

Genetic Diversity and Estimated Geographical Origin of Critically Endangered Captive Black Rhinoceros in Japan: Implications for Future Conservation and Breeding

Authors: Rijja, Mohamed M.S., Sato, Yu, Ramadan, Sherif I., Nonoue, Noriyuki, and Inoue-Murayama, Miho

Source: Zoological Science, 43(6)

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zs260015>

The BioOne Digital Library (<https://bioone.org/>) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (<https://bioone.org/subscribe>), the BioOne Complete Archive (<https://bioone.org/archive>), and the BioOne eBooks program offerings ESA eBook Collection (<https://bioone.org/esa-ebooks>) and CSIRO Publishing BioSelect Collection (<https://bioone.org/csiro-ebooks>).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Genetic Diversity and Estimated Geographical Origin of Critically Endangered Captive Black Rhinoceros in Japan: Implications for Future Conservation and Breeding

Mohamed M.S. Rijja^{1,2,3}, Yu Sato³, Sherif I. Ramadan⁴, Noriyuki Nonoue⁵,
and Miho Inoue-Murayama^{3*}

¹Graduate School of Science, Kyoto University, Sakyo, Kyoto 606-8224, Japan

²Forensic Wildlife DNA Laboratory, Government Chemist Laboratory Authority,
Dar es Salaam, Tanzania

³Wildlife Research Center, Kyoto University, Sakyo, Kyoto 606-8203, Japan

⁴Faculty of Veterinary Medicine, Benha University, Moshtohor 13736, Egypt

⁵Hiroshima City Asa Zoological Park, Hiroshima 731-3355, Japan

Black rhinoceros (*Diceros bicornis*) is critically threatened with extinction in the wild due to illegal horn poaching and habitat loss. Captive breeding programs for this species have become crucial insurance for endangered wild populations. Understanding the geographical origin of captive black rhinoceros in Japan and revealing their genetic diversity can aid breeding and conservation strategies. This study included nearly all available individuals in Japan to evaluate their genetic diversity and geographical origin. DNA samples of 21 animals were extracted from samples provided by 10 Japanese zoos. We analyzed haplotypes of the mtDNA D-loop (477 bp) and the diversity of 11 microsatellite (STR) loci, comparing them with previous reports on African wild populations. Our results indicated that the Japanese captive population has slightly smaller genetic diversity ($h = 0.65$) compared to other populations ($h = 0.72\text{--}0.94$) in mtDNA D-loop, while STR markers maintain diversity ($H_o = 0.66$) similar to that of the African wild populations ($H_o = 0.66$). Among four haplotypes found, one and three haplotypes of mtDNA D-loop matched sequences from the Southern and Eastern African haplogroups, respectively. The combination of mtDNA and STR data leads to the conclusion that the Japanese population likely originated from Kenya, Tanzania, and the Democratic Republic of Congo. The genetic diversity of black rhinoceros in Japan is considered satisfactory, with the potential to further enhance genetic variation among breeding combinations without needing external supplementation. Based on these results, caution should be exercised when selecting this population for international translocation or reintroduction to safeguard captive breeding of the species.

Key words: conservation genetics, *Diceros bicornis*, ex situ population, geographic provenance, captive breeding management

INTRODUCTION

Wildlife conservation interventions are essential for safeguarding biodiversity by tackling threats to species' survival. They play a critical role in protecting and preserving ecosystems, habitats, and individual species from extinction. One key approach is ex situ conservation, which is ideal to support the recovery of threatened species (Ogden et al., 2020). The principal goal of ex situ management emphasizes creating a sustainable population with robust genetic diversity through controlled selective breeding (Willoughby et al., 2015; Klimova et al., 2022). This strategy aims to prevent inbreeding and captive adaptations (Ivy et al., 2009; El Alqamy et al., 2012), which is crucial for ensur-

ing the successful restoration of the wild population in the event of reintroduction (Foose et al., 2006). In this way, ex situ populations effectively act as insurance for the wild populations, particularly for critically endangered species (Aliaga-Samanez et al., 2023). It is essential to recognize that most captive populations are prone to genetic drift and inbreeding (Willoughby et al., 2015). These issues are typically driven by smaller population size (Conde et al., 2013), low genetic diversity (Atkinson et al., 2018), and skewed breeding success (Edwards et al., 2015). However, long-term viability and self-sustainability for captive animals can be achieved through the movement of individuals among sites, the periodic addition of new individuals, and assisting gene flow through selective breeding (Witzenberger and Hochkirch, 2011). On the other hand, concerns about the geographical origin and possibility of cross-breeding individuals of different subspecies in captive settings have

* Corresponding author. E-mail: murayama.miho.5n@kyoto-u.ac.jp
doi:10.2108/zs260015

received increasing attention among conservation biologists (Hvilsom et al., 2013).

Genetic analyses have advanced the study of captive populations (Ogden et al., 2020; Farré et al., 2022). These analyses simplify the understanding of genetic variation in captive facilities by comparing them to their wild counterparts (El Alqamy et al., 2012; Svengren et al., 2017). They also help to quantify levels of relatedness and inbreeding (Svengren et al., 2017; Atkinson et al., 2018), ascertain geographical origin, and evaluate their taxonomy (Vega et al., 2022). Such insights are particularly valuable for designing effective conservation and management strategies for critically endangered species in small captive conditions threatened by a loss of genetic diversity (Sato et al., 2017; Farré et al., 2022).

Illicit wildlife trafficking and poaching in Africa fueled the unprecedented loss of charismatic megaherbivores in the 20th century (Harley et al., 2005; Berti et al., 2020). Most flagship mammal species, such as African elephant (*Loxodonta africanus*), and black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros (Harper et al., 2013), endured the severest consequences. The black and white rhinoceros species were declared critically endangered and near threatened, respectively (Ferreira et al., 2024) prompting the implementation of stringent plans for their protection. The population of the black rhinoceros declined by 96% from ~65,000 individuals in 1970 (Ferreira et al., 2024) to a minimum of 2354 by the 1990s (Sanchez-Barreiro et al., 2023), leading to fragmented populations across the continent (Harley et al., 2005). This decline was especially notable considering historical estimates of nearly 100,000 individuals at the start of the 20th century (Emslie and Brooks, 1999). The survival of such small in situ populations is increasingly threatened partly due to demographic, environmental, anthropogenic, and genetic factors such as bottleneck and founder effect, which lead to inbreeding, affecting conservation strategies (Lacy, 1995; Frankham et al., 2009; Klimova et al., 2022). With approximately 6487 black rhinoceros remaining in their natural habitat (Ferreira et al., 2024), and with a projected accelerated risk of extinction by 2036 (Haas and Ferreira, 2016), captive breeding remains a crucial intervention (Canessa et al., 2016).

Over the decades, captive breeding management has emerged as a powerful alternative for protecting critically endangered species from extinction, revitalizing declining wild populations, and supporting biological conservation (Frankham, 2008). Approximately 61 zoos worldwide host black rhinoceros, with most captive programs located in Europe, followed by North America, with a smaller proportion in Asia. In Europe, a subspecies of black rhinoceros (*D. b. michaeli*) recorded an increase in captive individuals from 43 in 1985 to 90 in 2024 (Elsner-Gearing et al., 2024).

Understanding genetic variation is fundamental in planning future conservation strategies for both captive and wild populations (Ogden et al., 2020, for scimitar-horned oryx; Muya et al., 2011, for eastern black rhinoceros; Luo et al., 2024, for golden snub-nosed monkey). Several studies have assessed the genetic diversity of wild populations of critically endangered black rhinoceros to inform conservation management (Muya et al., 2011; Anderson-Lederer et al., 2012; Kotze et al., 2014; Moodley et al., 2017; Mellya et al.,

2023; Stanbridge et al., 2023). For captive individuals, Brown and Holden (2000) examined the genetic diversity of captive black and white rhinoceros in Australia. More recently, Elsener-Gearing et al. (2024) reported on the genetic variation of captive critically endangered eastern black rhinoceros in European zoos. Given the significant survival threats facing the wild populations (Harley et al., 2005), genetic studies of captive black rhinoceros should be prioritized, since the earliest breeding programs established for large mammals involved rhinoceros taxa (Foose, 1983).

Japan has made a remarkable contribution to wildlife conservation efforts over the past several decades, with over 139 zoos and aquarium facilities forming the Japanese Association of Zoo and Aquariums (JAZA). Nearly 150 endangered and native species sourced worldwide are collectively managed by Japanese zoos and aquariums, including wild animals from African savannah ecosystems. They include mammal species such as the black and white rhinoceros, African and Asian elephants, lions, cheetahs, leopards, giraffes, zebras, and hippopotamuses, each with varying IUCN conservation statuses. Notably, captive propagation of the critically endangered black rhinoceros in Japan began in the mid-20th century. Originating from four presently deceased wild-born founders, the size of this captive population has fluctuated over time due to natural mortality and the exchange of individuals with the international facilities within the integrated conservation program. Currently, there are 21 individuals distributed across 11 Japanese zoos, with evidence of breeding spanning four generations.

