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Abstract: The cheetah (*Acinonyx jubatus*) is unusual among felids in exhibiting near genetic uniformity at a variety of loci previously screened to measure population genetic diversity. It has been hypothesized that a demographic crash or population bottleneck in the recent history of the species is causal to the observed monomorphic profiles for nuclear coding loci. The timing of a bottleneck is difficult to assess, but certain aspects of the cheetah's natural history suggest it may have occurred near the end of the last ice age (late Pleistocene, approximately 10,000 years ago), when a remarkable extinction of large vertebrates occurred on several continents. To further define the timing of such a bottleneck, the character of genetic diversity for two rapidly evolving DNA sequences, mitochondrial DNA and hypervariable minisatellite loci, was examined. Moderate levels of genetic diversity were observed for both of these indices in surveys of two cheetah subspecies, one from South Africa and one from East Africa. Back calculation from the extent of accumulation of DNA diversity based on observed mutation rates for VNTR (variable number of tandem repeats) loci and mitochondrial DNA supports a hypothesis of an ancient Pleistocene bottleneck that rendered the cheetah depauperate in genetic variation for nuclear coding loci but would allow sufficient time for partial reconstitution of more rapidly evolving genomic DNA segments.

Dating the genetic bottleneck of the African cheetah

(DNA fingerprint/mtDNA)

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ABSTRACT The cheetah is unusual among felids in exhibiting near genetic uniformity at a variety of loci previously screened to measure population genetic diversity. It has been hypothesized that a demographic crash or population bottleneck in the recent history of the species is causal to the observed monomorphic profiles for nuclear coding loci. The timing of a bottleneck is difficult to assess, but certain aspects of the cheetah's natural history suggest it may have occurred near the end of the last ice age (late Pleistocene, approximately 10,000 years ago), when a remarkable extinction of large vertebrates occurred on several continents. To further define the timing of such a bottleneck, the character of genetic diversity for two rapidly evolving DNA sequences, mitochondrial DNA and hypervariable minisatellite loci, was examined. Moderate levels of genetic diversity were observed for both of these indices in surveys of two cheetah subspecies, one from South Africa and one from East Africa. Back calculation from the extent of accumulation of DNA diversity based on observed mutation rates for VNTR (variable number of tandem repeats) loci and mitochondrial DNA supports a hypothesis of an ancient Pleistocene bottleneck that rendered the cheetah depauperate in genetic variation for nuclear coding loci but would allow sufficient time for partial reconstitution of more rapidly evolving genomic DNA segments.

The African cheetah (*Acinonyx jubatus*), best known as the world's fastest land animal, numbers fewer than 20,000 individuals in its dwindling range of sub-Saharan Africa. During the Pleistocene and Pliocene, at least four paleontological species and four subspecies of cheetahs had a distribution that included North America, Europe, Asia, and Africa (1–5). Toward the end of the Pleistocene, some 10,000–12,000 years ago, a monophyletic cheetah species emerged with a range restricted to portions of central, eastern, and southern Africa. During this period toward the end of the last ice age, nearly 75% of all large mammals that existed in North America, Europe, and Australia abruptly became extinct (6–8). The causes of the late Pleistocene mammalian extinctions are not known; environmental cataclysm or human hunting pressures are possible causes. Modern human occupation of suitable habitats has reduced the cheetah's range and numbers even further (estimated at 10,000–25,000 individuals) to the level where the cheetah has been classified as "Appendix 1, endangered species" by the Convention on International Trade in Endangered Species (CITES) (9).

In the early 1980s, difficulties in breeding of cheetahs in captivity prompted a combined reproductive and genetic analysis of the species (10–12). Several measures of population genetic variation available at that time indicated that the two major subspecies of cheetah (*A. jubatus jubatus* from southern Africa and *A. jubatus raineyi* from eastern Africa) displayed markedly reduced levels of genetic variation rela-

tive to other species (11–15). These measurements included: (i) electrophoretic variation of allozymes and cell proteins resolved by two-dimensional gel electrophoresis; (ii) immunological (surgical skin graft) and molecular [restriction fragment length polymorphism (RFLP)] variation at the feline major histocompatibility (MHC) locus, one of the most polymorphic loci in mammals; and (iii) morphological variation of cranial characteristics (11–15). The results of each of these approaches showed that the cheetah had levels of variation comparable to that of deliberately inbred strains of laboratory mice or livestock. These studies lent support to the hypothesis that the cheetah's ancestors had survived a historic period of extensive inbreeding, the modern consequences of which are 90–99% reduction in measurable allelic variation and remarkable physiologic impairments including increased spermatozoa abnormalities, decreased fecundity, high infant mortality, and increased sensitivity to disease agents (10–16).

