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Natural dispersal is better than translocation for reducing risks of inbreeding depression in eastern black rhinoceros (*Diceros bicornis michaeli*)

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Due to increasing anthropogenic impacts, many species survive only in small and isolated populations. Active conservation management to reduce extinction risk includes increasing habitat connectivity, translocations from captive populations, or intensive surveillance of highly protected closed populations. Advances in sequencing technology mean that it is now possible to consider the genomic impacts of such strategies, as a proxy for variation in individual fitness. Using whole genome sequences from critically endangered eastern black rhinoceros (*Diceros bicornis michaeli*), we compare the consequences of different types of conservation efforts, based on cohorts of offspring resulting from parents from different sources. Based on the fraction of the genome in runs of homozygosity (ROH) of different lengths, we found lower inbreeding in offspring of individuals that had either been translocated from ex-situ populations ($F_{ROH>1Mb}$ = 0.047) or dispersed between proximate native populations ($F_{ROH>1Mb}$ = 0.065) compared to the intensively managed closed population from which the migrant moved ($F_{ROH>1Mb}$ = 0.112). However, the benefit of such movement was removed after only a few generations of closed breeding ($F_{ROH>1Mb}$ = 0.149). Although sample size restricted power to detect significance of differences, the relative abundance of highly deleterious mutations was higher for offspring resulting from translocation compared to the other cohorts and this load was sheltered by higher heterozygosity, which could increase risks of inbreeding depression if inbreeding subsequently occurs. In contrast, native dispersers reduced the negative effects of inbreeding without compromising the benefits of past purging of deleterious mutations. Our study highlights the importance of natural dispersal and reiterates the importance of maintaining habitat corridors between populations.

conservation management | endangered species | whole genome sequencing | inbreeding | genetic load

Significance

A common strategy for conservation of small populations of endangered species that have experienced high levels of inbreeding is "genetic rescue" from genetically distinct populations. However, there is a potential trade-off between the fitness benefits of increasing genetic variation and reducing inbreeding and the potential costs of introducing negative mutations that were not previously present in the population. Using the eastern black rhinoceros (*Diceros bicornis michaeli*), here we demonstrate the power of assessing the genomic impacts of past management strategies to inform such conservation decisions. Focusing on cohorts of individuals whose parents had come from different sources, we find that allowing natural dispersal between populations with a long history of inbreeding could be more effective than more expensive long-distance translocations.

Many highly threatened animal species persist in small, isolated patches that are susceptible to inbreeding and loss of genetic variation due to drastic reductions in population size (i.e., bottlenecks or founder effects), which are warning signals of populations at threat of extinction (1). When too few individuals remain in the wild or when there is insufficient genetic variation for natural dispersal to have a substantial impact, active human interventions have been used for genetic rescue (2–4). This includes translocation of individuals between native populations (in situ) or (re)-introduction from captive populations (ex situ) (3). Whole genome sequencing data has the potential to revolutionize genetic rescue because of the expanded inferences possible compared to single-locus approaches. Genomic-scale data can not only inform selection of individuals that could be most beneficial to move for genetic rescue but can also be used to assess the genome-wide consequences of particular management practices (5–14). Additionally, inferences about demographic history can be modeled more accurately based on millions of single nucleotide polymorphism (SNP) loci compared to markers like microsatellites. This is important to enable assessment of the potential impacts of previous bottlenecks to purge deleterious recessive mutations (7, 9, 15–19). Genomic data are also opening up new controversies about the best approach to genetically informed conservation management. For example, artificial management strategies designed to boost wild populations such as establishing founder subpopulations or reintroducing captive animals into the wild could inadvertently negate the long-term benefits of purging deleterious alleles in surviving populations (20), but it has been argued that maximizing genetic diversity is more important to reduce extinction risks than minimizing introduction of potentially harmful mutations (21).

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Whole genome sequencing also has the potential to assess impacts of management on functional, rather than overall diversity, but this also has been controversial, due to the difficulties of directly relating mutations at particular genes to inbreeding depression and fitness (22, 23). Nevertheless, assessment of genome-wide deleterious mutation load as a proxy for fitness can provide powerful insights into the impacts that historical processes have had on small and isolated populations (7, 11, 19, 24–26). Most studies have tended to focus on predicting population-level impacts rather than tracing the effects of individuals with different ancestries due to variation in management practices. A notable exception is a study on kakapos, where crowd-source funding enabled sequencing of nearly every individual in the recovering population and the descendants of individual founders could be traced (13). Most conservation genetics projects will not have such large-scale funding available but even sampling a small number of individuals representing different past management practices has the potential to inform which strategies could be most sustainable for particular species groups.

Here, we use critically endangered eastern black rhinoceros (*Diceros bicornis michaeli*) populations in Tanzania as a model for predicting the relative fitness impacts of ex situ conservation (i.e., translocations from captive or geographically distant populations to the wild) compared to natural dispersal, but focusing on ancestry cohorts of individuals rather than whole populations. The species is critically endangered due to extensive poaching across their natural range, with most surviving individuals restricted to highly protected areas such as zoos, closed sanctuaries, and intensive protection zones (IPZ) (27–30). Active management has so far succeeded in preventing their extinction in the short term, but previous translocations in Tanzania have not been informed by genetics (31) and long-term consequences remain unknown.

Our main aim was to investigate the potential trade-offs between: 1) increasing adaptive potential by introducing new variation into threatened populations; and 2) increasing risks of inbreeding depression (reduction in fitness due to inbreeding) caused by introducing deleterious mutations (genetic load). We hypothesize that the genetic load might have been purged from inbred wild populations (due to severe past bottlenecks and ongoing inbreeding) but "hidden" in captive populations [due to reduced selection under the benign conditions under which they are kept or increased heterozygosity due to mixing individuals from different sources (14)]. We consider the impacts of various management practices on: i) genetic diversity; ii) genome-wide inbreeding; iii) relative deleterious mutational load across cohort classes; and iv) frequency of homozygous and heterozygous deleterious alleles compared to synonymous variation (proxy for realized load based on a model where deleterious mutations are recessive) across the genome of individuals, as well as considering allele frequencies under an additive model. We provide recommendations for managing eastern black rhinoceros populations, as well as more general strategies for genetic rescue of other highly endangered species.

Results

Observational Pedigrees and Cohort Classifications. Based on observational pedigree data (*SI Appendix*, Figs. S1 and S2) from each of the populations in the Serengeti-Mara ecosystem (Fig. 1*A*), we categorized individuals into five cohorts (Fig. 1*B* and Table 1): 1) native, no dispersal (NN)—individuals with two wild parents from the same native population; 2) recent natural dispersal (rND)—first-generation offspring of individuals who dispersed from a native population and mated with an individual in their new resident population; 3) old natural dispersal (oND)—2nd or 3rd generation offspring of a parent that had dispersed into a different native population and mated with residents but where there has been no subsequent gene flow into that lineage; 4) assisted dispersal (AD)—individuals where one parent was native to the population and the other was born in captivity (here defined as an ex situ managed population); and 5) translocated captive (TC)—individuals that were translocated to Tanzania but whose parents were both born in captivity.

