subspecies would suggest that merging of the 2 lineages may remain the only viable strategy, since any concerns due to potential outbreeding depression (Braude and Templeton 2009) are outweighed by the need to prevent the species as a whole from going extinct. We conclude this reluctantly, because our failure to detect gene flow between the 2 subspecies, and the deep separation between them (Steiner et al. 2017) would make it very difficult to justify treating Sumatran rhinos as a single conservation unit, were the surviving numbers not extremely low.

The decision to maintain all Sumatran rhinoceros as a single stock will involve considerations beyond population genetics, such as whether some breeding centers prove to have greater experience and success at breeding rhinos, and whether some rhino individuals prove highly successful at producing offspring. For example, although multiple facilities would reduce the impact of catastrophic events such as natural disasters or disease outbreaks, at least initially it may be better to consider a single facility that was greatly

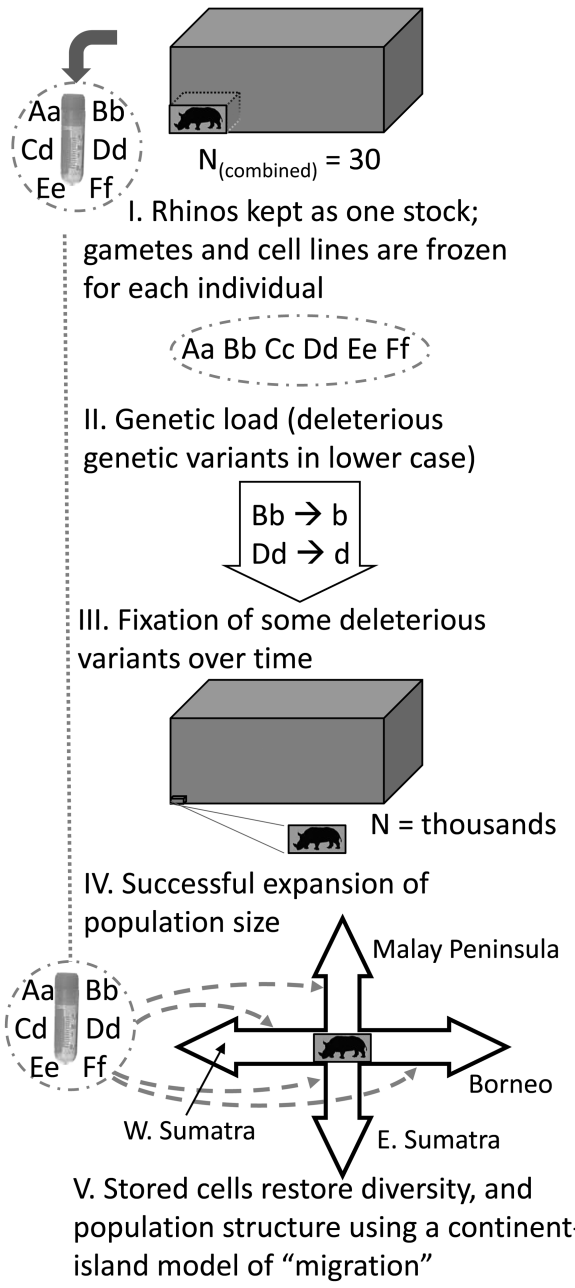

Figure 6. Proposed management of Sumatran rhinoceros. The wording within the figure refers to items immediately above the text. (I) All individuals and populations of Sumatran rhinoceros will be combined for management as a single stock, if current recommendations are implemented (Havmoller et al. 2016; IUCN 2013; Nardelli 2014). The larger box illustrates the size of the surviving population (ca. 30) (Hance 2017), in which a single rhino is proportionately represented by the size of the smaller box (with rhino silhouette). Gametes and cell lines are stored from every individual (illustrated by the arrow pointing to a cryotube), to preserve the current allelic diversity (loci labeled A, B, etc.) present across Sumatran rhinos. (II) Regardless of management strategy, the remaining rhinoceros are likely to carry deleterious alleles (genetic load) across their genomes. Each letter represents a different gene or genetic locus, with lower case letters representing deleterious variants. (III) Alleles become fixed at loci over time, and in very small populations even deleterious alleles (lower case b and d) may become fixed (Ohta 1992) due to stochastic effects, permanently lowering the genetic health of the species. The rate of fixation is higher in populations of small size. Although even as population size increases, deleterious alleles can become fixed. (IV) If the *ex situ* programs are successful, the species will undergo a large increase in population size. In this illustration, the larger box represents a very large population size, with the box representing a single individual proportionately very small (the box with a single illustrated rhino is an expanded view of the smaller box). (V) Gametes or cell lines frozen in earlier generations can then be used to restore genetic diversity to that present in the generation in which the cells were collected (Hendriks et al. 2015; Nakaki et al. 2013). This would be especially beneficial in the case of genes such as those of the immune system where diversity increases fitness. This would also reverse the fixation deleterious genetic variants. Thus, the immediate storage of cell lines is a critical step for future conservation management of the rhinoceros. Using the cell lines collected from rhinos before the populations were merged for management, it would also be possible to restore population structure, including local genetic adaptations such as resistance to endemic pathogens. Cells collected from rhinos before populations were merged could be used to emulate a continent-island unidirectional migration model (dashed arrows) to shift allele frequencies towards those present in distinctive ancestral populations (using cell lines collected from individuals in Borneo, the Malay Peninsula, East Sumatra and West Sumatra before all populations were merged for management).