Although the evidence for a demographic contraction or population bottleneck(s) followed by inbreeding in the cheetah's history is compelling, the precise timing of such a bottleneck is more difficult to assess (15–17). Because the loci studied previously evolve very slowly (allozymes, structural genes for fibroblast proteins determined by two-dimensional electrophoresis, MHC class I-coding loci), the contraction event could be very recent (e.g., due to overhunting or habitat destruction within the last few hundred years), during the late Pleistocene as part of the large-mammal extinction or even millions of years earlier (i.e., during the Pliocene or Miocene). It does appear that the principal bottleneck(s) preceded the geographic separations between *A. j. raineyi* and *A. j. jubatus* because the two subspecies are markedly similar in both the pattern of genetic monomorphism and consequent reproductive characteristics (10, 15). This similarity is best interpreted as a consequence of historic genetic inbreeding that preceded geographic separation of the subspecies estimated minimally as 200–500 years ago (18). Because nuclear coding genes have selective constraints on mutational accumulation, it would take on the order of millions of years to restore allelic variation that had been reduced to the extent seen in the cheetah (19, 20).

To estimate the timing of the postulated population bottleneck, we examined rapidly evolving molecular genetic loci with the expectation that they might exhibit a measurable level of recovery from a historic monomorphism. Mitochondrial DNA (mtDNA) evolves at a rate 5–10 times faster than nuclear genes in primates and other mammals (21–24). In addition, because of its recombination-free pattern of maternal inheritance, mtDNA is very sensitive to demographic partitions and perturbations such as we believe the cheetah has experienced. Analysis of minisatellite or variable number tandem repeat (VNTR) nuclear DNA (also called DNA fingerprinting) has also proved useful for demonstrating

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Abbreviations: MHC, major histocompatibility complex; VNTR, variable number of tandem repeats; APD, average percent difference; H, heterozygosity.

population contraction as well as historic geographic partitions in free-ranging populations (25–27). Furthermore, minisatellite loci have an unusual source of allelic variation—namely, DNA replication slippage and *inter se* recombination, producing mutation rates 100–1000 times faster than conventional nuclear coding loci (28–32). The extent of measured allelic variation was used to estimate the time required to recover variation from a state of reduced variation. The results support an ancient bottleneck 6000–20,000 years before the present, consistent with a hypothesis of a late Pleistocene near-extinction of the founders of the modern cheetah.

MATERIALS AND METHODS

DNA Analysis. Total genomic DNA was obtained from blood or skin fibroblast cell cultures (11, 12, 15, 33, 34). For estimates of overall genetic diversity within and between species, unrelated individuals were typed on the basis of knowledge of pedigree in captive animals or family structure in free-ranging individuals (15, 34–36). For mtDNA, 1 μ g of genomic DNA was digested with a panel of 28 restriction enzymes including *Acc* I, *Apa* I, *Ava* I, *Ava* II, *Bam*HI, *Bcl* I, *Bgl* II, *Bst*EII, *Bst*VI, *Cla* I, *Dra* I, *Eco*RI, *Eco*RV, *Hind*II, *Hind*III, *Hpa* I, *Hpa* II, *Kpn* I, *Nco* I, *Nde* I, *Pst* I, *Pvu* II, *Sal* I, *Sst* I, *Sst* II, *Stu* I, *Xba* I, and *Xho* I. Digestion products were electrophoresed in 1% agarose gels, capillary-blotted to nylon membrane (Gelman Biotrace, Ann Arbor, MI), and baked for 2 hr at 70°C. After incubation for 2 hr at 37°C in prehybridization solution (50% formamide/1 M NaCl/10 mM Na₂EDTA/50 mM Pipes, pH 6.4/1% SDS/0.02% denatured salmon sperm DNA/0.1% bovine serum albumin/0.1% Ficoll-400/0.1% polyvinylpyrrolidone-360), 1 \times 10⁶ cpm of a ³²P-labeled full-length domestic cat mtDNA clone (37) per ml was added, hybridized overnight, and washed as reported (37). mtDNA fragments were visualized after autoradiography (37, 38). Nuclear minisatellite (also termed VNTR) variation was determined by using cat-specific FCZ8 and FCZ9 minisatellite clones (26) and was quantified as the average percent difference (APD) in band-sharing plus the estimated average heterozygosity (H) (39).