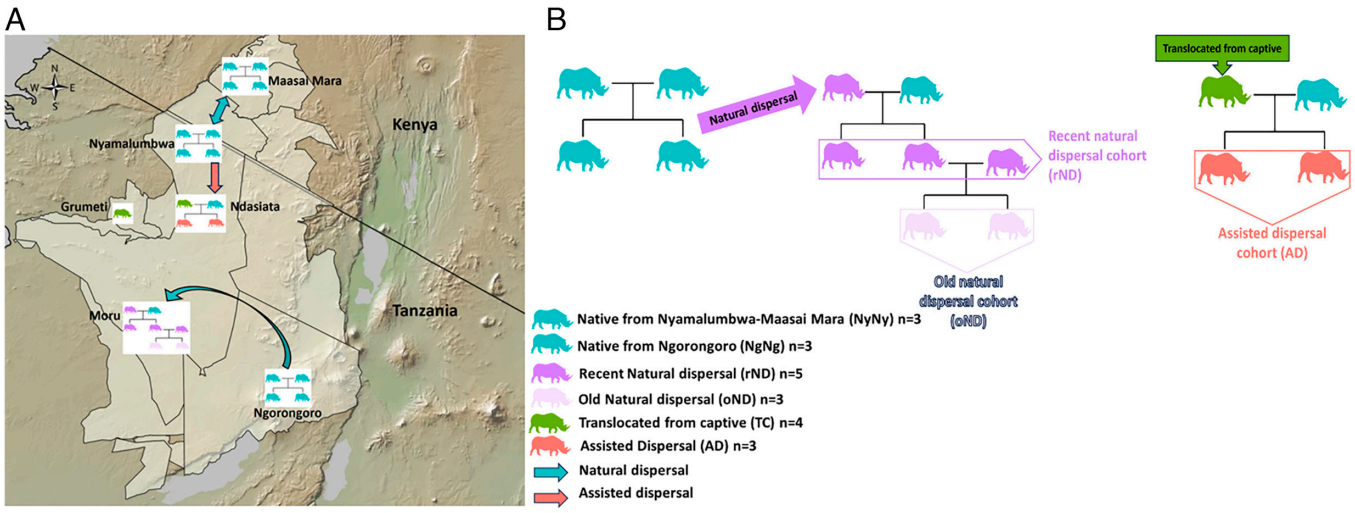

Fig. 1. Sampling details of black rhinoceros used in the study. (*A*) an illustrative map of the Serengeti-Mara ecosystem, situated on the Tanzania–Kenya border in East Africa. *Inset* shows the number (n) of individuals sampled for each cohort, as defined in (*B*), which is a schematic diagram defining the ancestry cohorts based on observational pedigrees. The native cohort comprises indigenous black rhinoceros that remained in the area following poaching incidents in the 1970s and 1980s. The two natural dispersal categories are intended to investigate the impacts of individuals moving naturally between native populations, assessed across multiple generations after the dispersal event. Both cohorts descend from a single male that dispersed from Ngorongoro to Moru in 1994, mated with the two remaining females in that population and remained dominant across multiple generations; he mated with his daughters, grand-daughters, and great-granddaughters. rND denotes the first-generation offspring, along with a mating between the dominant male and a daughter produced by his mating with the resident females. oND encompasses the 2nd generation offspring resulting from matings between the first-generation offspring of the founders, along with an offspring produced by the founding male mating with his grand-daughter. The Translocation Cohort (TC) are individuals reintroduced to Ndasiata and Grumeti from captive populations in South Africa between 2007 and 2022, while the AD Cohort represents offspring resulting from mating between an individual reintroduced from captivity after translocation and native individuals (effectively, hybrids between captive and native).

Table 1. Ancestry of sampled cohorts, indicating the source population of the parents, the type of ancestry (NN = native, no dispersal; rND = recent natural dispersal; oND = old natural dispersal; AD = assisted dispersal; TC = translocated individuals whose parents were from captive populations), the population from which the individual was sampled, and the number of individuals sequenced

Sire	Dame	Ancestry type	Population	N
Ngorongoro	Ngorongoro	NgNg	Ngorongoro	3
Ngorongoro	Moru	rND	Moru	5
Ngorongoro	Moru	oND	Moru	3
Nyamalumbwa	Nyamalumbwa	NyNy	Nyamalumbwa	3
Nyamalumbwa	Ndasiata	AD	Ndasiata	3
Captive	Captive	TC	Grumeti/Ndasiata*	4

*Since only a single translocated individual was available from Ndasiata, this was combined with the adjacent Grumeti population, which was established from the same captive population in South Africa (Thaba Tholo Game Farm).

Individuals presently in Moru are the most inbred because the population had only three founders and the pedigree clearly demonstrates transgenerational mating (*SI Appendix*, Figs. S1*A* and S2*A* and Table S1). However, the single founding male (Rajabu; R21) had dispersed naturally from the native Ngorongoro population and remained the dominant male across multiple generations in Moru, meaning that all rND and oND individuals descended from that sire. The native Ngorongoro population (Ng) experienced a milder bottleneck than Moru but is still characterized by multiple generations of inbreeding (*SI Appendix*, Figs. S1*B* and S2*B*). The records for the other native population in Tanzania (Nyamalumbwa) are not as complete because it is a transborder population connected to the Masai Mara National Reserve in Kenya (*SI Appendix*, Fig. S1*C*), and there has not yet been a common system of monitoring developed between the two countries. However, all individuals were classified as native (NyNy). For the Ndasiata population, founded by translocated South African captive individuals, the samples sequenced were offspring of a native male who had dispersed from Nyamalumbwa (Msafiri; not sequenced) and mated with the translocated females from Thaba Tholo; these were classified as AD (*SI Appendix*, Fig. S1*D*). We also had a sample available from one of the captive founders (Lunar; R9) from Ndasiata, but this was grouped with the translocated captive (TC) individuals from the adjacent Grumeti population (Grumeti Game Reserve) for subsequent analyses because they were sourced from the same captive population (Thaba Tholo).

Genetic Diversity. Using whole genome sequencing (aiming for a range of at least 10× coverage, with an average sequencing depth range after filtering of 7-21×) of 3 to 5 individuals per cohort (21 samples in total), genetic diversity was highest for the TC individuals (Grumeti/Ndasiata), based on both pairwise nucleotide diversity (Fig. 2*A*) and allelic richness (*SI Appendix*, Fig. S3). These individuals had been translocated from a large managed population in Thaba Tholo, South Africa, which includes animals from multiple geographic sources (26), but individuals selected to transfer were based on pedigrees to avoid potentially admixed individuals. The transborder native cohort (NyNy) showed higher genetic diversity than the other cohorts that included only native individuals (NgNg, oND, rND) but was more similar to the hybrids between a male from NyNy and translocated individuals (AD).

There was a significant effect of ancestry cohort on pairwise relatedness within cohorts ($F_{5, 22} = 5.31$, $P = 0.002$; Fig. 2*B*), which confirms expectations from the pedigree: relatedness was significantly higher (based on Tukey's tests) for the AD cohort, which are all siblings, compared to individuals native to the transborder Nyamalumbwa population (NyNy; difference = −0.295) and the two natural dispersal cohorts (oND = −0.217; rND = −0.193). None of the other pairwise differences between ancestry cohorts were significant.

The Principal Components Analysis (PCA) (Fig. 2*C*) indicated separation of the two native populations along both PC1, which explained 19% of the variation, and PC2, which explained 9% of the variation. As might be expected given the geographic proximity of the Ngorongoro and Moru populations (Fig. 1), there was little separation of the NgNg, oND, and rND cohorts from one another, but they were separated from the AD cohort along both axes. The TC cohort was separated from all other cohorts along PC2 but overlapped with both the AD and NyNy cohorts along PC1.

Although the lowest cross-validation error (CV) in an admixture analysis considering complexity values of K from 1 to 5 was at K = 2, there was not strong evidence of genetic structure across the cohorts (*SI Appendix*, Fig. S4). At K = 2 individuals from the native Ngorongoro (NgNg) and Moru (oND and rND) populations showed a different dominant cluster than individuals from the native transborder Nyamalumbwa (NyNy) population and those with ancestors from Thaba Tholo in South Africa (*SI Appendix*, Fig. S5). Although not supported by CV, the increasing complexity at higher values of K did not suggest more admixture in the individuals with ancestors from South Africa (TC, AD) compared to the all-native cohorts. In fact, the "hybrid" cohort (AD), whose father came from Ny and mother from Thaba Tholo, is the only cohort not showing evidence of admixture.