successful at breeding rhinos, than to consider multiple facilities that may not be as successful.

Such considerations should not influence or delay the urgent need, proposed by current management plans, for bringing isolated and fragmented populations and individuals together into breeding centers (GMPB 2010; IUCN 2013; Havmoller et al. 2016). Whether or not the Borneo and Sumatra subspecies are managed as separate stocks, it would still be of utmost importance to locate and bring together surviving rhinos into managed ex situ facilities, not only to reverse pedigree inbreeding, but also for circumventing locally skewed sex ratios, and to enable mating, especially given that lack of pregnancy can render the female infertile and lead to reproductive pathologies (Khan 1989; GMPB 2010; IUCN 2013; Nardelli 2014; Havmoller et al. 2016).

The very low number of remaining individuals means that genetic erosion is accelerating (Khan 1989). Genetic diversity is lost at a rate of 1/2Ne per generation, where Ne is the effective population size (Wright 1931). Given that the effective population size is likely to be smaller than the census size, this suggests that the remaining populations, and the species as a whole, have been losing genetic diversity at a fast rate, which accelerates as the population size further declines (Wright 1931; Khan 1989). This trend is even more worrisome given that the Sumatran rhinoceros has a low reproductive potential, so that numbers are unlikely to rise quickly and founder contributions will be difficult to equalize, making it even more difficult to slow genetic erosion. Given the low numbers and fast genetic erosion that is now inevitable among the remaining populations, it would only make sense to maintain any population in situ if 1) the reproductive rate of rhinos in situ could be established as being substantially higher than those ex situ and 2) the collection of cell lines (and possibly gametes) could still be conducted for any rhinos kept in situ, for use in eventual genetic restoration of quickly eroding local gene pools.

The low numbers also mean that it will be difficult to prevent deleterious mutations from becoming fixed across the entire species (Figure 6). As population size declines, a greater proportion of deleterious genetic variants become effectively neutral, increasing the probability that even a highly deleterious genetic variant may become fixed across a population (Ohta 1992). The fixation of deleterious alleles in small populations would be analogous to what occurred in the Florida panther, a subspecies of puma that became isolated with a small population size for many generations, resulting in an increase in the frequencies of deleterious alleles, causing harmful traits such

as heart valve defects and cryptorchidism (Roelke et al. 1993). The "genetic restoration" of the Florida panther by translocation of individuals from another subspecies led to reversal of inbreeding and improvement of genetic health (Johnson et al. 2010). However, in the case of the Sumatran rhinoceros, with rhinos kept as a single stock, the only way to reverse the loss of alleles through drift, reverse the fixation of deleterious genetic variants, and reverse the effects of pedigree inbreeding would be through the use of stored gametes and cell lines as reservoirs of otherwise lost genetic diversity.