Before this study, the genetic variations within the Japanese zoo population of black rhinoceros were unknown. This lack of information meant that well-informed selective breeding aimed at preserving unique wild genetic variants was impossible (Ballou et al., 1995), likely contributing to further genetic erosion in captivity. In addition, there has been limited insight into the potential wild geographical origins of the captive black rhinoceros in Japan. Notably, neither local nor international studbook records provide conclusive information regarding the wild ancestry of the Japanese population. Historical editions of the international studbook published between 1981 and 2001 contain inconsistent details about the origin of one of the founding individuals of this captive population. These revisions were possibly motivated by the earlier efforts to taxonomically classify these captive individuals, consequently introducing uncertainty regarding their subspecies category and wild place of origin. This information gap has hindered efforts to enhance genetic diversity by incorporating wild sources (where possible) and to identify and select ideal candidates for translocation or reintroduction from the Japanese zoo population. In Japan, black rhinoceroses are managed as a single conservation unit, with pedigree-assisted dispersal of individuals across captive settings for possible mating.

To date, no genetic studies have evaluated genetic variations in Japan's zoo population of black rhinoceros to determine their conservation value. This information gap persists despite the well-established fact that smaller captive animal groups are highly vulnerable to lower genetic diversity due to bottleneck effects, diminished survival viability, and inbreeding (Frankham et al., 2017). Furthermore,

efforts to uncover the potential wild habitats of captive black rhinoceros in Japan have been lacking, which impedes developing and integrating ex-situ—in-situ management plans in one plan approach. The current study aims to improve the overall understanding of conservation genetics for captive critically endangered black rhinoceros to support this species recovery program. Documenting conservation genetic parameters of black rhinoceros in Japan for the first time can assist in determining effective conservation interventions for this species and may also provide insights for other endangered species. In this study, we utilized the same mitochondrial DNA (mtDNA) control region and micro-satellite (STR) panels previously employed to examine wild black rhinoceros (Moodley et al., 2017), to characterize the genetic diversity and population genetic structure, and to estimate the geographical origin of the captive individuals in Japan.

We hypothesized that (i) the captive breeding population of black rhinoceros in Japan originated from a wild population in Kenya (East Africa), based on available local studbook data and (ii) the genetic diversity of the captive population in Japan is less than that of the wild population in Kenya due to small founding size. Our findings provide valuable genetic insight into ex-situ conservation of black rhinoceros in Japan, offering a basis for future conservation and breeding approaches by relying on genetic information.

MATERIALS AND METHODS

Ethics statement

All methods were carried out in accordance with relevant guidelines and regulations. The study was performed in compliance with the ARRIVE guidelines. All experimental protocols were approved by the ethics committee of the Wildlife Research Center of Kyoto University, and all sample collection and experiments were conducted with permission from the ethics committee (Approval number: WRC-2023-010A).

Study area and target population

Japan is not a natural habitat for the black rhinoceros, but captive propagation of this species dates back to 1952. Originating from Africa, black rhinoceroses were introduced to Japan as a protection strategy in response to unprecedented uncertainties in their wild habitat. We obtained samples from 10 Japanese zoos, each managing between one and three black rhinoceroses as a single population with cohesive reproductive strategies.

Sample collection

We collected a total of 21 samples of black rhinoceros (20 alive and 1 dead) kept in Japanese zoos between March and August 2023. One individual was not sampled; however, samples from its parent were obtained. We included one tissue sample collected from a female who died in 2019. All of the sampling was conducted by zookeepers at each zoo. In total, our sample set included blood ($n = 11$), feces ($n = 9$), and tissue ($n = 1$), representing 20 living individuals who provided blood and fecal specimens and one dead individual for the tissue specimen. All of the samples were collected following proper veterinary protocols. Of the 20 live individuals sampled, nine were males and 11 were females. The samples included 13 mother-offspring and eight father-offspring relationships (see Supplementary Table S1).

DNA extraction

DNA from blood and tissue samples was isolated using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. Fecal DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA). Extracted DNA samples were stored at 4°C until further down-

stream processing.

Genotyping

A 477 bp fragment of the mtDNA control region (also known as D-loop) was amplified using the primers mt15996L (5'-TCCACCAT CAGCACCCAAAGC-3', Campbell et al., 1995) and mt16502H (5'-TTTGATGGCCCTGAAGTAAGAACCA-3', Moro et al., 1998) as described by the workflow of Moodley et al. (2017). We also surveyed nuclear DNA (nDNA) diversity by genotyping 11 STR loci following previous research (Moodley et al., 2017). Special attention was given to the genotyping of fecal samples, with each analysis replicated three times to confirm allele calls (see Supplementary Text S1 for more details on PCR conditions and capillary electrophoresis).

mtDNA D-loop analysis

Diversity metrics for the mtDNA D-loop were calculated by determining haplotype diversity (h) (Nei, 1987) and nucleotide diversity (π) (Nei and Tajima, 1981) using the DnaSP v6 software (Rozas et al., 2017). Moreover, the evolutionary relationships among mtDNA haplotypes identified in this study and those previously reported in African wild populations were estimated by reconstructing a phylogenetic tree using maximum likelihood (ML) analyses. HKY+G+1 was selected and used as the best nucleotide substitution model (Hasegawa, 1990), estimated with MEGA to build our ML tree. Bootstrapping with 1000 replicates was conducted to estimate the robustness of internal nodes, assuming an equal substitution rate per site for estimating genetic distance (Kumar et al., 2018). The maternal genetic structure of haplotypes recovered in the Japanese population was determined and compared with African haplotypes (Moodley et al., 2017) to infer the putative geographical origin of the Japanese haplotypes, based on the country of origin of the African sequences. A neighbor-joining network was visualized in PopArt v1.7 (Leigh and Bryant, 2015) using default settings with epsilon = 0 and a constant transition/transversion ratio. This comparison relied on mtDNA haplotypes reported by Moodley et al. (2017) only, because haplotypes reported by Muya et al. (2011) were all included in the comprehensive datasets reported by Moodley et al. (2017), who sampled all of the sites that Muya et al. (2011) studied, and museum specimens, whereas Melly et al. (2023) did not report any new haplotype different from the ones reported by Moodley et al. (2017).

STR analysis

The level of nDNA variation at 11 STR markers was inferred by calculating the number of different alleles per locus (N_a), number of effective alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), and average pairwise relatedness between different mtDNA haplotype groups. These calculations were performed in GenAlEx 6.501 (Peakall and Smouse, 2012). A comparative analysis of nDNA diversities was performed between this study and previous reports (Kotze et al., 2014; Moodley et al., 2017) based on 11 overlapping STR markers. We computed the heterozygosity ratio of each sample as an indicator of individuals' diversity by dividing the total number of heterozygous loci by the count of 11 loci analyzed (Heterozygosity ratio = $\sum [\text{heterozygous loci}] / 11$).

Similar to mtDNA D-loop data, nDNA STR datasets obtained in this study evaluated the evolutionary relationship, population structure, and geographical origin by comparing them with a comprehensive dataset of wild populations as classified by a previous study (Moodley et al., 2017). A genetic distance-based approach using Principal Coordinate Analysis (PCoA) was first implemented on the multilocus data using GenAlEx to assess the demographical structure of the Japanese subpopulation relative to the wild African populations. We performed a Bayesian inference analysis in STRUCTURE v 2.3.4 (Hubisz et al., 2009) to determine the origin of the captive black rhinoceros in Japan based on partitioning. This involved running 10 replicates for each assumed population (K), ranging from 1 to 10, with 10^6 Burn-in periods and MCMC repetitions

per run. The analysis was guided by prior knowledge of 12 population clusters and their geographical assignments (Moodley et al., 2017). The simulation was repeated three times under identical conditions to ensure consistency of results.

The most probable number of population clusters (K) was determined using STRUCTURE SELECTOR (Li and Liu, 2018) following Evanno's method (Evanno et al., 2005), which is based on Delta K using the rate of change in the log posterior probability of the data, LnP (D) (Pritchard et al., 2000) between successive K -estimates. Due to sample size discrepancies among the reference populations, Puechmaillie's (2016) method for selecting the best-fit model (MedMedK, MedMeanK, MaxMedK, and MaxMeanK) for even and uneven datasets was also considered. STRUCTURE output and plots of population clusters were generated using CLUMPAK (Kopelman et al., 2015) through the Structure Selector web interface.

After establishing the number of clusters based on STRUCTURE results, we computed the global genetic differentiation coefficient (F_{ST}) for pairwise genetic distances (Weir and Cockerham, 1984) to assess the extent of genetic differentiation between subpopulations. Additionally, molecular variance (AMOVA) analysis was performed to examine genetic variation within and among subpopulations. All of the computations were performed on Arlequin 3.5 (Excoffier and Lischer, 2010) under the default setting. This analysis was used to supplement the STRUCTURE results using the same datasets. In addition, we estimated the effective population size based on the linkage disequilibrium (LD) method (Waples et al. (2008) using the Ne Estimator software 2 (Do et al., 2014).