Genome-Wide Inbreeding. Based on runs of homozygosity (ROH) longer than 100 kb, reflective of historical timescales, the native cohorts showed higher evidence of inbreeding than cohorts involving captive parents (AD and TC; *SI Appendix*, Fig. S6*A*). Although there was a significant effect of ancestry cohort on ROH > 100 kb ($F_{5,15} = 3.25$, $P = 0.0344$), no individual pairwise comparisons were significant based on Tukey's tests, perhaps reflecting the small sample sizes and large individual variation observed (32). However, for ROH > 1 Mb, evidence for historical inbreeding ($F_{5,15} = 3.18$, $P = 0.039$) was significantly higher for the oND cohort compared to the AD cohort; interestingly, the means for the TC and rND cohorts were similar, suggesting that natural dispersal can also reduce impacts of past inbreeding (Fig. 3*A* and *SI Appendix*, Fig. S6*B*). Levels of historical inbreeding in the native individuals were ranked as predicted by the observational pedigrees, with Nyamalumbwa individuals (NyNy), which have ongoing gene flow from the Maasai Mara, showing less historical inbreeding than Ngorongoro individuals (NgNg). However, the offspring of older dispersal from Ngorongoro to Moru (oND) were as inbred as the offspring of native Ngorongoro individuals (NgNg).

Considering lengths of ROH reflecting more recent inbreeding (ROH > 10 Mb and 20 Mb; Fig. 3*B* and *SI Appendix*, Fig. S4 *C* and *D*), while there was no significant effect of ancestry cohort on their frequencies ($F_{5,15} = 1.88$, $P = 0.157$; $F_{5,15} = 2.14$, $P = 0.116$), the patterns suggest that the highest levels of inbreeding remain in the oND cohort, whereas the rND cohort showed a substantially lower level of inbreeding that is similar to the

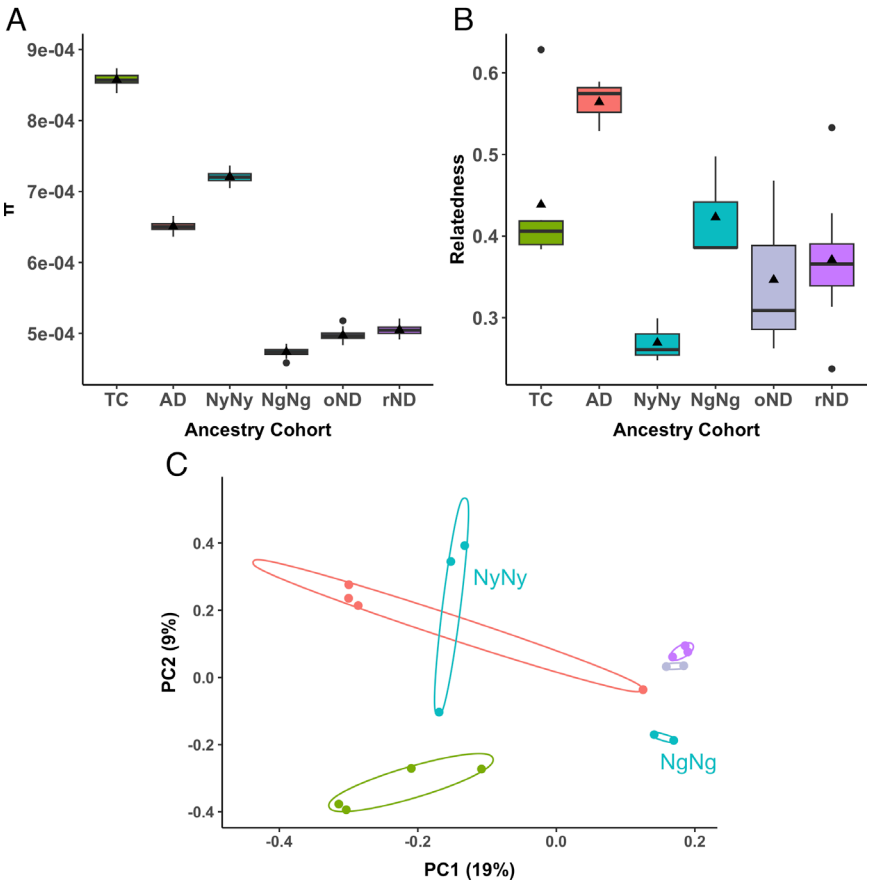

Fig. 2. The impact of different translocations on genetic diversity within and between cohorts. Boxplots showing: (*A*) average number of pairwise differences (π) between individuals in a cohort, with variances estimated based on bootstrap resampling; and (*B*) Pairwise relatedness between individuals. The length of the box signifies the interquartile range, the horizontal line represents the median value within each cohort group, triangles indicate means, whiskers extending from the box depict the range of the majority of the data in each cohort, and black circles beyond the whiskers represent outlier data points. (*C*) PCA based on SNPs of all individuals in the dataset. Ellipses indicate variation among individuals within cohorts. TC = green; AD = red; oND = light purple; rND = dark purple; NN = teal, with the populations (NyNy and NgNg) labeled.

translocated cohorts (TC and AD). This is consistent with the biology: oND are the descendants of a single dominant male who mated with his daughters and grand-daughters across generations, which could have reduced the benefits of natural dispersal apparent in the first generation (rND). At ROH > 10 Mb the rND cohort also showed less inbreeding than the native population that the sire had moved from (NgNg).

Although there was no overall significant effect of cohort ancestry on the proportion of single nucleotide variants (SNVs) that are heterozygous ($F_{5,15} = 2.63$, $P = 0.067$), there were some interesting patterns (Fig. 3*C*). Both recent (rND) and oND cohorts showed higher heterozygosity than individuals that had not dispersed from the population that the dispersing sire had come from (NgNg), which included individuals with the lowest values. However, while AD did not substantially alter heterozygosity compared to the native NyNy cohort from which one of the parents had dispersed, the cohort involving only translocated individuals (TC) had higher average heterozygosity than the others. A sliding window analysis of observed heterozygosity across the genome indicated extensive variation between individuals within cohorts in the distribution of tracks of reduced or zero heterozygosity but more extended tracks in the native compared to translocated cohorts (*SI Appendix*, Fig. S7). However, while the AC cohort showed consistently high heterozygosity, the TC cohort included some extended homozygous tracks, despite overall high heterozygosity across most of the genome.

Recent Demographic History. Recent demographic history was estimated using the distribution of linkage disequilibrium as implemented in the program GONE (33), using only the individuals with two native parents (NN, rND, oND cohorts). The results suggest that eastern black rhino populations had an effective population size less than 3,500 in the last 350 generations (8,400 y) (*SI Appendix*, Fig. S8). Additionally, the population faced a severe bottleneck 80 generations (~1,900 y) ago and then again seven generations (~168 y) ago.

Accumulation of Genetic Load. Based on loss of function mutations (LOF), identified as transcript ablation/splice donor/splice acceptor/stop gained/frameshift/insertion/deletion/splice region variants, relative genetic load (as measured by Rxy) differed between ancestry cohorts (Fig. 4). Notably, although the oND cohort had the highest levels of inbreeding (Fig. 3), it showed a significantly reduced load compared to all of the other cohorts, including native individuals from the sire's source population (NgNg; Fig. 4*A*). Although the earlier generation relatives of the oND individuals (rND) showed a lower load than the cohorts involving individuals translocated from captive populations (TC and AD) and a slightly higher load than one of the native cohorts (NyNy), they did not differ from the native cohort of their founding father (NgNg; Fig. 4*B*). Both the AD and TC cohorts showed increased LOF loads compared to the native cohorts, which did not differ substantially from one another, but the "hybrid" AD cohort had lower load than the first-generation translocated individuals (TC; Fig. 4*C*).

For derived missense mutations, whose impacts are less clear but are often assumed to be more mildly deleterious (19), there were fewer significant differences between cohorts (Fig. 4). The exception was the cohort from the intensively managed ("closed") native population (NgNg), which showed significantly lower missense loads than the transborder ("open") native population (NyNy), both natural dispersal cohorts (oND and rND) and both cohorts involving captive individuals (AD and TC).