The use of reproductive interventions such as artificial insemination have been successfully carried out in rhinoceros (Hermes et al. 2009; Stoops et al. 2016), yet even successful reproductive programs would only slow the erosion of genetic diversity in a species surviving in such low numbers. Although increasing the population growth rate of rhinos would tend to slow the loss of variation and reduce fixation of deleterious variants, it would not completely prevent it. Thus, the collection and storage of gametes is absolutely necessary to prevent and eventually reverse the otherwise inevitable loss of genetic diversity over time and the potential fixation of deleterious alleles (Figure 6) (GMPB 2010; Howard et al. 2016). In other endangered species such as the black-footed ferret, frozen sperm stored for 20 years has been used to reverse the loss of genetic diversity in the species (Howard et al. 2016). Recent studies suggest that fibroblasts may provide a potential means for maintaining or restoring genetic diversity through the generation of "artificial gametes" (Nakaki et al. 2013; Hendriks et al. 2015). In addition, fibroblasts would enable genetic, cytogenetic, and immune system research; for example, enabling the use of genomic methods to identify long regions of homozygosity that reduce genetic fitness (Kardos et al. 2018), or identifying the footprints of natural selection (Oleksyk et al. 2010).

The inevitable and accelerating loss of genetic diversity means that the collection of gametes and cell lines from all remaining Sumatran rhino individuals cannot be overemphasized, above all from individuals with reproductive pathologies that prevent them from otherwise contributing to the gene pool of future generations. Given the low numbers of remaining rhinos, it is likely that each surviving rhino pedigree carries many alleles not found in other surviving members of the species. If the Sumatran rhinoceros population can be expanded within *ex situ* facilities, stored gametes and cell lines (whether collected from rhinos kept *ex situ* or *in situ*) would provide a mechanism for restoring lost genetic diversity and for reversing any fixation of deleterious variants across the species. The currently surviving rhino individuals, although few in number, carry allelic diversity from different populations and lineages, representing genetic diversity considerably higher than what is likely to survive many generations after all rhinos have been combined into a single pedigree for *ex situ* management. Although increasing population size would slow the loss of genetic diversity and fixation of deleterious alleles, it would not prevent these from occurring. Thus, the loss of genetic diversity among Sumatran rhinos is inevitable.

Although it is far from a certain outcome, it may be possible to greatly increase the Sumatran rhinoceros population over many generations. Such an outcome would not be unprecedented. After proper conservation management measures were enacted, the southern white rhinoceros population rose across a century from fewer than 100 to over 20 000 (Emslie 2012), before the current poaching crisis unfolded. Should conservation efforts for the Sumatran rhinoceros prove as successful, further loss of genetic diversity would occur at a much slower rate in the larger population (e.g., loss of diversity is reduced 100-fold if the population is 100 times larger). The fixation of additional deleterious alleles would be of little concern in a greatly expanded population, because selection removes deleterious alleles in large populations to a much greater extent than in small populations (Ohta 1992). Additionally, a repository of gametes and cell lines (preferably stored in multiple facilities across nations) would be available indefinitely. The genetic erosion that occurs between the generation in which rhino cells are collected and future generations of their descendants could be restored through the use of "artificial gametes" made from the cell lines. These would also reverse fixation of deleterious alleles, since non-deleterious variants are likely to be present in one or more of the distinct populations alive today. Furthermore, non-deleterious alleles introduced in this manner would increase in frequency due to greater relative role of selection over drift in larger populations (Ohta 1992). Finally, from 4 rhino populations (Malay Peninsula, West Sumatra, East Sumatra, Borneo), cell lines are already collected, and could be used to generate four genetically differentiated stocks. For example, "artificial gametes" made from the cells of Bornean rhinos could be used, generation after generation, to produce "continent-island" model of gene flow (Wright 1931; Bodine and Martinez 2014). This would gradually alter the genetic composition of a target stock of rhinos, shifting their allele frequencies to that of the Bornean rhinos at the time the cell lines were collected, thus reversing the loss of local adaptations that can occur when divergent populations are combined (Allendorf et al. 2001; Edmands 2007), notably restoring immune system alleles adapted to fight local pathogens.

Frozen cell collections already include individuals from regions where rhinos have since been extirpated. Additional cells can be collected from all surviving individuals, including those currently alive that cannot reproduce. The greater the number of cell lines stored from living rhinos today, the greater the number of "founders" available to reverse the effects of inbreeding or drift and maintain the long term genetic health of the Sumatran rhinoceros in the future.