RESULTS AND DISCUSSION

mtDNA. Cellular DNA was extracted from leukocytes or tissue specimens from the free-ranging eastern subspecies *A. j. raineyi* collected in Tanzania and Kenya and from unrelated captive individuals of the southern subspecies *A. j. jubatus* collected from Kruger Park, Transvaal, or Namibia. A total of 91 restriction sites were scored in 74 individuals, representing 505 nucleotides or 3.2% of the 16,500 base pairs in feline mtDNA. Six polymorphic sites were observed from which seven different mtDNA haplotypes were found. The restriction-site pattern and a parsimony network of haplotypes are presented in Fig. 1.

Four of the mtDNA haplotypes, each separated by one restriction site, were found among 35 *A. j. jubatus* specimens. The other haplotypes were found among 39 *A. j. raineyi* samples. The two subspecies did not share any of the haplotypes. The *A. j. raineyi* mtDNA haplotypes were at least two sites apart from each other and the intermediate type in each case was a haplotype found in *A. j. jubatus*. The simplest interpretation of this pattern is that the intermediate *A. j. jubatus* haplotypes “D” and “F” were ancestral to both subspecies and were retained in *A. j. jubatus*, although it is also possible that D and F were derived during isolation of the progenitors of *A. j. jubatus*. The geographic pattern of mtDNA haplotype relationships reflects the observed geographic isolation of the two subspecies. *A. j. jubatus* retained central (and likely primitive) haplotypes, while the *A. j.*

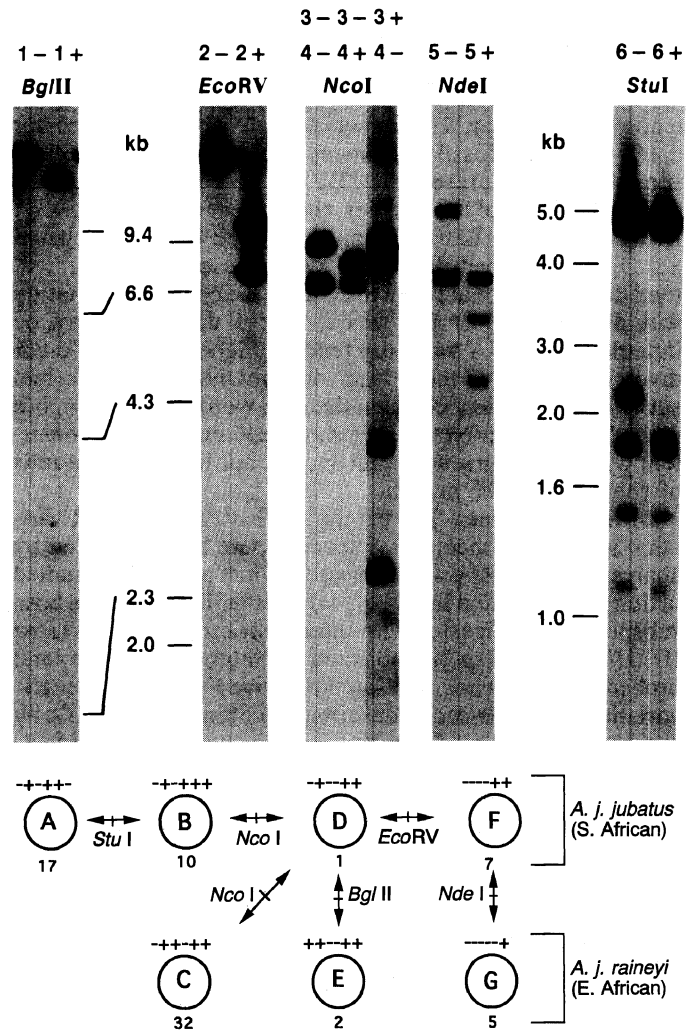


FIG. 1. (Upper) Composite autoradiogram of mtDNA RFLPs observed from the digestion patterns of 28 restriction enzymes in 74 animals. Molecular size is shown in kilobases. (Lower) Phylogeny of cheetah mtDNA haplotypes (A–G) in cheetah subspecies. The number of individuals exhibiting the haplotype is indicated below the circles. The seven mitochondrial DNA haplotypes are interrelated by single site changes. The presence (+) or absence (–) of six polymorphic restriction sites is indicated above each circled haplotype; from left to right these six polymorphic sites are *Bgl* I (first), *Eco*RV (second), *Nco* I (third and fourth), *Nde* I (fifth), and *Stu* I (sixth).

raineyi has lost certain primitive types possibly as a consequence of recent demographic fluctuation or founder effects (15).