RESULTS

mtDNA haplotype distribution and diversity of captive population in Japan

A 477 bp fragment of the mtDNA control region was successfully amplified and sequenced for all genotyped samples. The sequences featured four haplotypes based on 22 polymorphic sites characterized by transition nucleotide substitutions ($A \leftrightarrow G$ or $C \leftrightarrow T$). No evidence of deletion or insertion was observed, retaining 455 monomorphic sites.

A significant evolutionary divergence between white rhinoceros (*C. simum*, an outgroup) and black rhinoceros (*D. bicornis*) clusters was suggested based on a maximum likelihood phylogenetic tree (see Supplementary Figure S1). The haplotypes 2J, 3J, and 4J identified in this study were closely related, separated by a few nucleotides. Haplotype 1J was far from the other three haplotypes (see Supplementary Figure S2).

Among the 21 genotyped individuals, 47% shared haplotype 1J, and 38% shared haplotype 3J, while haplotype 2J was shared by 10% of the individuals, with a small fraction (5%) aligning with haplotype 4J. When analyzing the frequency and distribution of the observed haplotypes across Japanese zoos, the highest number of haplotypes present in a zoo was two across seven zoos, while three zoos exhibited only one haplotype, although the maximum number of individuals in a zoo is three (one zoo) and the average is two.

All of the haplotypes observed in Japan were haplotypes previously reported in the wild population in Africa (Moodley et al., 2017) based on a comparative analysis aligned to previously reported mtDNA haplotypes (see Supplementary Table S2).

Haplotype 3J ($n = 8$), which was common within the captive black rhinoceros population in Japan, was also widely distributed among the wild population in Africa ($n = 24$, haplotype AH19 in the previous report). In contrast, Japan's most common haplotype 1J ($n = 10$), was the least common in the wild sequences ($n = 5$, haplotype AH47). Haplotype 2J ($n = 2$) and haplotype 4J ($n = 1$) were observed in 23 and eight wild samples (Haplotype AH1 and AH14), respectively (Table 1). Moreover, three haplotypes (2J, 3J, and 4J) matched a mixture of historic and modern sequences from East African countries classified as East African (EA) haplogroup. In contrast, haplotype 1J was uniquely similar to historic mtDNA sequences sampled only from Southern Africa (SN) haplogroup, tracing back from historic samples in the late 18th century to the mid-19th century (Moodley et al., 2017).

mtDNA sequence variation was highly structured into three distinct clusters or lineages based on a phylogenetic reconstruction including 67 haplotypes from both Africa and Japan (Fig. 1). This result was consistent with the classification demonstrated in a previous study (Moodley et al., 2017). When examining the geographic origin of haplotypes found under captivity in Japan relative to the wild populations in Africa, the maternal lineage of haplotype 1J matched sequences purported to originate geographically from Zimbabwe. In contrast, haplotype 2J corresponded with mtDNA sequences from Kenya, Tanzania, the Democratic Republic of Congo, Zambia, and Uganda. On the other hand, both haplotypes 3J and 4J were detected in samples from Kenya and Tanzania, with haplotype 3J appearing in a few sequences from Zambia (Table 1; see also Supplementary

Table 1. Distribution of haplotypes recovered in the Japanese captive black rhinoceros population with reference mtDNA dataset retrieved from GenBank.

Japanese haplotype	n in Japan	Properties of African wild sequences matched Japanese captive sequences					
		Previously reported haplotype	n^*	Nature of sequences	Country of origin	Haplogroup	Subpopulation category proximal to Zambezi-Chobe River ecosystem
1J	10	AH47	5	Historic	Zimbabwe	SN	Southern side
2J	2	AH1	23	Historic & Modern	Kenya, Tanzania, DR Congo, Zambia & Uganda	EA	Northern side
3J	8	AH19	24	Historic & Modern	Kenya, Zambia & Tanzania	EA	Northern side
4J	1	AH14	8	Historic & Modern	Kenya & Tanzania	EA	Northern side

*Column n represents the number of wild individuals whose haplotypes matched those of the protocols described in Moodley et al. (2017). Historic describes sampled specimens from museums, and Modern suggests extant species sampled from the wild.

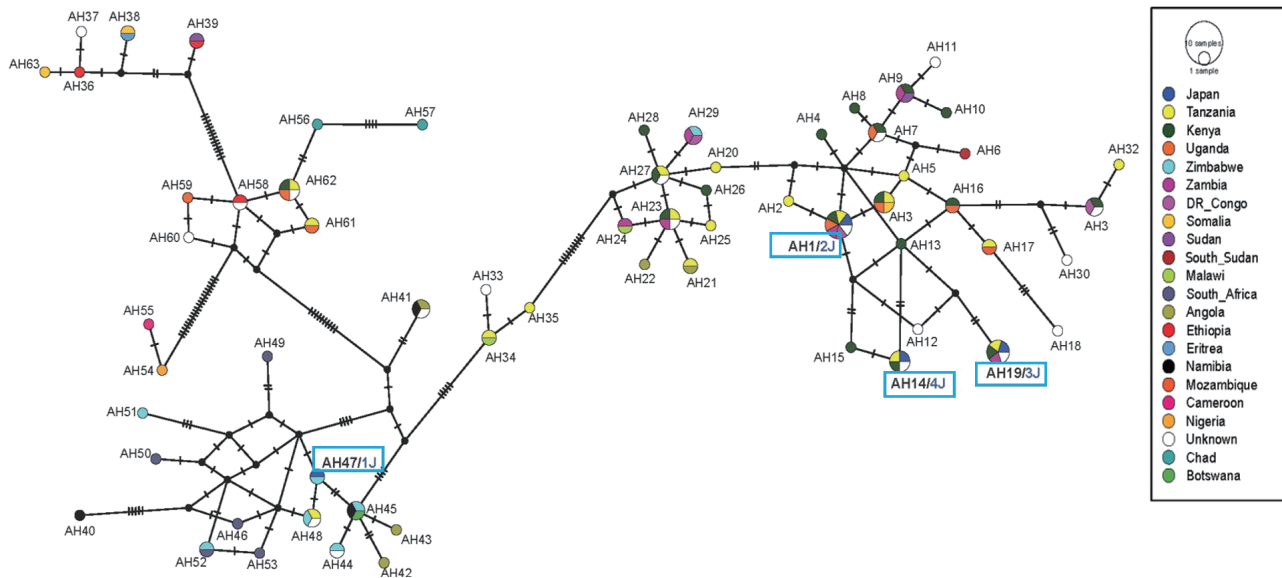


Fig. 1. A neighbor-joining network of 64 combined mtDNA control region haplotypes representing Africa's wild haplotypes reported by Moodley et al. (2017), alongside the haplotypes found across Japanese zoos. Circles represent number of sequences or individuals within each haplotype, with the size proportional to the relative frequency of occurrence of that haplotype in African countries and Japan. Colors represent country of origin of sampled individuals, and hatch marks indicate number of mutations separating the haplotypes. Haplotypes with blue rectangles represent Japanese haplotypes and their complementary haplotypes reported previously in Africa. See Supplementary Table S2 for further description of haplotype names.

Table S2). Thus, Japanese haplotype 2J is the most widely distributed haplotype in the wild.

The overall mtDNA haplotype diversity (h) and nucleotide diversity (π) in the Japanese captive black rhinoceros population were 0.65 and 0.02 respectively (Table 2). This result suggests smaller haplotype diversity in the Japanese captive population compared to that of the historical and modern samples from the EA haplogroup ($h = 0.94$) (Moodley et al., 2017).

Nuclear genetic polymorphism and diversity

In this study, 11 STR markers were genotyped for captive black rhinoceros in Japan ($n = 21$). In addition, no STR genotyping errors were detected in the dataset except that the B1RH37D locus failed for six fecal samples despite three attempted repeats (see Supplementary Table S3). All analyzed STR loci were polymorphic, with the number of alleles detected per locus ranging from two to six, and the effective number of alleles per locus ranged from 1.1 to 4.4 (mean = 2.76 ± 0.26 SE, Table 3). The three markers DB1, DB44, and BR4 exhibited the highest number of alleles per locus, each with six alleles, while DB14, SW35, and RHI32A had the fewest, with two alleles each.

Expected heterozygosity and observed heterozygosity varied considerably across all loci, ranging from 0.09 to 0.79 and 0.1 to 1.00, respectively, with averages of 0.56 ± 0.07 and 0.66 ± 0.09 (Table 3). Although DB14, SW35, and RHI32A had only two alleles each, making them unsuitable for genetic diversity estimation according to standard STR marker criteria (Barker, 1994; Li et al., 2010), we retained all markers to facilitate comparative analysis with wild African data (Moodley et al., 2017). This decision was guided by the sufficient allelic diversity observed across all loci with a mean number of alleles (MNA) of 4.10 ± 0.55 SE (Table 3).

Table 2. mtDNA control region diversity metrics of the captive black rhinoceros population in Japan ($n = 21$) compared to previous studies on wild populations.