All of our findings and suggestions should be considered within and not outside of the context of current conservation efforts. Current efforts call for strict measures against poaching and trafficking, local capacity building and community engagement, monitoring of and increasing populations, and transfer of individuals, especially isolated individuals or small groups into *ex situ* facilities, and potentially the building of additional such facilities (IUCN 2013; Nardelli 2014; Havmoller et al. 2016). Due to continued decline in the population, currently all Sumatran rhinos exist as isolated individuals or small groups, further emphasizing the need to collect remaining rhinos into *ex situ* facilities to encourage reproduction. Previous analyses of Sumatran rhino conservation needs have recognized the value of every surviving individual (IUCN 2013), and noted that declining population size accelerates the loss of genetic diversity (Khan 1989). Our findings and suggestions do not detract from but rather emphasize these points. As the population declines, genetic erosion accelerates and the fixation of deleterious mutations becomes increasingly likely, making collection and storage of gametes and cell lines from all accessible surviving Sumatran rhinoceros of paramount importance.

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Data Availability

The primary data underlying these analyses are available at Dryad (https://datadryad.org//resource/doi:10.5061/dryad.8n00jc6).

Literature Cited

Allendorf FW, Leary RF, Spruell P, Wenburg JK. 2001. The problems with hybrids: setting conservation guidelines. *Trends Ecol Evol*. 16:613–622.

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

Arandjelovic M, Thalmann O. 2012. *Microsatellites in historic and ancient DNA*. Chichester: John Wiley & Sons, Ltd.

Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol*. 16:37–48.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F. 2004. "*GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations*. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France)." Available from: http://www.univ-montp2.fr/~genetix/genetix/genetix.htm.

Bodine EN, Martinez MV. 2014. Optimal genetic augmentation strategies for a threatened species using a continent-island model. *Lett Biomath*. 1:23–39.

Brandt JR. 2016. *Conservation and population genetics of African and Asian Rhinoceros*. Ph.D. Thesis, University of Illinois at Urbana-Champaign, Urbana, IL.

Braude S, Templeton AR. 2009. Understanding the multiple meanings of 'inbreeding' and 'effective size' for genetic management of African rhinoceros populations. *Afr J Ecol*. 47:546–555.

Campbell NJ, Harriss FC, Elphinstone MS, Baverstock PR. 1995. Outgroup heteroduplex analysis using temperature gradient gel electrophoresis: high resolution, large scale, screening of DNA variation in the mitochondrial control region. *Mol Ecol*. 4:407–418.

Christman J. 2010. *Sumatran Rhinoceros International Studbook*. Available from: http://www.rhinoresourcecenter.com/pdf_files/130/1302735710.pdf.

Crandall KA. 2009. A multifaceted approach to species conservation. *Anim Conserv*. 12:105–106.

Cui Y, Lindo J, Hughes CE, Johnson JW, Hernandez AG, Kemp BM, Ma J, Cunningham R, Petzelt B, Mitchell J, *et al*. 2013. Ancient DNA analysis of mid-Holocene individuals from the Northwest Coast of North America reveals different evolutionary paths for mitogenomes. *PLoS One*. 8:e66948.

Dinerstein E. 2011. Family Rhinocerotidae (Rhinoceroses). In: Wilson DE, Mittermeier RA, editors. *Handbook of the mammals of the world: Hoofed Mammals*. Vol. 2. Barcelona: Lynx Ediciones.

Earl DA, vonHoldt BM. 2012. Structure Harvester: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet Resour*. 4:359–361.

Edmands S. 2007. Between a rock and a hard place: evaluating the relative risks of inbreeding and outbreeding for conservation and management. *Mol Ecol*. 16:463–475.

Emslie RH. 2012. *Ceratotherium simum*. IUCN Red List of Threatened Species 2012: e.T4185A16980466. doi:10.2305/IUCN.UK.2012.RLTS.T4185A16980466.en. [cited 2018 March 08]. Available from: http://www.iucnredlist.org.

Emslie RH, Miliken T, Talukdar B. 2013. *African and Asian rhinoceroses—status, conservation and trade*. IUCN Species Survival Commission (IUCN/SSC) African and Asian Rhino Specialist Groups and TRAFFIC.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol*. 14:2611–2620.

GMPB. 2010. "Sumatran rhino global management and propagation board (GMPB) meeting: meeting minutes." Bogor, Indonesia. Available from: http://www.rhinoresourcecenter.com/pdf_files/145/1453634922.pdf.

Goudet J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. *J Hered*. 86:485–486.

Groves CP. 1965. Description of a new subspecies of Rhinoceros, from Borneo, *Didermocerus sumatrensis harrissoni*. *Säugetierkd Mitt*. 13:128–131.