The overall amount of nucleotide diversity (π) for cheetahs, estimated by using the maximum likelihood method of Nei and Tajima (40), was 0.182%, a rather low value for outbred mammal species including carnivores similarly studied (24). For example, mtDNA nucleotide diversity estimated by using RFLPs is 1.29% for leopards, 0.35% for pumas, 8.0% for black-back jackals, 3.65% for orangutans, 2.9% for deer mouse, 0.57% for humans, and 0.25% for humpback whales (refs. 24, 37, 41, and 42; S. Miththapala and S.J.O., unpublished data). The relatively low mtDNA diversity for the cheetah would suggest that a historic bottleneck had occurred and that either multiple haplotypes survived or the bottleneck occurred long enough ago to permit mutational accumulation or both.

To estimate the time necessary to produce this level of variation, we first hypothesize that at the time of the principal bottleneck, the ancestors of modern cheetahs were genetically homogeneous for mtDNA variation as well as for nuclear markers (11–14). We then compared the amount of

accumulated mtDNA diversity in the cheetah to the level of mtDNA divergence between feline species for which fossil calibration dates were available. The great cats (genus *Panthera*) consist of five species: lion, tiger, leopard, snow leopard, and jaguar. These species share a common ancestor dated by using fossil specimens recorded 1.6–2.0 million years before the present (1, 43).

For this group of five species, the average pairwise mtDNA RFLP divergence is 10.4% (range 5.4–15.3%) (P. Dratch and S.J.O., unpublished data). If a molecular clock is assumed, then the proportionate time to achieve the cheetah's variation would be 28,000–36,000 years. These durations may be inflated as mtDNA variation tends to decelerate after 8.0% divergence (21). If more than one mtDNA haplotype had survived the bottleneck, then the data would support a more recent bottleneck. Nevertheless, the calculations are consistent with the occurrence of an ancient bottleneck, thousands of years before the present.

DNA Fingerprints. The extent and pattern of VNTR variation in the two cheetah subspecies were also estimated by analysis of feline-specific minisatellite loci previously studied in domestic cats and lions (26, 44). Genomic DNA from both subspecies was digested with three restriction enzymes (*Hae* III, *Hinf* I, and *Msp* I), subjected to Southern analysis, and probed with the feline minisatellite probe FCZ8 (26). Genetic variation was assessed as described by Stephens *et al.* (39) by computation of the APD in band sharing between individuals and the estimated average H. APD and H are highly correlated with each other for DNA fingerprinting data ($r = 0.986$) and with other measures of overall genomic variation collected in our laboratory (13, 25, 26, 39). Results of a comparison of cheetahs from both subspecies are illustrated in Fig. 2 *Top* and tabulated in Table 1.

The cheetah displays an appreciable level of VNTR genetic variation (mean APD = 42.5%; H = 0.435) that is only slightly lower than that of domestic cats (*Felis catus*), outbred lions (*Panthera leo*), or California Channel Island foxes (*Urocyon littoralis*) (Table 1). For each restriction enzyme sampled, the two cheetah subspecies have nearly equivalent levels of VNTR variation. Cheetah polymorphism levels are in marked contrast to VNTR results from three previously described populations with documented severe bottlenecks in their recent history: Asiatic lions in the Gir Forest Sanctuary, Channel Island foxes from San Nicolas Island, and naked mole rats (*Heterocephalus glaber*) (Table 1) (25–27, 47).