Population	n	H	P	h	π	Source
Japanese samples	21	4	22	0.65	0.02	This study
Historic & Modern wild African samples	146	34	50	0.94	0.021	Moodley et al., 2017
Modern Kenya samples	170	16	16	0.73	0.007	Muya et al., 2011
Modern Tanzania samples	102	6	25	0.72	0.017	Mellya et al., 2023

n Number of individuals H number of haplotypes; P number of polymorphic sites h haplotype diversity; π nucleotide diversity. Estimates of wild samples in Moodley et al. (2017) were obtained from the EA haplogroup.

The level of nDNA variation in the captive Japanese population of black rhinoceros ($H_o = 0.75$, $H_e = 0.62$; Table 3) was considerably higher than that reported for the EA haplogroup ($H_o = 0.66$, $H_e = 0.70$; Moodley et al., 2017), both of which were based on the same set of eight STR markers. Moreover, it was also slightly higher than the nDNA diversity reported for the black rhinoceros population in Zimbabwe ($H_o = 0.54$; $H_e = 0.52$; based on 11 markers) (Kotze et al., 2014). The average pairwise relatedness between different mtDNA haplotype groups was < 0.5 (see Supplementary Table S4).

Guided by the hypothesis that different Kenyan subpopulations contributed to this gene pool, as suggested by the mtDNA results, we used the heterozygosity ratio from STR data to examine whether a pattern of genetic diversity similar to that of mtDNA haplotypes would emerge. Our data

revealed that the heterozygosity ratio was higher, at 0.69, among 12 individuals carrying mtDNA haplotype 1J and 2J each, followed by 0.55 for one individual possessing mtDNA

Table 3. Diversity metrics of the captive black rhinoceros population in Japan ($n = 21$) reported based on eight and 11 STR loci as compared with reports of eight STR loci from the wild population. African wild estimates are based on the EA haplogroup in Moodley et al. (2017).

Locus	N_a	N_A	H_o	H_e	F_{IS}
DB1 #	6	4.35	1	0.79	-0.23
DB23 #	4	2.26	0.62	0.58	-0.11
DB44 #	6	2.92	0.67	0.66	-0.01
BR4 #	6	3.87	0.91	0.74	-0.22
BR6 #	5	3.6	0.91	0.72	-0.25
BR17 #	6	4.14	0.91	0.76	-0.19
SW35 #	2	1.21	0.19	0.17	-0.11
RHI32A #	2	1.99	0.62	0.55	-0.14
DB14 ‡	2	1.1	0.1	0.09	-0.05
B1RH2B ‡	3	2.91	0.81	0.66	-0.23
B1RH37D ‡	3	2.02	0.53	0.54	0.02
This study (11 Loci)	4.1	2.76	0.66	0.56	-0.14
This study (8 Loci) #	5.13	3.14	0.75	0.62	-0.16
Wild data (8 Loci) #	10	1.7	0.66	0.7	0.39

N_a number of different alleles; N_A number of effective alleles; H_o observed heterozygosity; H_e expected heterozygosity; F_{IS} inbreeding coefficient

#Markers (8) used for comparison with African wild estimates

‡STR marker was removed for subsequent comparative analysis

haplotype 4J. The lowest heterozygosity ratio, at 0.48, was observed in eight individuals associated with mtDNA haplotype 3J. This analysis considers the six samples that failed at locus B1RH37D, which are homozygous.

The overall mean expected heterozygosity was lower than the mean observed heterozygosity, suggesting an overall deviation from Hardy-Weinberg equilibrium (Sharma et al., 2016). This deviation was due to excess heterozygotes contributed by importing genetically different individuals from the wild in this captivity; however, it is important to note that our 21 animals included some family groups. Furthermore, none of the loci showed a significant deviation from Hardy-Weinberg equilibrium (HWE) after applying sequential Bonferroni correction at $P < 0.05$ (Table 3). The mean inbreeding coefficient (F_{IS}), which indicates the level of inbreeding based on the variance among individuals in a population, was -0.14 ± 0.03 SE (Table 3).

An N_e estimates outcome was 27.4 with 95% CI (Confidence intervals) of 18.2–62.6.

Nuclear genetic structure and phylogeography

Given the uncertain origin of captive black rhinoceros across Japanese zoos, two analytical approaches, individual pairwise genetic distance and Bayesian admixture method, were explored with previously wild-reported STR data (560 individuals) from Moodley et al. (2017) and 21 Japanese captive individuals to clarify the population's origin.

The output of PCoA of 581 total datasets partitioned the samples into two groups with the Japanese population situated mid-way between these two groups (Fig. 2). Specifically, one cluster defined as the “south of the Zambezi-Chobe population” included samples from South Africa,

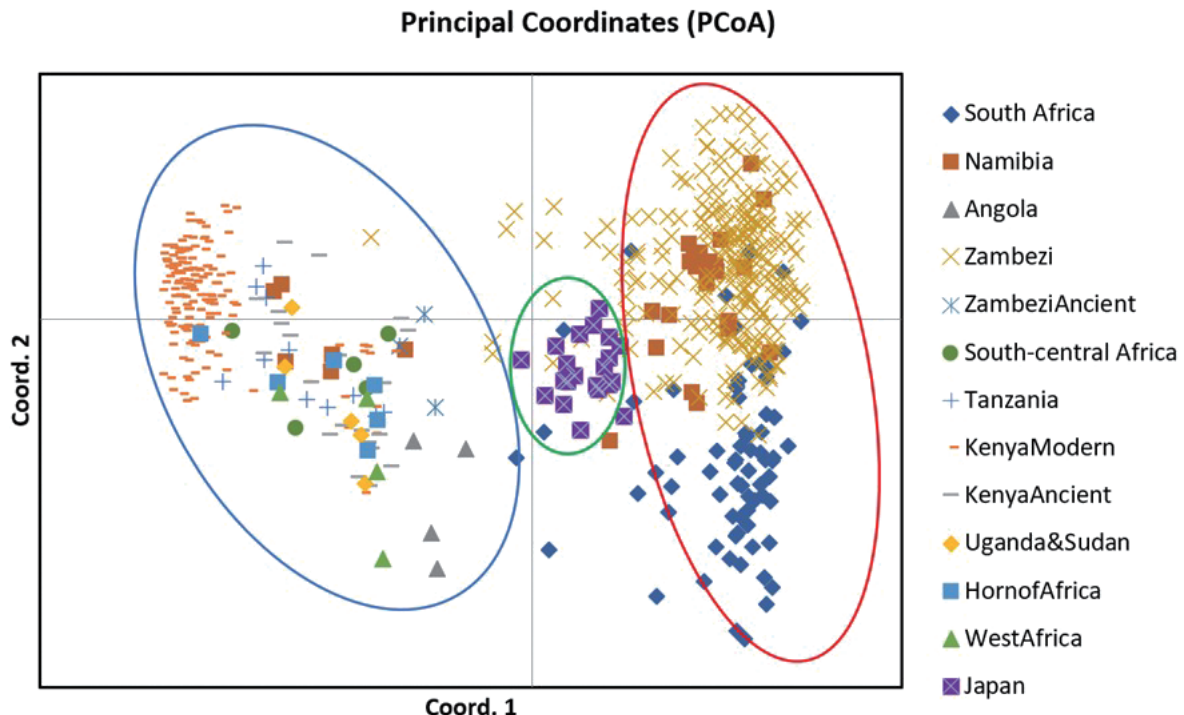


Fig. 2. PCoA result of combined 560 wild and 21 Japanese captive Black rhinoceros STR data. Blue and red circles represent samples derived from North and South of the Zambezi-Chobe River, respectively. The green circle shows the Japanese captive population.

Namibia, South-western Angola, and Zambezi (Zimbabwe). The second cluster, “North of Zambezi-Chobe population”, comprised samples from Zambia, Malawi, Mozambique, Tanzania, Kenya, Malawi, North-eastern Angola, Uganda, South Sudan, Chad, Cameroon, Ethiopia, Somalia, and Sudan (Moodley et al., 2017). Consequently, the PCoA scatterplots were insufficient to assign the Japanese population exclusively to either cluster and did not clarify the captive black rhinoceroses’ ancestry relative to contemporary wild populations.

The best model of the number of genetic clusters (K) in STRUCTURE was evaluated, yielding mixed results for each evaluation method. Evanno’s method (Evanno et al., 2005), which relied on maximum delta (ΔK) values, suggested two distinct clusters as the optimal model, supported by a sharp peak at $K = 2$ (see Supplementary Figure S3). In this method, 13 pre-defined subpopulations, including the Japanese captive subpopulation, were grouped into two genetic clusters, with the Japanese population showing higher genetic affinity to the second cluster (Fig. 3A). This result contradicted the PCoA results (Fig. 2), which placed the Japanese population mid-way. In contrast, estimation based on K plot values by the Puechmaile method (Puechmaile, 2016) indicated that the optimal K was at six clusters based on all four estimators at K values of 6 and 7 (see Supplementary Figure S4). The bar plots at $K = 6$ and 7 coincided with the actual number of clusters observed (Fig. 3B, C).