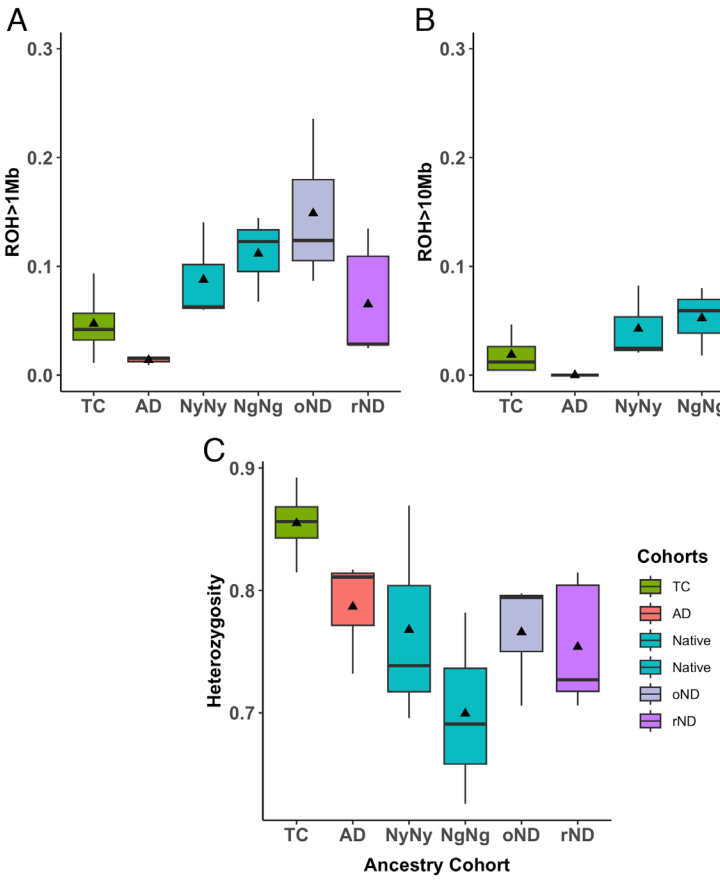

Fig. 3. Boxplots of estimates of inbreeding within ancestry cohorts based on genome wide sequencing. Fraction of the genome in ROH of varying lengths, with (*A*) ROH > 100 kb reflective of historical inbreeding and (*B*) ROH > 10 Mb reflective of recent inbreeding. (*C*) Proportion of heterozygous SNVs in polymorphism-containing loci in relation to ancestry cohorts. Although there was no overall significant effect of ancestry cohort, the translocated cohort showed higher genome-wide heterozygosity than the others, whereas one of the native cohorts included individuals with the lowest values (NgNg).

Annotations of all the missense mutations using the Polyphen2 method (34) revealed that out of the 6,023 total missense mutations tested, at least 27% (1,659 mutations) were predicted to be potentially damaging, while about 8% (534 mutations) had unknown impact and the rest were potentially benign (*SI Appendix*, Table S2), highlighting the potential severity of the situation.

Analysis of the LOF mutations showed the highest enrichment ratios associated with myosin complex and energy-related functions, as well as cytoskeletal functions and anion binding (Table 2). Missense mutations showed higher associations with olfactory and chemical stimulus related functions, as well as cytoskeletal functions. Based on knockout mutations in mice provided in the FUSIL database (35), out of the 729 genes that we classified as LOF and that had survival records available, 155 (21%) were found to be lethal and 82 (11%) were found to be subviable in the homozygous state.

Realized Genetic Load. As a proxy for realized load based on a model where deleterious mutations are recessive (36), there was no overall significant effect of ancestry cohort on the proportion of derived LOF variants that were homozygous (Fig. 5*A*), but the patterns were generally similar to missense (Fig. 5*B*) and synonymous (Fig. 5*C*) variants. Homozygosity of derived variants was highest for the native NgNg cohort and descendants of the male who had moved from that population to Moru (rND and oND) and lowest for the first-generation cohort translocated from captive populations (TC). However, the differences were only significant for TC vs. NgNg for missense (difference = −0.075, P = 0.017; Fig. 5*B*) and synonymous variants (difference = −0.083, P = 0.0384: Fig. 5*C*). There were no significant differences for any of the mutation classes between the AD cohort and the population that their native father came from (NyNy; Fig. 5). The sheltered load (*SI Appendix*, Fig. S9) also closely tracked the synonymous variation, with the highest LOF and missense load in the TC cohort and the lowest in the NgNg cohort; however, the differences were not significant. Similarly, although the additive model (*SI Appendix*, Fig. S9) showed less pronounced differences between cohorts, patterns closely tracked synonymous variation for both LOF and missense loci.

Discussion

Overall, our results suggest that past translocations from captive populations have achieved the goals of genetic rescue of increasing the overall population size (see chapter 4 in ref. 32), as well as the genetic variation and heterozygosity, but at what cost? Introducing new alleles and increased heterozygosity of beneficial alleles could increase adaptive potential (23) but our results also emphasize that introduction of deleterious alleles that have been sheltered in heterozygotes could result in increased inbreeding depression if exposed as homozygotes in the wild; i.e., if active management does not prevent subsequent inbreeding after translocations. This is consistent with the observation from meta-analyses that translocation of captive-born individuals is often less successful than translocation of wild-born individuals (3, 37). Although the molecular measures of genetic load that we used can only provide a proxy for fitness until validated against empirical measures of inbreeding depression (11) and our sample sizes are modest, our results and others (13, 38, 39) emphasize the critical importance of monitoring potential fitness consequences for multiple generations after translocations.

An important contribution of our study is comparison of the two cohorts from the Moru population that shared a male ancestor who had migrated from Ngorongoro: the noted differences in