The amount of pairwise divergence between members of different cheetah subspecies is higher for all three enzymes (mean APD = 48.2%) than that of either subspecies (Table 1). These data are consistent with a history of recent geographic separation. Of 167 polymorphic fragments tracked with the three restriction enzymes, 49 (29%) were unique to only one of the subspecies while 71% of the fragments were common to both. To explore further the pattern of phylogeographic partition, minimum-length parsimony networks based on minisatellite DNA fragments for individuals from each subspecies were constructed (Fig. 2 *Middle* and *Bottom*). For the *Hae* III- and *Msp* I-based DNA fingerprints, cheetahs from each geographic subspecies were clustered together, indicating that sufficient divergence had occurred at these VNTR families to reflect the known geographic separation in a phylogenetic analysis. The consistency indices (CI) for each of these topologies are low (CI = 0.35 for *Hae* III and 0.47 for *Msp* I), indicating a requirement for a high degree of homoplasy or parallel changes (due to allelic segregation within and between subspecies) to produce minimum-length trees. A large fraction of this homoplasy (81% for *Hae* III analysis; 74% for *Msp* I) involved polymorphic sites found in both *raineyi* and *jubatus* lineages, suggesting that the changes were plesiomorphic or shared ancestral characters. This high

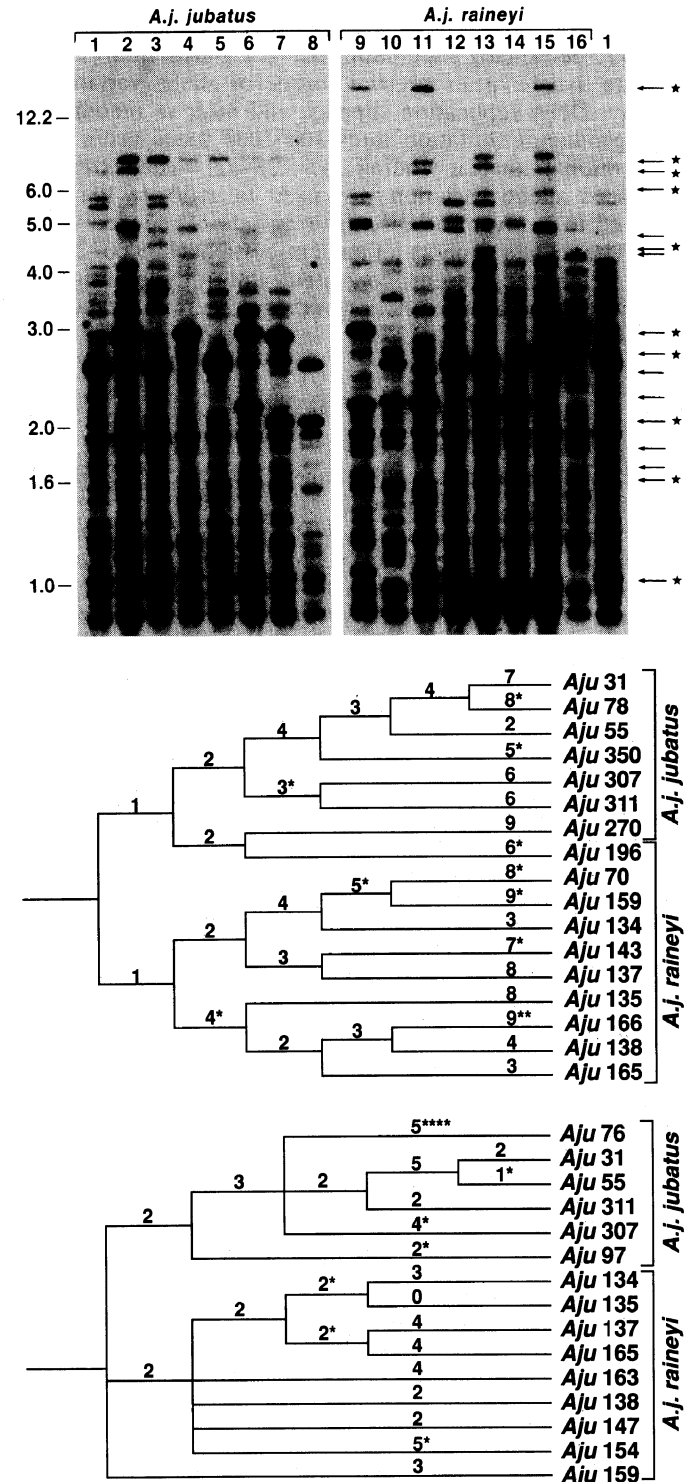


FIG. 2. (*Top*) DNA fingerprint patterns observed in two subspecies of cheetah. Genomic DNA was digested with *Hae* III and probed with a feline-specific minisatellite FCZ8. Arrows mark polymorphic fragments specific to subspecies; stars are specific to *A. j. raineyi*. DNA from the same animal was run in the outside lanes to aid in band scoring. Numbers to the left indicate size in kilobases. (*Middle*) Most parsimonious tree of the cheetah based on a presence-absence matrix of fingerprint fragments after *Hae* III digestion. A single most parsimonious tree was generated by using the branch-and-bound option contained in version 2.4 of the PAUP program (45) and was rooted at the midpoint of the longest path connecting any pair of taxa. Significant partitions according to subspecies designation are evident. Length of tree = 151 steps; consistency index = 0.351, indicating 65% homoplasy or parallel changes required. The numbers of fragment changes for each limb are indicated. Asterisks indicate the position of synapomorphies. (*Bottom*) Strict consensus parsimony tree from analysis with *Msp* I fragments: steps length = 62; consistency index = 0.468.

Table 1. Levels of DNA fingerprint variation in cheetahs and other mammals

Species	Subspecies	No. individuals	Probe	Restriction enzyme	APD \pm SD, %	Average H, %	References	