Groves CP, Kurt F. 1972. *Dicerorhinus sumatrensis*. *Mamm Species*. 21:1–6.

Guo SW, Thompson EA. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*. 48:361–372.

Hance J. 2017. "Worst-case scenario: there could be only 30 wild Sumatran rhinos left." Mongabay. Available from: https://news.mongabay.com/2017/11/worst-case-scenario-there-could-be-only-30-wild-sumatran-rhinos-left/.

Hanke M, Wink M. 1994. Direct DNA sequencing of PCR-amplified vector inserts following enzymatic degradation of primer and dNTPs. *Biotechniques*. 17:858–860.

Havmøller RG, Payne J, Ramono W, Ellis S, Yoganand K, Long B, Dinerstein E, Williams AC, Putra RH, Gawi J, *et al*. 2016. Will current conservation responses save the Critically Endangered Sumatran rhinoceros *Dicerorhinus sumatrensis*? *Oryx*. 50:355–359.

Heaney LR. 1991. A synopsis of climatic and vegetational change in Southeast-Asia. *Clim Change*. 19:53–61.

Hendriks S, Dancet EA, van Pelt AM, Hamer G, Repping S. 2015. Artificial gametes: a systematic review of biological progress towards clinical application. *Hum Reprod Update*. 21:285–296.

Hermes R, Göritz F, Saragusty J, Sós E, Molnar V, Reid CE, Schwarzenberger F, Hildebrandt TB. 2009. First successful artificial insemination with frozen-thawed semen in rhinoceros. *Theriogenology*. 71:393–399.

Howard JG, Lynch C, Santymire RM, Marinari PE, Wildt DE. 2016. Recovery of gene diversity using long-term cryopreserved spermatozoa and artificial insemination in the endangered black-footed ferret. *Animal Conserv*. 19:102–111.

IRF. 2016. "International Rhino Foundation: Sumatran Rhino (*Dicerorhinus sumatransis*)." Available from: https://rhinos.org/species/sumatran-rhino/.

Ishida Y, Roca AL, Fratpietro S, Greenwood AD. 2012. Successful genotyping of microsatellites in the woolly mammoth. *J Hered*. 103:459–464.

IUCN. 2013. *Bandar Lampung Declaration*. International Union for Conservation of Natural and Natural Resources.

Johnson WE, Onorato DP, Roelke ME, Land ED, Cunningham M, Belden RC, McBride R, Jansen D, Lotz M, Shindle D, *et al*. 2010. Genetic restoration of the Florida panther. *Science*. 329:1641–1645.

Kalinowski ST. 2005. HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Mol Ecol Notes*. 5:187–189.

Kardos M, Åkesson M, Fountain T, Flagstad Ø, Liberg O, Olason P, Sand H, Wabakken P, Wikenros C, Ellegren H. 2018. Genomic consequences of intensive inbreeding in an isolated wolf population. *Nat Ecol Evol*. 2:124–131.

Khan M. 1989. *Asian Rhinos: an action plan for their conservation*. Gland, Switzerland: International Union for Conservation of Nature and Natural Resources (IUCN).

Leonard JA, den Tex RJ, Hawkins MTR, Munoz-Fuentes V, Thorington R, Maldonado JE. 2015. Phylogeography of vertebrates on the Sunda Shelf: a multi-species comparison. *J Biogeogr*. 42:871–879.

Leonard JA, Vilà C, Wayne RK. 2005. Legacy lost: genetic variability and population size of extirpated US grey wolves (*Canis lupus*). *Mol Ecol*. 14:9–17.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*. 25:1451–1452

Maynard T. 1993. *A rhino comes to America*. London, UK: Franklin Watts.

Mays HL Jr., Hung CM, Shaner PJ, Denvir J, Justice M, Yang SF, Roth TL, Oehler DA, Fan J, Rekulapally S, *et al*. 2018. genomic analysis of demographic history and ecological niche modeling in the endangered Sumatran Rhinoceros *Dicerorhinus sumatrensis*. *Curr Biol*. 28:70–76.e4.

Miller W, Hayes VM, Ratan A, Petersen DC, Wittekindt NE, Miller J, Walenz B, Knight J, Qi J, Zhao F, *et al*. 2011. Genetic diversity and population structure of the endangered marsupial *Sarcophilus harrisii* (Tasmanian devil). *Proc Natl Acad Sci USA*. 108:12348–12353.