These outputs highlighted strong genetic differentiation within the wild subpopulations, with the Japanese population showing similar genetic similarity to subpopulations forming the second cluster at $K = 6$ (Fig. 3B). Therefore, the genetic ancestors of the Japanese subpopulations likely

originated from wild individuals in Kenya, Tanzania, Uganda, Zambia, Mozambique, Malawi, Angola, South Sudan, Ethiopia, Somalia, Sudan, Chad, or Cameroon. When the upper K value was considered based on all estimators at $K = 7$ (Fig. 3C), the Japanese population appeared to be genetically isolated from the wild populations.

Estimates from AMOVA revealed the highest genetic variation (80.69%) within the subpopulations compared to the lower diversity expressed among subpopulations (19.31%) (see Supplementary Table S5A). Moreover, significant genetic differentiation was found across all of the subpopulations, with an overall F_{st} value of 0.19 (see Supplementary Table S5B). F_{st} value lower than 0.15 was observed between the Japanese subpopulation and the subpopulations mainly sampled from north of the Zambezi-Chobe River ecosystem (Moodley et al., 2017) (see Supplementary Table S5B). The lack of significant genetic differentiation between Japanese captive individuals and wild populations from subpopulations in Tanzania, Kenya, Uganda, South-central Africa, Horn of Africa, and West Africa further suggests a possible common genetic ancestor among these wild subpopulations and the current Japanese population.

DISCUSSION

The management of the black rhinoceros population in Japan has encountered two common issues that have been prevalent in many captive breeding programs for years (Farre et al., 2022). These issues are a) undefined genetic diversity of the current gene pool and b) uncertainty regarding the geographical wild origin of the founders and subsequently imported individuals that make up the current

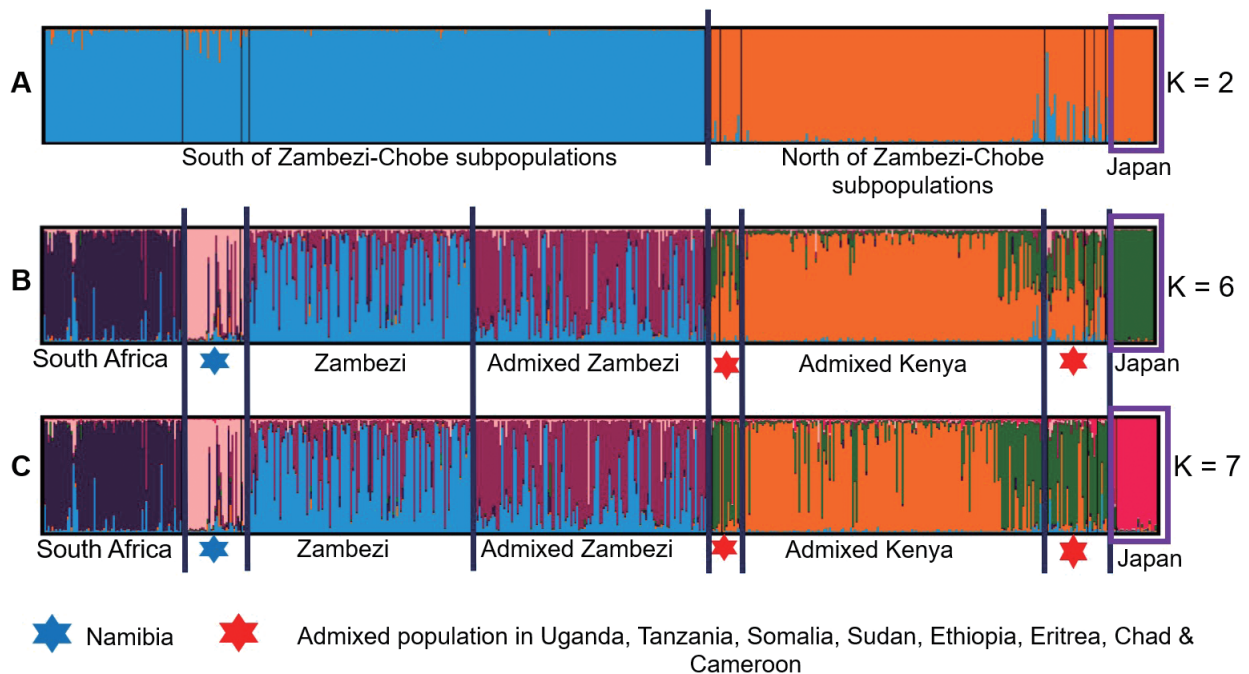


Fig. 3. STRUCTURE plots of black rhinoceros with both African wild and Japanese captive. In a total of 560 wild black rhinoceros samples from Africa (Moodley et al., 2017) supplemented with 21 Japanese captive individuals showing (A) bar plot with two clusters at $K = 2$ suggested based on Evanno’s method, (B) and (C) showing bar plots with six or seven clusters at $K = 6$ or 7, respectively, based on four estimators of the Puechmaile method.

population. These uncertainties have the potential to jeopardize future conservation management and breeding intervention if left unresolved. Harnessing the advances in genetic analyses can help to address these gaps in critical information valuable for conservation breeding management (Ogden et al., 2018). This study assessed levels of genetic variation within the captive population of black rhinoceros in Japan using mtDNA D-loop and nDNA STR markers. To better understand the genetic diversity in the current gene pool, we integrated wild black rhinoceros metapopulation datasets dating back to the 18th century into our analyses. Additionally, we have identified the potential geographical origin of this captive population based on scientific evidence generated from genetic information.

mtDNA D-loop and STR diversity

The mtDNA D-loop diversity within Japan's captive black rhinoceros population is lower than the values reported for the wild populations in Africa (Table 2; Moodley et al., 2017), which is consistent with our expectations. Three possible reasons explain this overall reduction: first, founding members of this captive breed had a predisposed low mtDNA D-loop diversity caused by decreased effective population size due to past genetic bottlenecks in the wild. Given that, successive generations emerging from a severely bottlenecked population most often experience reduced genetic variation (Moodley et al., 2017). Second, sampling bias upon comparing a small captive Japanese population ($n = 21$) to a larger wild population ($n = 146$; Moodley et al., 2017) potentially affects estimates of variation based on differences in the number of examined individuals. Third, the inclusion of historic (museum) samples in the reference metapopulation database of black rhinoceros by Moodley et al. (2017) led to the observed highest variation in the wild, relative to the Japanese population. To address potential sampling bias and the impact of historical samples on mtDNA diversity analysis, we compared our findings with the findings of studies conducted with extant populations with large sample sizes to counteract sampling bias and offset the impact of historical samples (Table 2; Muya et al., 2011; Mellya et al., 2023). Interestingly, comparable mtDNA D-loop diversity levels were observed in this comparative analysis. This suggests that historical samples had a significant effect in comparison with the metapopulation database, which implies that the current gene pool of black rhinoceros in Japan and the extant wild populations share similar modern mtDNA gene variants, with historical variants being lost due to genetic drift that emerged spontaneously from past poaching disasters in the wild.

In contrast, STR loci revealed higher genetic diversity in the study population than in the wild populations based on eight STR loci (Table 3). However, the use of eight STR loci, which revealed a slightly high nuclear diversity, may not fully reflect the true extent of observed estimates of nDNA diversity in Japan. Thus, we performed a comparative analysis similar to mtDNA analysis using the extant wild subpopulation of black rhinoceros based on exactly 11 STR loci reported herein ($n = 301$, $H_o = 0.54$; Kotze et al. [2014]). Surprisingly, slightly high STR diversity persisted with the high number of loci involved despite differences in sampling sizes between the two subpopulations, suggesting that

nDNA was not spared from the negative consequences of past genetic bottlenecks, which contributed to the loss of historical nDNA gene variants. On the other hand, a slightly higher estimated effective population size ($N_e \approx 27$) than the current census population size of 21 individuals reflects more on the retained genetic diversity in Japan from a historically large or diverse founder population. Another possibility is that the Japanese population was a mixture of various genetic backgrounds. This reinforces our hypothesis that the Japanese population originated from multiple regions of Africa, as illustrated in Fig. 3. Thus, immediate genetic management interventions, such as the periodic introduction of genetically diverse individuals and the pairing of diverse individuals, would potentially help this population to avoid future increases in inbreeding and rapid declines in genetic diversity.

Overall, sample size differences in this analysis had minimal effect on both mtDNA D-loop and STR diversity comparison with the extant wild subpopulations, because these captive individuals exhibited relatively high mtDNA diversity with fewer individuals than the extant wild population, which suggests that genetically polymorphic founders or different subspecies that were captured from wider natural habitat ranges in the wild were recruited by chance in the establishment of Japanese captive breeding of black rhinoceros. Similarly, slightly high haplotype diversity was also reported in the founders of the captive endangered Japanese golden eagle (*Aquila chrysaetos japonica*) (Naito-Liederbach et al., 2021), a raptor species in Japan with a wider habitat range (Sato et al., 2017). However, nine founding individuals of a captive Asian lion (*Panthera leo leo*) in Europe from the restricted home range exhibited low nDNA genetic diversity (Atkinson et al., 2017) suggesting that the genetic diversity of the captive population can be adversely influenced by the founders' home range distribution.