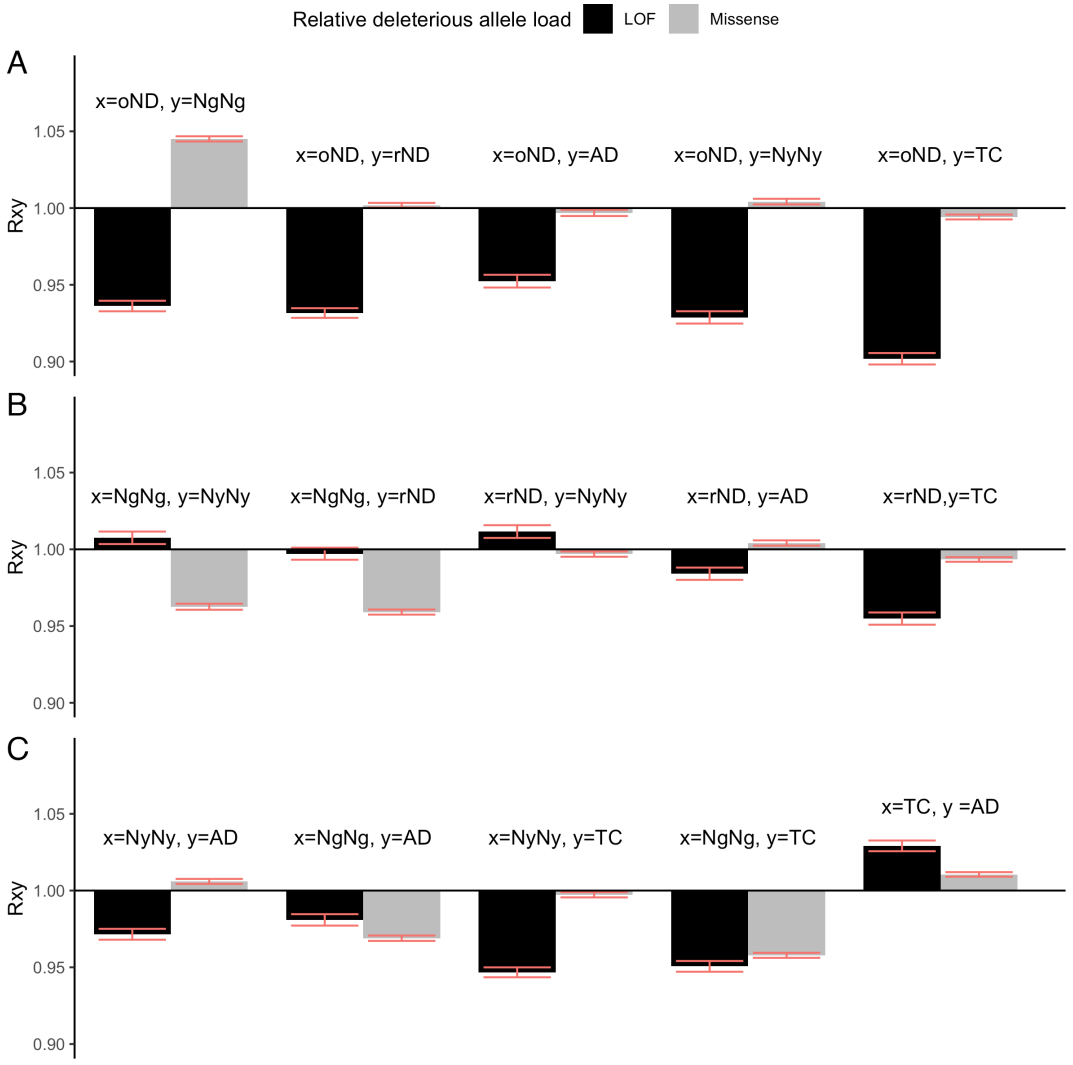

Fig. 4. Relative deleterious allele load (Rxy) for both mildly (missense; gray bars) and LOF (black bars) derived alleles, comparing cohorts of individuals with different ancestries. For each pair of cohorts, relative load is assessed by comparing cohort "x" compared to "y," with values less than one indicating a lower load in the former compared to the latter and values above one vice versa. (*A*) The oND cohort showed significantly lower genetic load for LOF than any of the other cohorts, consistent with purging of the most deleterious mutations. However, missense mutations were similar to the other cohorts, except for NyNy, which showed lower load than the oND cohort. (*B*) The rND cohort (earlier generation relatives of the oND cohort) did not show evidence of purging of LOF compared to the native cohorts (and in fact had a slightly higher load than NyNy) but they did show reduced load compared to the cohorts including captive individuals (AD and TC). The NgNg cohort showed a slightly higher LOF load than the NyNy cohort but again showed a significantly lower missense load compared to both NyNy and rND. (*C*) Native cohorts showed a consistently lower LOF load than both cohorts including captive individuals but the load was higher for both LOF and missense mutations in the first-generation translocated individuals (TC) compared to offspring that were hybrids between translocated and native individuals (AD). The NyNy cohort showed a slightly higher missense load than the AD cohort but not the TC cohort. As for the other comparisons, the NgNg cohort showed significantly lower missense load than both AD and TC.

levels of inbreeding and reduction of genetic load across only a few generations emphasize the initial benefits of allowing natural movement of individuals between native populations but also the potential consequences of allowing ongoing mating between close relatives after any type of genetic mixing. The very severe bottleneck experienced by the Moru population (down to only two females and the one migrant male in 1994) could explain the strong evidence we found for purging of mutations expected to have deleterious consequences (7, 9, 21, 40) several generations of inbreeding after the natural dispersal event. Since fitness is challenging to assess in natural populations, such assessment of genome-wide genetic loads could provide important management perspectives (41), particularly as genomes for more species become better annotated to predict the functional consequences of particular mutations (13). Because of their long generation times, the pedigrees for most black rhinoceros are too shallow to directly estimate fitness after translocations but reproductive rates have been higher in the Moru population than any of the others, despite the bottleneck to a single male and two females (32). The impact that individual males who have sired a disproportionate number of offspring can have on the recovery of highly bottlenecked species has been recorded for other species, such as kakapo (13) and Isle Royale wolves (6). Nevertheless, the short-term benefits of introducing genetic variation from different sources could be compromised in the long term unless there are sustained efforts to reduce subsequent inbreeding and monitor impacts on fitness (40). Critically, our results emphasize the value of monitoring multiple generations of offspring resulting from translocated individuals rather than just focusing on population-level parameters when considering costs and benefits of different management interventions.

Long-distance translocations are expensive not only in terms of financial costs and logistics but also can come at a cost to animal welfare (42). Encouragingly, at least for highly threatened eastern

Table 2. Gene enrichment analysis for LOF and missense mutations, showing the enrichment ratio for particular Biological Process, Cellular Component and Molecular Function categories, the significance of the enrichment, and the mutation type

Function	Enrichment ratio	*P* value	Mutation type
Myosin complex	4.68	4.9E-06	LOF
ATPase activity	2.40	6.4E-09	LOF
ATPase activity, coupled	2.27	1.8E-06	LOF
Actin binding	2.207	2.0E-06	LOF
Nucleoside-triphosphatase activity	1.787	4.7E-06	LOF
Protein-containing complex binding	1.747	6.1E-07	LOF
Cytoskeletal protein binding	1.73	4.9E-06	LOF
Cytoskeletal part	1.560	6.5E-07	LOF
Cytoskeleton	1.52	2.9E-07	LOF
Anion binding	1.38	6.6E-06	LOF
Detection of chemical stimulus involved in sensory perception of smell	2.19	<2.2e-16	Missense
Olfactory receptor activity	2.19	<2.2e-16	Missense
Sensory perception of smell	2.19	<2.2e-16	Missense
Detection of chemical stimulus involved in sensory perception	2.11	<2.2e-16	Missense
Detection of chemical stimulus	2.05	<2.2e-16	Missense
Sensory perception of chemical stimulus	2.03	<2.2e-16	Missense
Detection of stimulus involved in sensory perception	1.20	<2.2e-16	Missense
Detection of stimulus	1.81	<2.2e-16	Missense
Sensory perception	1.55	1.8E-13	Missense
Cytoskeleton	1.32	2.0E-11	Missense

black rhinoceros, we found that both assisted and natural dispersal reduce inbreeding in the target population. However, natural movement of individuals between bottlenecked populations with potentially similar fitness landscapes has resulted in a lower high-impact genetic load than mating between individuals from the potentially different fitness landscapes of natural and captive environments. Native cohorts also consistently showed a lower LOF load than cohorts involving captive individuals. Our demographic analysis suggests that there have been both historic (~1,900 y ago) and recent (~170 y ago) bottlenecks in the native eastern black rhino populations, which is consistent with global analyses suggesting that black rhino populations have been persisting in small populations for at least the last 2,500 y and have been declining for the last 200,000 y (29). Repeated bottlenecks could facilitate purging of deleterious alleles but also increase the realized load (i.e., homozygosity of remaining damaging mutations) (43). Since occasional immigration can be sufficient to maintain genetic diversity, reducing the effects of drift and increasing adaptive potential in small populations (23), we suggest that in severely bottlenecked species such as black rhinoceros, increasing corridors to dispersal could be more sustainable than relying only on costly translocations. Although the fitness impacts of missense mutations are harder to predict (44), there was also evidence of purging for alleles expected to be under weaker selection in the native Ngorongoro cohort compared to all others, even though this was not observed for LOF. In a study comparing captive scimitar-horned oryx that have undergone different genetic rescue strategies, Humble et al. (11) emphasized that weakly deleterious mutations might be expected to be maintained at higher frequencies than lethal mutations and so could accumulate through genetic drift more rapidly after a bottleneck. Therefore, by allowing animals from Ngorongoro to disperse to other parts of the Serengeti ecosystem the load of mildly deleterious alleles could be reduced.

Enrichment analysis of LOF loci provides a warning that potential fitness consequences should be monitored in wild populations that hybridize with captive-bred individuals. This is because deleterious mutations can be "hidden" both by heterozygosity in captive populations and because the selection pressures on wild individuals is very different from captive populations (14), emphasizing the role of the environment in expression of inbreeding depression (45). For example, deleterious alleles associated with the myosin and energy-related functions that we identified could potentially affect muscle-related activities, including heart disease, developmental defects, or anatomical anomalies (46, 47). Intriguingly, the postmortem report for an individual that had been translocated to Grumeti documented evidence of muscle attrition, which resulted in abnormal posture, difficulties to get up and lack of mobility before death (Idrissa Chuma, Godfrey Mwakyusa, and Iddi Lipende, personal observation 2022). Unfortunately, we were not able to obtain a sample for this individual. Such health problems might not be apparent in captive populations due to supplemental feeding or benign environmental conditions under which they are kept, as well as increased heterozygosity resulting from mixing of individuals from different source populations. However, there also could be impacts of the physical stress of translocating individuals to a novel environment. For example, hemosiderosis, which results from accumulation of iron deposits (hemosiderin) in tissues, was found to be prevalent in captive black rhinos from a UK zoo but also in individuals that had been translocated within Zimbabwe from the wild to managed ranches (48). In contrast, high levels of