Morales JC, Andau PM, Supriatna J, Zainuddin ZZ, Melnick DJ. 1997. Mitochondrial DNA variability and conservation genetics of the Sumatran rhinoceros. *Conserv Biol*. 11:539–543.

Morley RI, Flenley IR. 1987. Late Cainozoic vegetational and environmental changes in the Malay Archipelago. In: Whitmore TC, editors. *Biogeographical evolution of the Malay Archipelago*. Oxford: Oxford University Press.

Nakaki F, Hayashi K, Ohta H, Kurimoto K, Yabuta Y, Saitou M. 2013. Induction of mouse germ-cell fate by transcription factors in vitro. *Nature*. 501:222–226.

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

Ohta T. 1992. The Nearly Neutral Theory of Molecular Evolution. *Annu Rev Ecol Syst*. 23:263–86.

Oleksyk TK, Smith MW, O'Brien SJ. 2010. Genome-wide scans for footprints of natural selection. *Philos Trans R Soc Lond B Biol Sci*. 365:185–205.

Oliver P, Keogh JS, Moritz C. 2014. New approaches to cataloguing and understanding evolutionary diversity: a perspective from Australian herpetology. *Aust J Zool*. 62:417–430.

Peakall R, Smouse PE. 2006. GenAlEx 6: genetic analysis in excel. Population genetic software for teaching and research. *Mol Ecol Notes*. 6:288–295.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*. 28:2537–2539.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics*. 155:945–959.

Pusparini W, Sievert PR, Fuller TK, Randhir TO, Andayani N. 2015. Rhinos in the parks: an island-wide survey of the last wild population of the Sumatran rhinoceros. *PLoS One*. 10:1–16.

Raymond M, Rousset F. 1995. GENEPOP (version-1.2) - population-genetics software for exact tests and ecumenicism. *J Hered*. 86:248–249.

Roelke ME, Martenson JS, O'Brien SJ. 1993. The consequences of demographic reduction and genetic depletion in the endangered Florida panther. *Curr Biol*. 3:340–350.

Rookmaaker LC. 1984. The taxonomic history of the recent forms of Sumatran rhinoceros (*Dicerorhinus sumatrensis*). *JMBRAS*. 57:12–25.

Schwartz MK. 2009. Uniting ecological and genetic data for the conservation of wild ibex. *Anim Conserv*. 12:103–104.

Scott C, Foose T, Morales C, Fernando P, Melnick DJ, Boag PT, Davila JA, De Groot PJV. 2004. Optimization of novel polymorphic microsatellites in the endangered Sumatran rhinoceros (*Dicerorhinus sumatrensis*). *Mol Ecol Notes*. 4:194–196.

Steiner CC, Houck ML, Ryder OA. 2017. Genetic variation of complete mitochondrial genome sequences of the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). *Conserv Genet*. 19:397–408.

Stoops MA, Campbell MK, DeChant CJ, Hauser J, Kottwitz J, Pairan RD, Shaffstall W, Volle K, Roth TL. 2016. Enhancing captive Indian rhinoceros genetics via artificial insemination of cryopreserved sperm. *Anim Reprod Sci*. 172:60–75.

Toews DP, Brelsford A. 2012. The biogeography of mitochondrial and nuclear discordance in animals. *Mol Ecol*. 21:3907–3930.

Tsangaras K, Ávila-Arcos MC, Ishida Y, Helgen KM, Roca AL, Greenwood AD. 2012. Historically low mitochondrial DNA diversity in koalas (*Phascolarctos cinereus*). *BMC Genet*. 13:92.

van Strien NJ, Manullang B, Sectionov, Isnan W, Khan MKM, Sumardja E, Ellis S, Han KH, Boeadi, Payne J, Martin BE. 2008. *Dicerorhinus sumatrensis*. IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1. Available from: http://www.iucnredlist.org.

Wright S. 1931. Evolution in Mendelian populations. *Genetics*. 16:97–159.

Zafir AWA, Payne J, Mohamed A, Lau CF, Sharma DSK, Alfred R, Williams AC, Nathan S, Ramono WS, Clements GR. 2011. Now or never: what will it take to save the Sumatran rhinoceros *Dicerorhinus sumatrensis* from extinction? *Oryx*. 45:225–233.