The Japanese population may have experienced genetic rescue as 57% of the captive individuals (mtDNA haplotype 1J and 2J) showed greater genetic diversity, among all of the individual categories surveyed. Moreover, individuals possessing mtDNA haplotype 1J, which were more polymorphic than individuals with the remaining haplotypes (Fig. 1), also displayed greater genetic diversity in STR estimations (data not shown), potentially being the source of the observed greater microsatellite genetic diversity in Japan.

On the other hand, comparable nucleotide diversity despite low haplotype diversity (Table 2) illustrates the long-term effects of past genetic bottlenecks. Such bottlenecks often result in reduced genetic variation due to a small number of contributing individuals, leading to similar maternal haplotypes with comparable levels of nucleotide diversity. Additionally, the geographical separation of black rhinoceroses in Japan from their wild counterparts has restricted gene flow, leading to the observed consistent nucleotide diversity. The reduced mitochondrial genetic variation in the Japanese captive population compared to historical and contemporary wild populations is likely due to a historical genetic bottleneck affecting the genetic signatures of Japanese founding animals. Despite this, the captive black rhinoceros population in Japan has maintained good levels of mitochondrial D-loop and STR diversity after several decades of breeding. This outcome reflects effective captive

breeding management in Japan that avoids pairing between closely related individuals.

Degree of inbreeding

The negative mean inbreeding coefficient ($F_{IS} < 0$) presented in this study (Table 3) may possibly suggest excess heterozygosity or non-random mating of inbred individuals according to Kardos et al. (2016). Typically, excess heterozygosity in a small population suggests limited relatedness among the individuals (Kardos et al., 2016). However, this expectation deviates from our findings, as a close familial relationship is observed within the Japanese gene pool (see Supplementary Table S1).

This may only reflect more on the idea that the founders with varying levels of heterozygosity were selected by chance from either the same or different wild subpopulations, since a small number of successful breeding events occurred to significantly contribute to excess heterozygosity or non-random mating system, as substantiated by our data. Moreover, it is important to note that STR markers are less powerful than whole genome sequences in detecting inbreeding (Sato et al., 2023) rendering our STR data not able to define the current inbreeding levels clearly. Pedigree-based inbreeding coefficient offers a precise estimation of inbreeding levels (Pemberton, 2008); however, lack of samples from certain dead individuals within the pedigree hinders its application. Since inbreeding depression is a strong fitness parameter for the survival of a small, captive population, it is important to harness the benefits of genomic assessment through analysis of Runs of Homozygosity (ROH) to quantify inbreeding in captive black rhinoceroses in Japan.

Geographical origin of captive black rhinoceros in Japan

The possible geographical origin of the critically endangered black rhinoceros in Japan was estimated by examining its genetic differentiation relative to the wild population of known origin (Moodley et al., 2017). According to Frankham et al. (2002), an F_{ST} value ≥ 0.15 indicates significant population differentiation whereas an F_{ST} value ≤ 0.15 specifies insignificant population differentiation. The Japanese population showed low F_{ST} values (0.1–0.14) (see Supplementary Table S5B) and strong Bayesian genetic clustering (Fig. 3) with subpopulations of natural habitat originating north of the Zambezi-Chobe River ecosystem as classified by Moodley et al. (2017). The weak population differentiation observed between these groups, as estimated using these methods, suggests a shared genetic ancestor. African countries with individuals that displayed strong genetic affinity to the Japanese captive population were identified based on F_{ST} values and bar plot at $K = 6$ (Fig. 3B).

Furthermore, the mtDNA D-loop phylogeny (Fig. 1), suggested a common maternal ancestor between Japanese haplotypes and wild samples of known geographical origins. The fact that all Japanese haplotypes were detected in both historical and modern wild samples (Table 1) further substantiates this conclusion. A similar approach was used by McDonald et al. (2023) in a recent study to trace the geographic origin of the captive Angolan colobus monkey (*Colobus angolensis*).

By analyzing historical and modern datasets, we identified the geographical origin of the Japanese samples through overlapping results from the genetic markers examined. Both mtDNA D-loop (Fig. 1) and STR marker (Fig. 3) suggested that the founding black rhinoceros in Japan most likely originated from Kenya, Tanzania, and the Democratic Republic of Congo (as shown in Fig. 4). This result could be explained by two factors: first, the genetic variants currently found in Japan were once widely distributed in Eastern-Central African countries but were lost in the Democratic Republic of Congo due to rampant illegal poaching (Harley et al., 2005; Moodley et al., 2017). Japanese mtDNA haplotypes 2J, 3J, and 4J still exist in the wild populations of Tanzania and Kenya, based on a report by Melly et al. (2023) (see Supplementary Table S2). Second, African countries with historically large populations of wild black rhinoceros (Kenya and Tanzania) provided valuable breeding animals for captivity, likely due to their presumed high genetic diversity (Moodley et al., 2017).

On the other hand, our data do not provide overall evidence on classifying the Japanese population into two subspecies as the mtDNA comparative analysis suggested. STR analysis supports the Japanese gene pool as one population north of the Zambezi-Chobe River (Fig. 4). Moreover, both mtDNA and STR data suggested haplotypes 2J, 3J, and 4J to be of *D.b michaeli* subspecies, which aligns with JAZA studbook records of Japan hosting eastern black rhinoceros. In our analysis, we failed to accurately confirm mtDNA-based classification of haplotype 1J to *D.b nyasae* due to a lack of STR data for the five sequences (AH 47) matching the haplotype 1J (see Supplementary Table S2), which are currently absent in the wild (Moodley et al., 2017). However, this finding does not rule out the possibility of genetic admixture in Japan, but rather warrants further investigations based on robust genomic approaches to accurately assess the genetic purity of this population.

It is important to note that, scientific discourse surrounding the classification of black rhinoceros subspecies presented by Rookmaaker (2011) has been debated for many years, prompting anticipated re-assessment of black rhinoceros taxonomy (Ferreira et al., 2024). Nevertheless, further analysis based on the whole genomic data is necessary to validate whether this subspecies classification results from historical misclassification (*D.b nyasae* subspecies name is dated back to the 18th century as per Moodley et al. [2017]) or represents true admixture within the existing population. Additionally, genomic analysis could help clarify the genetic relationships among individuals sharing the same mtDNA haplotype, particularly where microsatellite-based pairwise relatedness estimates are inconsistent with historical studbook records (Supplementary Table S4).

Moreover, we could not accurately examine the geographic origin of individuals with haplotype 1J using STR genotypes, as nDNA genotypes for the five ancient sequences corresponding with haplotype 1J were unavailable (Moodley et al., 2017). However, no significant private alleles were found for haplotype 1J individuals in the STR genotypes, implying that these individuals were indistinguishable from others based on STR data (Fig. 3A, B). Therefore, further investigation using whole genome data to resolve the alignment of haplotype 1J with wild sequences of



Fig. 4. Map of Africa showing Sub-Saharan African countries, with marked countries in red plus sign as the possible natural habitats of the captive population of black rhinoceros in Japan.

Zimbabwe origin (SN haplogroup) is recommended.

The PCoA result was excluded from the geographical inference analysis due to clear genetic separation from the wild datasets. This may be attributed to a sample size discrepancy in our comparative analysis, as the PCoA method is sensitive to sample size (Allberry et al., 2024). However, reducing the number of samples for each wild subpopulation to ~25, corresponding to the Japanese data did not change the outcome (data not shown), which limited our ability to determine genetic affinity using this method.

STR data limitations in ascertaining the geographical origin

The wild genotypes data integrated into this comparative analysis were not calibrated beforehand due to international restrictions on the movement of samples between countries for species under CITES Appendix I. The lack of data calibration may introduce an element of machine-based and population-specific allele differences between the two datasets. It is crucial to recognize that seemingly disparate outputs may arise with calibrated genetic data. Therefore, the conclusion regarding geographical origin using STR data should be approached with a high degree of caution.

Future conservation and breeding implications

This study demonstrates that the small captive population of black rhinoceros in Japan has higher genetic diversity

than expected, given the severe demographic decline in the wild and its small population size. That is because selective breeding strategies coupled with periodic pedigree-assisted importation of animals have sustained gene flow and minimized the effects of genetic drift, ultimately restoring genetic diversity (Klimova et al., 2022). This supports Lacy's (1995) argument that breeding practices that mitigate inbreeding within a particular gene pool often contribute to greater genetic diversity. On the other hand, identifying the wild counterparts provides critical genetic baseline data to guide implementation of in situ and ex situ management strategies, which can support this vulnerable small, ex situ population or threatened in-situ population.