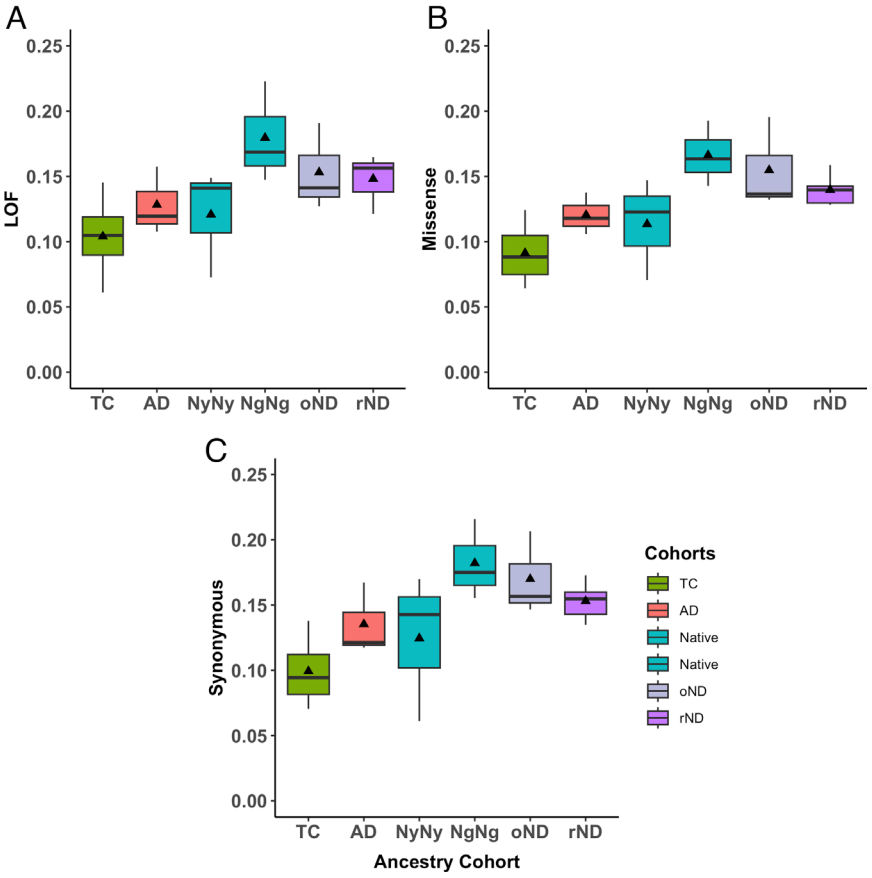

Fig. 5. Proportion of genotyped genic variants of different types that are homozygous (as a proxy for the expressed load) in various ancestries for: (*A*) LOF mutations, expected to be under the strongest selection; (*B*) missense mutations, with unknown effects on fitness; and (*C*) synonymous mutations. There was a significant effect of ancestry cohort for missense ($F_{5,15}$ = 4.023, P = 0.0163) and synonymous mutations ($F_{5,15}$ = 3.15, P = 0.0385) but this was driven predominantly by the difference between the NgNg and TC cohorts. LOF mutations showed the same pattern but the differences were not significant.

hemosiderin were not observed in free-ranging individuals. Such diseases could also be exacerbated by the diet in captive populations. For example, a serious skin disease (similar to necrolytic dermatitis) was identified in nearly 50% of captive black rhinoceros individuals across the 21 zoo populations that were held in the United States in the late 1990s (49). Since the disease had not been reported in wild individuals, the authors suggested that it could have resulted from metabolic changes due to the rich captive diet. The small size of the native populations could also lead to introduced deleterious alleles reaching fixation rapidly, which may push populations to extinction (50). Moreover, a further caution comes from the history of the South African captive rhinoceros that have been used for translocations to Ndasiata and Grumeti: there are reports of hybridization between eastern black rhinoceros (*D. b. michaeli*) from Kenya and southern black rhinoceros (*D. bicornis minor*) from Zululand (51), which diverged between 0.73 and 1.22 Mya (26). Such admixture between subspecies could have introduced deleterious alleles or contributed to sheltering of the load due to increased genome-wide heterozygosity. Although the signal was weak, our admixture analysis suggests that the translocated individuals included in our cohorts were not more admixed than native individuals but is consistent with our previous mtDNA analysis suggesting that their maternal lineages were originally east African in origin (31). Interestingly, the clustering of the Nyamalumbwa individuals with translocated individuals (in both the admixture analysis and along PC1 in the PCA) and the lack of admixture in the AD cohort (whose father was from that native population) suggest that the translocated individuals we sequenced are descendants from individuals who had been moved to South Africa in 1961 from Kiboko in the Simba district west of Tsavo National Park, Kenya before active management (e.g., fences) restricted movements between the east African populations (51).

Even though the Serengeti-Mara ecosystem consists of continuous, unfenced protected areas where movement of individuals with the large home ranges typical of rhinoceros should be possible (52), the IPZ strategy, in which animals are artificially pushed back into specific areas of the landscape where they can be easily monitored, reduces any prospect of natural dispersal between different populations (28). Thus, the current management strategy would require modification so that animals are allowed to mix across management boundaries. Previous translocations of eastern black rhinoceros have not considered genetics but our results suggest the potential benefits of capitalizing on the existing variation in the local native populations, rather than relying only on long-distance translocations. This is further emphasized by the observation that the transborder Nyamalumbwa population, for which mixing is allowed with the Kenyan Maasai Mara population, shows lower levels of inbreeding, within cohort relatedness and homozygosity of deleterious mutations than the other Serengeti populations. The observational pedigrees could be used to identify unrelated individuals for translocation (30), but the approach would be more powerful if combined with an assessment of the genetic load. For example, removing dominant males that have contributed multiple generations of offspring (e.g., in Moru and Ngorongoro) could allow a wider range of individuals to breed, as has been suggested for southern white rhinoceros managed in a metapopulation structure in Botswana (53). Genome-wide sequencing data then could be used to model what impacts such a strategy could have on the deleterious mutation load. Nevertheless, our results suggest that sustainable strategies for inbreeding reduction through natural dispersal may be more important than supplementing variation (e.g., increasing heterozygosity) through translocation and reintroduction of captive animals.

Our results showed little effect of management strategies on genetic diversity within cohorts, with a substantial increase in pairwise nucleotide diversity only for individuals whose parents were from captive populations. This is consistent with a recent study using the D-loop of mitochondrial DNA, which suggested that some of the historical maternal diversity in Tanzania had been restored in the populations that included translocated individuals from South Africa or European zoos, rather than introducing completely new variants (31). The study also confirmed that the translocated individuals were from maternal ancestors originally captured from wild populations in Kenya, where many of the lineages still persist. Since Kenyan populations have been found to harbor higher genetic diversity than the Tanzanian populations (26, 30, 31) and there is no fence between the Serengeti (Tanzania) and Maasai Mara (Kenya) management areas, cross-border gene flow could enhance genetic variation. There has already been increased collaboration between rhinoceros management teams in the recent translocation of five eastern black rhinoceros from Ngulia Rhino Sanctuary in Kenya to Ngorongoro in Tanzania for the purpose of increasing diversity, but this was conducted without first obtaining genetic profiles of the translocated rhinos, which would have allowed for prediction of the genetic impacts.

In conclusion, facilitating natural dispersal could be the most sustainable strategy for managing threatened wild populations like black rhinos, which have been sufficiently bottlenecked to purge out some of the most serious genetic load. Corridors that facilitate animal dispersal have the combined benefits of reducing inbreeding without increasing the genetic load while maximizing breeding opportunities with unrelated individuals. While translocations from managed game reserves (similar to captive populations) do reduce inbreeding and increase genetic diversity, they risk increasing deleterious mutation loads, unless sustained efforts are made to ensure inbreeding avoidance (40). Although sample sizes were modest, we found that sheltered load of both LOF and missense mutations closely tracked neutral heterozygosity of derived mutations, suggesting that such risks could be higher for animals sourced from genetically more variable populations. Our comparison of multiple generations after a natural dispersal event emphasizes both the benefits that movement of even a few individuals can make but also the transient nature of genetic rescue approaches, without ongoing connectivity between isolated groups, facilitated by changing management practices to allow for natural mixing. Alternatively, targeted translocations in each generation could reduce the risk of inbreeding within reintroduced populations but this would be more costly both financially and in terms of animal stress. The power provided by whole genome sequence data offers the opportunity to move away from a common assumption of ex situ conservation that supplementation of any genetic variation will reduce extinction risks; instead, we should consider the functional consequences of population mixing on the re-emergence of deleterious alleles, particularly for highly threatened populations (54).