<i>A. jubatus</i>	<i>raineyi</i>	16	FCZ8	<i>Hae</i> III	45.7 \pm 12.6	56.0	This report	
	<i>jubatus</i>	14	FCZ8	<i>Hae</i> III	47.4 \pm 9.5	47.0	This report	
	<i>raineyi</i>	15	FCZ8	<i>Hinf</i> I	53.0 \pm 12.4	51.6	This report	
	<i>jubatus</i>	15	FCZ8	<i>Hinf</i> I	50.8 \pm 9.9	55.8	This report	
	<i>raineyi</i>	9	FCZ8	<i>Msp</i> I*	24.5 \pm 7.6	22.4	This report	
	<i>jubatus</i>	7	FCZ8	<i>Msp</i> I*	28.1 \pm 7.7	28.0	This report	
					Mean: 41.5	43.5		
		<i>raineyi-jubatus</i> genetic distance						
			17	FCZ8	<i>Hae</i> III	54.6 \pm 8.3	57.5	This report
			15	FCZ8	<i>Hinf</i> I	55.7 \pm 12.0	60.8	This report
		16	FCZ8	<i>Msp</i> I*	34.4 \pm 7.7	31.0	This report	
				Mean: 48.2	49.8			
<i>Felis catus</i>		17	FCZ8	<i>Msp</i> I	47.3 \pm 3.6	46.0	26	
		16	33.6	<i>Hinf</i> I	40.4 \pm 3.8	35.1	26	
<i>Panthera leo</i> (Serengeti)		76	FCZ8	<i>Msp</i> I	49.0 \pm 3.3	48.1	26	
	(Ngorongoro Crater) [†]	6	FCZ8	<i>Msp</i> I	51.5 \pm 10.5	43.5	26	
	(Gir Forest) [†]	16	FCZ8	<i>Msp</i> I	3.8 \pm 0.2	2.8	26	
<i>Urocyon littoralis</i> (S. Catalina)		16	33.6	<i>Hinf</i> I	25.3 \pm 3.8	31.0	25	
	(S. Nicolas) [†]	14	33.6	<i>Hinf</i> I	0.0 \pm 0.0	0.0	25	
<i>Heterocephalus glaber</i> [†]		50	M13	<i>Hae</i> III	1.0	—	27	
		50	33.6	<i>Hae</i> III	6.0	—	27	

*The level of APD and H should be equivalent for the same loci with different restriction enzymes because they cut outside of the minisatellite repeats (25, 26, 46). The lower value for both subspecies with *Msp* I compared with the other two enzymes is therefore unexpected but could represent the presence of *Msp* I site(s) inside the core repeats that produce invariant internal fragments. For computation of time required to produce the observed minisatellite variation, the *Msp* I values were not included.

[†]Populations with population bottleneck are followed by inbreeding in recent history. The Ngorongoro lion bottleneck was not as extreme but was apparent from documented observation, reduced allozyme, and MHC-RFLP variation (47, 48).

level of pleiomorphy for minisatellite allelic variation would imply that prior to geographic partitioning, the ancestral population possessed a sizable fraction of accumulated allelic polymorphism. An appreciable portion of this variation (70% of variation in common) was retained by both diverging subspecies (ancestral polymorphisms would be recognized in a parsimony analysis as pleiomorphic homoplastic changes). Additional stochastic losses or gains in minisatellite alleles since the subspecies split (the 30% of the variation specific to either subspecies) resulted in sufficient phylogenetic divergence to resolve the apparently monophyletic subspecies clustering represented in Fig. 2 *Middle* and *Bottom*.