Although the current level of genetic diversity in this captive population is promising, the small population size, potential high rate of relatedness, and captive conditions could compromise the survival of future generations. Therefore, preserving current levels of genetic variations and minimizing relatedness is crucial for the conservation of the captive black rhinoceros population in Japan. We propose the following conservation management interventions for the Japanese zoo population of black rhinoceros: (1) maintain periodic movement of genetically diverse individual (s) among local zoo sites to sustain gene flow and prevent loss of current diversity. This strategy can be specifically implemented to help preserve the rare Japanese haplotypes 2J and 4J, which are at high risk of disappearing from the Japanese gene pool if breeding efforts fail due to low repro-

ductive success (Edwards et al., 2015) or if the only individual carrying the haplotype dies; (2) for zoo sites holding individuals of the same sex with a single haplotype, a targeted pairing with individuals of different haplotypes from other Japanese zoos may improve future breeding success and support conservation efforts at these zoos; (3) individuals of Haplotype 1J whose genetic purity is doubtful (Fig. 1 versus Fig. 3 results) shall not be considered for internal or external supplementation based on this neutral genetic information. However, practical, integrative conservation management with other captive populations in Europe and North America can be implemented for individuals with haplotypes 2J, 3J, and 4J. Since the genetic background of the captive black rhinoceros in Japan has been elucidated in this study, collaboration with other global populations can further strengthen conservation efforts for the species.

In conclusion, the genetic survey presented in this study relied exclusively on putative-neutral genetic markers, insufficient to capture the adaptive potential of the population. Despite that, the current gene pool has the potential for self-sustainability, even if external supplementation becomes unrealistic. Minimal genetic changes suggest retention of wild gene variants and limited adaptation in captivity. Selective breeding practices in managing this population by pairing with genetically different individuals from local zoo facilities are likely the most effective strategy for maintaining future sustainability. Identified geographical wild origin supports informed future conservation and breeding interventions. This is the first genetic assessment of Japan's critically endangered captive population of black rhinoceros.

ACKNOWLEDGMENTS

Our special appreciation to all the Japan Zoological Gardens housing black rhinoceros for agreeing to participate in this study, granting us the permit to study this iconic species, and for the generous support in sample collection. These include Ueno Zoological Garden, Osaka Tennoji Zoo, Nagoya Higashiyama Zoo and Botanical Garden, Kanazawa Zoological Gardens, Hitachi City Kamine Zoo, Yokohama Zoological Gardens "Zoorasia", Hiroshima City Asa Zoo, Kumamoto City Zoo, Sendai Yagiya Zoological Park, and Tobe Zoological Park. We also express sincere gratitude to Ms. Hiromi Kobayashi, Wildlife Research Center, Kyoto University for her unconditional technical support and Ms. Kana Arai for English cross-checking. We are especially grateful to Prof. Rob Ogden for his valuable comments, which significantly contributed to the revision of our manuscript. The first author received financial support from the Government of Japan for his MSc studies through the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Scholarship and the Leading Graduate Program in Primatology and Wildlife Science (PWS).

COMPETING INTERESTS

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

MMSR conceptualized and designed the study, performed laboratory analysis with the help of SIR, analyzed and interpreted data with the assistance and discussion of YS. Discussion from the viewpoint of population management in Japanese zoos was added by NN, and MIM mobilized necessary resources and overall supervision. MMSR wrote the first draft of the manuscript, and all authors provided input on previous drafts and approved the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online (URL: <https://doi.org/10.2108/zs260015>)

Supplementary Text S1. PCR conditions, capillary electrophoresis, and data quality analysis information.

Supplementary Table S1. Detailed sample information of captive black rhinoceros in a Japanese zoo.

Supplementary Table S2. Haplotype information for the black rhinoceros samples retrieved from GenBank, and new samples for the captive individuals for this study.

Supplementary Table S3. Records of the microsatellite markers surveyed with corresponding allele for each marker and the 21 samples analyzed.

Supplementary Table S4. Pairwise relatedness table showing all of the 21 individuals with their respective mtDNA haplotype, whose overall mean pairwise relatedness among different groups was < 0.5.

Supplementary Table S5. (A). AMOVA results of 12 wild and 1 Japanese captive population based on 11 microsatellite loci. **(B).** Pairwise population genetic differentiation (F_{ST}) between 12 wild and 1 Japanese captive population based on 11 microsatellite loci.

Supplementary Figure S1. Maximum likelihood phylogenetic tree of combined extant haplotypes recovered in Japan and wild population in Africa ($n = 67$) and one haplotype representing the outgroup sequence.

Supplementary Figure S2. A median-joining haplotype network showing the distribution of four haplotypes observed across Japanese zoos.

Supplementary Figure S3. Evanno's ΔK graph shows the appearance of sharp peak at $K = 2$ as the best optimal model for the identification of genetic partitioning.

Supplementary Figure S4. Kplot graphs showing four estimators by the Puechmille method indicating the optimal K at 7 for MedMean estimator and K at 6 and 7 for the Max Med estimator.

REFERENCES

- Aliaga-Samanez GG, Javarotti NB, Orecife G, Chaves-Congrains K, Pissinatti A, Monticelli C, et al. (2023) Genetic diversity in ex-situ populations of the endangered *Leontopithecus chrysomelas* and implications for its conservation. *PLOS One* 18: e0288097
- Allberry K, Rovie-Ryan J, Ali Nanbg, Elias NA, Darmaraj MR, Thiam Wong CC, et al. (2024) Emerging patterns of genetic diversity in the critically endangered Malayan tiger (*Panthera tigris jacksoni*). *Biodivers Conserv* 33: 1325–1349
- 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
- Atkinson KE, Kitchener AC, Tobe SS, O'Donoghue P (2018) An assessment of the genetic diversity of the founders of the European captive population of Asian lion (*Panthera leo leo*), using microsatellite markers and studbook analysis. *Mamm Biol* 88: 138–143
- Ballou J, Lacy R (1995) Identifying genetically important individuals for management of genetic diversity in pedigreed populations. In "Population Management for Survival and Recovery" Ed by JD Ballou, M Gilpin, TJ Foose, Columbia University Press, New York, pp 76–111
- Barker JSF (1994) A global protocol for determining genetic distances among domestic livestock breeds. *Proc 5th World Congress on Genetics Applied to Livestock Production*, pp 501–508
- Berti E, Monsarrat S, Munk M, Jarvie S, Svenning JC (2020) Body size is a good proxy for vertebrate charisma. *Biol Conserv* 251: 108790

- Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele sizing methods. *Biotechniques* 31: 25–28
- 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
- Brown SM, Houlden BA (2000) Conservation genetics of the black rhinoceros (*Diceros bicornis*). *Conserv Genet* 1: 365–370
- Campbell NJH, 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
- Canessa S, Guillera-Aroita G, Lahoz-Monfort JJ, Southwell DM, Armstrong DP, Chades I, et al. (2016) Adaptive management for improving species conservation across the captive-wild spectrum. *Biol Conserv* 199: 123–131
- Conde DA, Colchero F, Gusset M, Pearce-Kelly P, Byers O, Flesness N, et al. (2013) Zoos through the lens of the IUCN Red List: A global metapopulation approach to support conservation breeding programs. *PLOS One* 8: e80311
- Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, Ovenden JR (2014) Ne Estimator v2: re-implementation of software for the estimation of contemporary effective population size (N_e) from genetic data. *Mol Ecol Resour* 14: 209–214
- Edwards KL, Walker SL, Dunham AE, Pilgrim M, Okita-Ouma B, Shultz S (2015) Low birth rates and reproductive skew limit the viability of Europe's captive eastern black rhinoceros, *Diceros bicornis michaeli*. *Biodivers Conserv* 24: 2831–2852
- El Alqamy H, Senn H, Roberts MF, McEwing R, Ogden R (2012) Genetic assessment of the Arabian oryx founder population in the Emirate of Abu Dhabi, UAE: an example of evaluating unmanaged captive stocks for reintroduction. *Conserv Genet* 13: 79–88
- Elsner-Gearing F, Kretzschmar P, Shultz S, Pilgrim M, Dawson DA, Horsburgh GA, et al. (2024) Admixture and reproductive skew shape the conservation value of ex-situ populations of the critically endangered eastern black rhino. *Conserv Genet* 25: 897–910
- Emslie R, Brooks M (1999) African Rhino. Status Survey and Conservation Action Plan. IUCN/SSC African Rhino Specialist Group. IUCN, Gland and Cambridge
- 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
- Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour* 10: 564–567
- Ferreira SM, Goodman P, Balfour D, Vigne L, Knight M, Mosweu K (2024) Conservation impacts and the future of the black rhinoceros (*Diceros bicornis*). *Afr J Wildl Res* 54: 81–91
- Farré M, Johnstone C, Hopper J, Kitchener AC, Roos C, King T, et al. (2022) Novel mtDNA haplotypes represented in the European captive population of the endangered François' langur (*Trachypitecus francoisi*). *Int J Primatol* 43: 533–537
- Foose TJ (1983) The relevance of captive populations to the conservation of biotic diversity. In "Genetics and Conservation" Ed by CM Schonewald-Cox, SM Chambers, B MacBryde, L Thomas, Benjamin/Cummings Publishing Company, Menlo Park, CA, pp 374–401
- Foose TJ, Wiese RJ (2006) Population management of rhinoceros in captivity. *Int Zoo Yearb* 40: 174–196
- Frankham R (2008) Genetic adaptation to captivity in species conservation programs. *Mol Ecol* 17: 325–489
- Frankham R, Ballou J, Briscoe DA (2002) Introduction to Conservation Genetics. Cambridge University Press, London
- Frankham R, Ballou JD, Briscoe DA, McInnes KH (2009) Introduction to Conservation Genetics. Cambridge University Press, London
- Frankham R, Ballou JD, Ralls K, Eldridge M, Dudash MR, Fenster CB, et al. (2017) Genetic Management of Fragmented Animal and Plant Populations, Oxford University Press, London
- Haas TC, Ferreira SM (2016) Conservation risks: When will rhinos be extinct? *IEEE Trans Cybern* 46: 1721–1724
- Harley EH, Baumgarten I, Cunningham J, O'Ryan C (2005) Genetic variation and population structure in remnant populations of black rhinoceros, *Diceros bicornis*, in Africa. *Mol Ecol* 14: 2981–2990
- Harper CK, Vermeulen GJ, Clarke AB, de Wet JI, Guthrie AJ (2013) Extraction of nuclear DNA from rhinoceros horn and characterization of DNA profiling systems for white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros. *Forensic Sci Int Genet* 7: 428–433
- Hasegawa M, Kishino H, Hayasaka K, Horai S (1990) Mitochondrial DNA evolution in primates: Transition rate has been extremely low in the lemur. *Mol Ecol* 31: 113–121
- Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. *Mol Ecol Resour* 9: 1322–1332
- Hvilsom C, Frandsen P, Børsting C, Carlsen F, Salle B, Simonsen BT, et al. (2013) Understanding geographic origins and history of admixture among chimpanzees in European zoos, with implications for future breeding programs. *Heredity* 110: 586–593
- Ivy JA, Lacy AM, DeWoody AJ (2009) Methods and prospects for using molecular data in captive breeding programs: an empirical example using parma wallabies (*Macropus parma*). *J Hered* 100: 441–454
- Kardos M, Taylor HR, Ellegren H, Luikart G, Allendorf FW (2016) Genomics advances the study of inbreeding depression in the wild. *Evol Appl* 23: 1205–1218
- Klimova A, Gutiérrez-Rivera J, Sánchez-Sotomayor V, Hoffman JI (2022) The genetic consequences of captive breeding, environmental change, and human exploitation in the endangered peninsular pronghorn. *Sci Rep* 12: 11253
- Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I (2015) CLUMPAK: A program for identifying clustering modes and packaging population structure inferences across K. *Mol Ecol Resour* 15: 1179–1191
- Kotze A, Dalton DL, du Toit R, Anderson N, Moodley Y (2014) Genetic structure of the black rhinoceros (*Diceros bicornis*) in south-eastern Africa. *Conserv Genet* 15: 1479–1489
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35: 1547–1549
- Lacy RC (1995) Clarification of genetic terms and their use in the management of captive populations. *Zoo Biol* 14: 565–578
- Leigh JW, Bryant D (2015) POPART: full-feature software for haplotype network construction. *Methods Ecol Evol* 6: 1110–1116
- Li HF, Song WT, Shu JT, Kuan-Wei C, Wen-Qi C, Wei H, et al. (2010) Genetic diversity and population structure of 10 Chinese indigenous egg-type duck breeds assessed by microsatellite polymorphism. *J Genet* 89: 65–72
- Li YL, Liu JX (2018) STRUCTURESELECTOR: A web-based software to select and visualize the optimal number of clusters using multiple methods. *Mol Ecol Resour* 18: 176–177
- Luo J, Cai Y, Xie Y, Jin X, Yu J, Xu M, et al. (2024) Genetic assessment of eight zoo populations of golden snub-nosed monkey (*Rhinopithecus roxellana*) implication to the conservation management of captive populations. *Evol Appl* 17: e13726
- McDonald MM, Cunneyworth PMK, Anderson AG, Wroblewski E (2023) Wild origins and mitochondrial genetic diversity of Angolan Colobus monkeys (*Colobus angolensis*) in AZA-

- accredited zoos and its implications for ex-situ population management. *Zoo Biol* 42: 668–674
- Mellya RVK, Hopcraft JGC, Eblate EM, Kariuki I, Otiende M, Chuma IS, et al. (2023) Mitochondrial DNA diversity of the eastern black rhinoceros (*Diceros bicornis michaeli*) in Tanzania: implications for future conservation. *Conserv Genet* 24: 905–919
- Moodley Y, Russo IR, Dalton D, Kotze A, Muya S, Haubensak P, et al. (2017) Extinctions, genetic erosion, and conservation options for the black rhinoceros (*Diceros bicornis*). *Sci Rep* 7: 41417
- Moro D, Campbell JH, Elphinstone NS, Baverstock MRP (1998) The Thevenard Island mouse: historic and conservation implications from mitochondrial DNA sequence variation. *Pac Conserv Biol* 4: 282–288
- Muya SM, Bruford MW, Muigai AWT, Osiemo ZB, Mwachiro E, Okita-Ouma, et al. (2011) Substantial molecular variation and low genetic structure in Kenya's black rhinoceros: implications for conservation. *Conserv Genet* 12: 1575–1588
- Naito-Liederbach AM, Sato Y, Nakajima N, Maeda T, Inoue T, Ogden R, et al. (2021) Genetic diversity of the endangered Japanese golden eagle at neutral and functional loci. *Ecol Res* 36: 815–829
- Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York
- Nei M, Tajima F (1981) DNA polymorphism detectable by restriction endonucleases. *Genetics* 97: 145–163
- Ogden R, Ghazali M, Hopper J, Čulík L, King T (2018) Genetic assessments for antelope reintroduction planning in four European breeding programs. *J Zoo Aquar Res* 6: 79–84
- Ogden R, Chuven J, Gilbert T, Hosking C, Gharbi K, Craig M, et al. (2020) Benefits and pitfalls of captive conservation genetic management: evaluating diversity in scimitar-horned oryx to support reintroduction planning. *Biol Conserv* 241: 108244
- 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
- Pemberton JM (2008) Wild pedigrees: The way forward. *Proc Biol Sci* 275: 613–621
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959
- Puechmaille SJ (2016) The program structure does not reliably recover the correct population structure when sampling is uneven: Subsampling and new estimators alleviate the problem. *Mol Ecol Resour* 16: 608–627
- Raymond M, Rousset F (1995) An exact test for population differentiation. *Evolution* 49: 1280–1283
- Rookmaaker K (2011) A review of black rhino systematics proposed in ungulate taxonomy by Groves and Grubb and its implications for rhino conservation. *Pachyderm* 50: 72–76
- Rozas J, Ferrer-Mata A, Sanchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, et al. (2017) DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol Biol Evol* 34: 3299–3302
- Sanchez-Barreiro F, De Cahsan B, Westbury MV, Sun X, Margaryan A, Fontseré C, et al. (2023) Historic sampling of a vanishing beast: population structure and diversity in the black rhinoceros. *Mol Biol Evol* 40: msad180
- Sato Y, Ogden R, Komatsu M, Maeda T, Inoue-Murayama M (2017) Integration of wild and captive genetic management approaches to support conservation of the endangered Japanese golden eagle. *Biol Conserv* 213: 175–184
- Sato Y, Humble E, Ogden R (2023) Genomic data reveal strong differentiation and reduced genetic diversity in island golden eagle populations. *Biol J Linn Soc* 143: 1–10
- Sharma R, Kumar B, Arora R, Ahlawat S, Mishra AK, Tandia MS (2016) Genetic diversity estimates point to immediate efforts for conserving the endangered Tibetan sheep of India. *Meta Gene* 19: 14–20
- Stanbridge D, O'Riain MJ, Dreyer S, Le Roex N (2023) Genetic restoration of black rhinoceroses in South Africa: conservation implications. *Conserv Genet* 24: 99–107
- Svengren H, Prettejohn M, Bunge D, Fundi P, Bjorklund M (2017) Relatedness and genetic variation in wild and captive populations of mountain bongo in Kenya obtained from genome-wide single-nucleotide polymorphism (SNP) data. *Glob Ecol Conserv* 11: 196–206
- Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30: 2725–2729
- Van Oosterhout C, Hutchinson WF, Wills D, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes* 4: 535–538
- Vega R, Hopper J, Kitchener AC, Catinaud J, Rouillet D, Robsomanitrandsana E, et al. (2022) The mitochondrial DNA diversity of captive ruffed lemurs (*Varecia* spp.): implications for conservation. *Oryx* 57: 649–658
- Waples RS, Do C (2008) A program for estimating effective population size from data on linkage disequilibrium. *Mol Ecol Resour* 8: 753–756
- Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358–1370
- Willoughby JR, Fernandez NB, Lamb MC, Iv JA, Lacy RC, DeWoody JA (2015) The impacts of inbreeding, drift, and selection on genetic diversity in captive breeding populations. *Mol Ecol* 24: 98–110
- Witzenberger KA, Hochkirch A (2011) Ex situ conservation genetics: a review of molecular studies on the genetic consequences of captive breeding programmes for endangered animal species. *Biodivers Conserv* 20: 1843–1861

(Received February 18, 2026 / Accepted June 21, 2026 /

Published online October 9, 2026)