Materials and Methods

Study Area. We focused on the Serengeti-Mara ecosystem on the border of Tanzania and Kenya because the extant populations represent a range of different management scenarios. Black rhinoceros are restricted to five IPZ regions (Fig. 1*A*), where individuals are free-ranging and unfenced but actively monitored by dedicated wardens (28). This intensive management strategy, along with past translocations, has allowed for population size to increase from a low of 24 in 1995 to 171 in 2021 (31). It also means that there are reliable details on population founders and observational pedigrees, as well as detailed information about reproductive rates and survival of all individuals. We collated these data and constructed observational pedigrees for each geographic location in our study using the R package visPedigree (https://github.com/luansheng/visPedigree). We used these pedigrees and detailed knowledge about the history of translocations and migrants to classify individuals into cohorts arising from different ancestries (Fig. 1*B*).

Three populations were established by a small number of native founders after a severe bottleneck: 1) Moru kopjes (Moru) in the central part of the Serengeti National Park (Serengeti), was founded by two females native to the area and one male that dispersed from the Ngorongoro Crater in 1994; 2) in 1990 the Ngorongoro Crater population consisted of 13 native individuals, with two females (mother and calf) being reintroduced from Addo Elephant National Park (South Africa) in 1997; 3) in 1999 Nyamalumbwa-Maasai Mara (Nyamalumbwa) in northern Serengeti (a transboundary population between Kenya and Tanzania) consisted of 10 native individuals (on the Serengeti side of the border). These two populations originated from native black rhinoceros that endured the poaching era. No rhinos have been artificially introduced into this region in either Tanzania or Kenya and there is continuous movement between the two sides of the border, with each individual being closely monitored by both countries. Two populations were formed by reintroduced semiwild and captive individuals: 4) Ndasiata, was founded by individuals translocated from Thaba Tholo Game Farm (South Africa) in 2010 but there has been subsequent mixing with native individuals who had migrated from Moru; and 5) Ikorongo-Grumeti (Grumeti), on the western border of the Serengeti, was founded by a young bull (Limpopo) and cow (Laikipia), born at Port Lympne Wild Animal Park in England. The individuals were translocated to Grumeti Reserve in 2007. Unfortunately, Limpopo was killed in a fight with a bull elephant in 2009. Therefore, in 2018, a young bull (Eric) from San Diego Zoo Safari Park (USA) joined the original female (Laikipia) in the sanctuary but they have not yet produced a calf. Furthermore, in 2019, nine eastern black rhinoceros from Thaba Tholo Game Farm (South Africa) were translocated and released to the wild, composed of five cows and four bulls (two of whom were calves). The Grumeti population does not yet have a pedigree as all the rhinos were part of the first generation resulting from translocations, with all of the individuals we sampled from the most recent translocation in 2019 from Thaba Tholo.

Sample Collection and Sequencing. Tissue samples from ears of rhinos were collected opportunistically when the individuals were chemically captured/immobilized for marking, translocation, ear notching operations, fitting of telemetric gadgets, health treatment, or rescue (*SI Appendix*, Table S1). The samples were stored in absolute ethanol in 30 mL vials and transported to a laboratory, where they were stored in −20 °C until further use. Blood samples were collected for three individuals from Grumeti and stored on FTA cards (Flinders Technology Associates) at 20 °C until further use. DNA from tissue samples was extracted using DNeasy® Blood & Tissue Kits, following the manufacturer's protocol (Qiagen Inc., Paisley UK). From the 102 samples collected (described in ref. 31), we selected 3 to 5 individuals per ancestry cohort for whole genome sequencing (Fig. 1*B*), based on DNA quality and quantity (*SI Appendix*, Table S1).

Libraries for Illumina short-read sequencing were prepared by Novogene, using their in-house DNA Library Prep Set kit. Briefly, genomic DNA was randomly sheared into short fragments (350 bp) which were end repaired, A-tailed, and further ligated with Illumina adapters. The fragments with adapters were amplified using PCR, size selected, and purified. The library was quality checked with a Qubit® 2.0 Fluorometer 2010 (Thermo Fisher Scientific, Cambridge), quantified using real-time PCR and then the size distribution was checked with a 2100 Bioanalyzer instrument (Agilent Technologies Inc., Cambridge). Libraries were sequenced on Illumina short-read platforms (NovaSeq PE 150) with an aim to obtain at least 30 Gbp data per sample. The raw data are available on the European Nucleotide Archive Database (55).

Data Processing, Variant Calling, and Filtering. The raw FASTQ reads obtained from the Illumina platform were end trimmed using default settings of Trim Galore for Illumina (https://github.com/FelixKrueger/TrimGalore). The trimmed reads were mapped to the black rhinoceros reference genome (https://www.dnazoo.org/assemblies/Diceros_bicornis, 2.6 Gbp), using bwa mem (56). The mapped reads were sorted and duplicates were marked with samtools (57). This created the final binary alignment map (BAM) files. The BAM files were then indexed and default settings of Strelka2 germline variant caller (9) was used for identifying

variants. We limited variant calling only to the long chromosomal scaffolds of the rhino genome assembly to avoid biases later when estimating ROH (58). This, however, covers more than 90% of the rhino genome. Overall, we obtained 98,736,095 SNP (97,157,299) and indel loci (1,578,796) before filtering.

The raw variants identified need stringent filtering for population genetic analysis to remove genotyping errors. They were filtered using vcftools (57). We removed all indel variants. Any base with a PHRED quality score less than 30 was removed and genotypes with quality score less than 30 were set as missing. Any site with a minor allele count less than 3 and deviating from Hardy–Weinberg equilibrium with a chi-squared *P*-value of less than 0.05 were removed. We also removed any individual that had more than 80% missing data. We then removed any loci that were missing in at least 25% of the individuals. We identified sex chromosomes, as described by Armstrong et al. (56), which were also removed from analyses to maintain consistency in the estimates for males and females. We then estimated the mean sequencing depth at each locus and retained only those loci that had the mid–95 percentile depth of sequencing. Although there was variation among individuals in terms of sequencing depth and retained loci (*SI Appendix*, Table S1), overall, we retained 1,649,646 loci with SNPs after all the filtering. We call this the filtered set of SNPs.

Estimating Genetic Diversity, Inbreeding, and Heterozygosity. In order to avoid confounding missing genotypes with monomorphic sites in estimation of pairwise nucleotide diversity (pi), an all site vcf was called using the bcftools *call –m –Oz –f GQ* as described in the pixy manual (59). The SNPs were only called for the autosomal chromosomes. Using vcftools, this file was filtered for Indels, PASS flag, minimum quality of 30, genotype quality 30, maximum missingness of 0.25, and Hardy–Weinberg equilibrium with a chi square *P* value of 0.001. Nucleotide diversity was estimated per SNP locus using the *--site-pi* option in vcftools (57) for each cohort; to standardize sample sizes, three random samples from each cohort were used. To bootstrap the estimates, we then sampled 10 million loci with replacement 100 times and estimated the average pi.

To estimate allelic richness, we used a rarefaction-based approach, as implemented in ADZE (60). The filtered set of SNPs were input to ADZE for a maximum haploid sample size G of seven and a TOLERANCE of 0.7. The populations were grouped by ancestry cohorts for the calculations. We used the filtered set of SNPs to estimate pairwise relatedness (PI_HAT). The filtered set of SNPs were input to PLINK v1.9 (61) and the option–genome was selected to estimate pairwise relatedness (PI_HAT). To visualize genetic differentiation between cohorts and populations, a PCA was conducted using the filtered set of SNPs, as implemented in PLINK v1.9 (61) using the option–pca.