The amount of VNTR variation present in modern cheetahs can also be used to estimate the time elapsed since the proposed cheetah bottleneck. The time to reconstitute genetic variation in a population that has been reduced to homozygosity by inbreeding depends upon the mutation rate of the gene class examined and the generation time of the species (19, 20). In general the number of generations required for genetic recovery is on the order of the reciprocal of the mutation rate. Thus, if the mutation rate is 10^{-6} per locus per generation (as it is for conventional allozyme or MHC loci), then it would take about 10^6 generations multiplied by the average generation time to reconstitute allozyme and MHC locus variation to levels observed in other feline species (11).

The mutation rates of the feline FCZ8 VNTR loci have not been estimated, but mutation rates of several vertebrate VNTR loci have been (28–31). The estimated mutation rates per locus per gamete, μ , for four VNTR families are as follows: (i) chicken M13, $\mu = 0.0017$; (ii) human 33.15, $\mu = 0.0010$; (iii) human 33.6, $\mu = 0.00051$; and (iv) human (CAC)₅/(GTG)₅, $\mu = 0.00047$. The modal cheetah generation time is 6 years (34). If the mutation rate of the feline FCZ8

family is comparable to the above four rates, the time required to produce the cheetah's level of variation would be estimated respectively at 3,529, 6,000, 11,765, and 12,766 years. If the cheetah's ancestors did experience a single extreme bottleneck that reduced all their minisatellite loci to homozygosity, along with their allozyme and MHC loci, then these time periods would approximate the time elapsed since such an event. Because demographic considerations would make the survival of a few individuals from such a catastrophe unlikely, a series of less severe bottlenecks spread over time and over geographic space is more realistic (17, 49). If there were several bottlenecks, the time of recovery here calculated would be an estimate of the most recent events. Although these estimates are subject to fluctuations in mutation rate, the assumption of a molecular clock, demographic effects, and statistical errors, they are still consistent with an ancient bottleneck on the order of 10,000 years ago that reduced the cheetah's genomic composition to remarkably limited genetic diversity at nuclear coding loci.

Conclusion. The genetic status of cheetahs previously studied for nuclear coding loci revealed 90–99% less genetic variation than is observed in other outbred felid species (11–15). Here we present evidence based on accumulated DNA variation in rapidly evolving mtDNA and VNTR loci that the population bottleneck that might have reduced coding locus variation was ancient, estimated at several thousand years before the present. The back calculation, based on relative divergence of mtDNA in felids and mutation rates of VNTR loci in other species, supports the placement of the bottleneck on the order of the end of the Pleistocene, about 10,000 years ago, when a major extinction of large vertebrates occurred (6–8). Our data do not limit the size or number of demographic contractions, but given the extreme reduction of allozyme, 2DE, and MHC variation, and the predictions of demographic modeling, it is likely that

several independent events perhaps extended over time and geographic space (17).

The DNA fingerprinting results recapitulate a more recent geographic partition of the eastern African subspecies *A. j. raineyi* and the southern subspecies *A. j. jubatus*. The derived subspecies retained large amounts of shared ancestral minisatellite genetic variation en bloc in both subspecies, indicating that the split occurred between large subdivisions of the ancestral population. Subsequent to that partition, temporal founder effects in each subspecies apparently led to stochastic loss of ancestral mtDNA haplotypes in *A. j. raineyi* (Fig. 1) and of allozyme variants in *A. j. jubatus* (15).

The results presented here emphasize that despite dramatic reduction in genetic variation previously reported for cheetahs, the diminution is neither complete nor permanent. A moderate level of variation has accumulated over time, and some residual adaptive genetic variation likely survived in modern cheetahs. Captive breeding management plans should continue to strive to minimize inbreeding effects. Finally experimental breeding between subspecies should be encouraged at least in captive settings, since evidence for increased survivorship and reduction in juvenile mortality among *raineyi-jubatus* crosses has been observed in European (12, 34) and in North American (35) breeding programs.

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