The filtered set of SNPs in the vcf file was converted to the bed format using the *–make-bed* option in PLINKv1.9. ADMIXTURE (62) was used to estimate population structure for complexity (K) values from 1 to 5 using default settings as explained in the manual. The option *–cv* was used to estimate the cross-validation errors.

Levels of inbreeding can be predicted across different historical time periods, based on the length of homozygous tracts spread throughout the genome (ROH), under the assumption that recombination will break up linked variants over time (63). We used BCFtools ROH (64) to estimate ROH and then calculate the fraction of the genome in ROH (F_{ROH}) to measure inbreeding, as described by Armstrong et al. (56). For each sample we used the ROH option of bcftools and set –G 30 and the allele frequencies were estimated on the "fly with by setting"*–e* -. We then estimated F_{ROH} for each size class using the formula F_{ROH} = length of genome in ROH/total autosomal length. The filtered set of SNPs were used for estimating ROH.

Overall estimates of heterozygosity of individuals in different cohort categories were defined as the number of heterozygous loci divided by the total loci with data for that individual, as calculated using default values of the vcfstats option of RTGtools (https://github.com/RealTimeGenomics/rtg-tools).

The distribution of heterozygous loci across the genome was estimated using the filtered set of SNPs. The *–window-pi* option along with *–indv* in vcftools was used to estimate heterozygosity in windows of 1,000 bp. The obtained values were then multiplied by 1,000 to obtain the number of heterozygous loci/Kb for each window.

We tested whether ancestry cohort significantly explained variation in the summary statistics using one-way ANOVA, followed by Tukey's tests, performed using the multcomp (65) and emmeans (66) packages, to determine which levels of the variables explained the variation. All statistical analyses were performed in R 4.3.3 (67). The R script and associated data files are available on the University of Glasgow Enlighten database (68).

Estimating Recent Demographic History. We estimated the recent demographic history of eastern black rhinoceros using only the native individuals, by combining the NN, oND, and rND cohorts. The analysis was based on the distribution of linkage disequilibrium as implemented using default parameters in the program GONE (*PHASE = 2, cMMb = 1, DIT = 1, NGEN = 2000, NBIN = 400, MAF = 0.0, ZERO = 1, maxNCHROM = −9, maxNSNP = 50000, hc = 0.05, REPS = 40, threads = 10*) (https://github.com/esrud/GONE) (33) using the filtered set of SNPs. We plotted the harmonic mean of effective population size (*Ne*) obtained for the first 350 generations, using a generation time of 24 y (29).

Identifying Ancestral Alleles to Estimate Relative and Realized Load of Damaging Mutations. We defined ancestral alleles as the most common variant present in taxa related to the black rhino. For this, we used the approach described by Khan et al. (7) and Kardos et al. (69). The genome assembly fasta files of the northern white rhinoceros (*Ceratotherium simum* genbank assembly: GCA_004027795.1), greater Indian rhinoceros (*Rhinoceros unicornis* genbank assembly: GCA_018403435.1), and Sumatran rhinoceros (*Dicerorhinus sumatrensis* genbank assembly: GCA_014189135.1) were downloaded, and the haploid data mapped to the black rhino genome (https://www.dnazoo.org/assemblies/Diceros_bicornis) were used to polarize the data. Specifically, the alleles that are most common in the three species were identified as ancestral, as described by Khan et al. (7).

Under the assumption that derived alleles are expected to more often be deleterious than ancestral variants (70), we used the approach described by Khan et al. (7) to quantify the genetic load. Briefly, the initial set of variants identified from Strelka2 (9) were filtered to remove indels, bases below PHRED quality 30 and genotypes below a score of 30. Individuals with more than 80% missing data were removed. Then, we removed any loci missing in at least 25% of individuals. Loci with minor allele count of at least 1 and falling within the mean depth across all loci in the mid-95th percentile were retained. Loci with F_{IS} values of $-0.5 > F_{IS} > 0.95$ were retained (to remove monomorphic sites and sites with only heterozygous loci) and sex chromosome scaffolds were removed.

The genetic load (i.e., potential relative fitness reduction due to accumulation of deleterious mutations) of pairs of donor and recipient populations was predicted for each cohort type based on both missense (i.e., amino acid changes that retain the function of a protein) and more serious LOF mutations (5, 56, 71). The SNPs obtained were annotated using the black rhinoceros assembly annotation files using Ensembl variant effect predictor (VEP) (72). Loci with missense or LOF mutations, as defined in ref. 29, were identified and the derived allele at these loci was classified as deleterious. All intergenic regions were classified as neutral sites. We randomly selected three individuals from each cohort for estimating mutation loads for all pairs of cohorts to control for the differences in sample size. The R_{XY} method, described by Do et al. (73) and as implemented by Xue et al. (74), was used to estimate load. SD were obtained by 100 rounds of bootstrap.

As a proxy for the realized genetic load within each cohort, we assessed both models considering deleterious mutations as recessive and additive models. To account for differences in the number of loci for each damaging mutation class as well as differences in coverage or genotyping efficiency across individuals, we standardized load estimates by the total number of loci of each class within an individual that could be genotyped. Although this is a simplification (19, 25), we first estimated the expressed load as the proportion of derived homozygous LOF and missense loci in genic regions, and compared these with standardized estimates of homozygosity of synonymous mutations. We estimated the sheltered load as the proportion of loci in each mutational class that were heterozygous. As a proxy for realized load under an additive model, we also calculated the frequency of LOF, missense, and synonymous mutations based on twice the number of loci with derived homozygous mutations plus the number with heterozygous mutations in genic regions, corrected by the total number of genotyped loci of each class. Differences associated with ancestry cohort were tested as for the genetic diversity summary statistics.

Enrichment Analysis. In order to determine what types of processes might be associated with deleterious mutations in the overall population, default settings of WebGestalt (75) (http://www.webgestalt.org) was used for gene enrichment

analysis of both the LOF and missense alleles. *Homo sapiens* was set as the organism of interest, the Over-Representation Analysis was chosen as the method of interest and gene ontology Biological Process, Cellular Component and Molecular Function was the functional database that was chosen. The black rhinoceros genome annotation was uploaded as the reference set. We then uploaded the list of genes containing LOF and missense mutations to run the analysis, with default parameters. The enrichment ratio compares the observed number of genes in the set of interest (e.g., genes with LOF mutations) for a particular category (e.g., myosin complex) to the expected number, which is calculated by dividing the overall number of genes in the set of interest by that in the reference set, multiplied by the number in the reference set for the particular category (76). A hypergeometric test is then used to estimate the significance of the enrichment.

In order to assess the potential for fitness impacts, the LOF variants were additionally compared with the Full Spectrum of Intolerance to Loss-of-function (FUSIL) database, which predicts the proportion of viable, subviable, and lethal mutations based on phenotyping screens of knockout mice by the International Mouse Phenotyping Consortium (35). For the genes hosting LOF mutations, the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC) codes were searched (www.genenames.org) and the obtained gene names submitted to the FUSIL database using the batch query submission form (https://www.mousephenotype.org/data/batchQuery).

The missense mutations were further classified into benign and damaging based on the Polyphen2 method for nonhuman species which is an in-silico prediction based on amino acid substitutions (34). We obtained the predicted protein sequence for each gene with a missense mutation from the *Diceros bicornis* genome assembly on the DNAzoo website (https://www.dnazoo.org/assemblies/Diceros_bicornis) and used the ensembl VEP to list the amino acid substitutions (72). These were then input into the Polyphen2 online server to obtain functional predictions (http://genetics.bwh.harvard.edu/pph2/).

Data, Materials, and Software Availability. The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB82674 (https://www.ebi.ac.uk/ena/browser/view/PRJEB82674) (55), with BioSample accession numbers as listed in *SI Appendix*, Table S1. Summary data files and R scripts are available on the University of Glasgow Enlighten database (https://www.gla.ac.uk/myglasgow/research/enlighten/), under the doi https://doi.org/10.5525/gla.researchdata.1957 (68).

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