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Genetics

East African cheetahs: Evidence for two population bottlenecks?

(spermatozoa/allozyme polymorphism/Pleistocene extinction)

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ABSTRACT **A combined population genetic and reproductive analysis was undertaken to compare free-ranging cheetahs from east Africa (*Acinonyx jubatus raineyi*) with the genetically impoverished and reproductively impaired south African subspecies (*Acinonyx jubatus jubatus*). Like that of their south African counterparts, the quality of semen specimens from east African cheetahs was poor, with a low concentration of spermatozoa (25.3×10^6 per ejaculate) and a high incidence of morphological abnormalities (79%). From an electrophoretic survey of the products of 49 genetic loci in *A. jubatus raineyi*, two allozyme polymorphisms were detected; one of these, for a nonspecific esterase, shows an allele that is rare (less than 1% incidence) in south African specimens. Estimates of polymorphism (2–4%) and average heterozygosity (0.0004–0.014) affirm the cheetah as the least genetically variable felid species. The genetic distance between south and east African cheetahs was low (0.004), suggesting that the development of genetic uniformity preceded the recent geographic isolation of the subspecies. We propose that at least two population bottlenecks followed by inbreeding produced the modern cheetah species. The first and most extreme was ancient, possibly late Pleistocene (circa 10,000 years ago); the second was more recent (within the last century) and led to the south African populations.**

Modern cheetah populations, which number less than 20,000 animals, are largely restricted to two zoogeographic subspecies: *Acinonyx jubatus jubatus* (range including portions of South Africa, Namibia, Mozambique, and Zambia) and the east-central African subspecies, *Acinonyx jubatus raineyi* (found in Kenya, Tanzania, Uganda, and some central African countries) (1–6). We recently discovered that the south African cheetah (*A. jubatus jubatus*) is unique among felids and other mammals in having an extreme paucity of genetic variation as estimated by electrophoretic surveys of allozymes and cell proteins resolved by two-dimensional gels (7). More unusual was the observation of allogeneic skin graft acceptance among unrelated cheetahs, revealing genetic monomorphism at the major histocompatibility complex, an abundantly polymorphic locus in nearly all mammals (8).

Captive reproduction of cheetahs historically has been poor and their infant mortality rate is high (8). A comparative analysis of cheetah ejaculates revealed a sperm count 1/10th of that observed in domestic cats and an extremely high frequency (71%) of morphological spermatozoal abnormalities (9, 10). In addition to these phenotypic observations, patterns of skeletal variation also show significant asymmetry of bilateral characters, a phenomenon generally common in inbred animals (11, 12). The combined genetic, reproductive, and morphological data placed the cheetah in a status reminiscent of deliberately inbred mice or livestock and prompted us to hypothesize that in its recent history the species had probably suffered a demographic contraction or population bottleneck necessarily followed by inbreeding.

An important caveat of the genetic and reproductive results was that all cheetahs studied were bred in captivity and derived from the south African subspecies (*A. jubatus jubatus*) (7–10). We present here the results of a genetic and reproductive analysis of free-ranging and captive cheetahs from the previously unstudied east African subspecies (*A. jubatus raineyi*). Blood and semen samples from 30 east African animals (20 free-ranging and 10 captive) were collected in Tanzania and Kenya in 1985. Three specific issues were addressed. First, the specific ejaculate characteristics of the two subspecies were compared to determine the extent of subspecies differentiation that may have occurred since their geographic isolation. The evaluation of reproductive norms in free-ranging animals (9, 10) also provided a unique opportunity to evaluate retrospectively the influence of captive rearing on reproductive potential. Second, although a hypothesis can be made for a population contraction in the cheetah's history, the precise timing of such a bottleneck is more difficult. A comparison of the genetic status (extent of polymorphism and allelic concordance between subspecies) of *jubatus* versus *raineyi* could be used to estimate the timing of the bottleneck. Allelic differences between east and south African populations would be consistent with a recent bottleneck [e.g., possibly through well-documented overhunting (13) by 19th-century farmers in southern Africa], while monomorphism and allelic identity of the two isolated populations would provide evidence for a more ancient (possibly late Pleistocene) population contraction. Third, because of the severe genetic consequences for captive breeding observed in southern animals, the possibility of detecting a subspecies line with a different genetic or evolutionary history may offer an opportunity for infusion of new germ lines into the captive-bred populations.

MATERIALS AND METHODS

Anesthesia. Captive cheetahs were immobilized for semen/blood collection by using one of two anesthetic regimens administered by blow dart or hand syringe. On 25 of 29 sampling occasions, males were given ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, NY; 14.2 mg/kg, intramuscular) and maintained in a surgical plane of anesthesia with supplemental ketamine injections (5.5 mg/kg, intravenous). In approximately one-half of these episodes the animal experienced one or more brief (15- to 60-sec) catatonic seizures while under ketamine anesthesia. At the onset of any third convulsion, diazepam (Valium, Hoffmann–La Roche, Nutley, NJ; 0.03 mg/kg) was admin-

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istered intravenously to control the seizures. The remaining four captive males were immobilized by using a combination of tiletamine hydrochloride and zolazepam hydrochloride (Telazol, Warner Lambert, Ann Arbor, MI; 4.6 mg/kg, intramuscular). In contrast to ketamine, Telazol resulted in a more relaxed anesthesia with less muscular tone and no convulsions. Ancillary sedative therapy was unnecessary, although a surgical plane of anesthesia was maintained with supplemental Telazol (2.0 mg/kg, intramuscular).

For immobilization of free-ranging animals, males were approached by a vehicle used routinely in the Serengeti National Park for cheetah observation (5). All animals were injected with Telazol (4.6 mg/kg, intramuscular) administered via a dart from a blowpipe extended through the vehicle window. Darted animals usually bolted 30–50 min before lying down and becoming recumbent within 10 min. A surgical plane of anesthesia was maintained throughout the blood sampling interval with supplemental Telazol (usually a single injection, 2.0 mg/kg, intramuscular).

Electrophoretic Procedures. Blood was separated into plasma, erythrocytes, and leukocytes as previously described and stored at −70°C (14–16). Field collections were stored in liquid nitrogen freezers until their delivery to the National Cancer Institute. Isozyme extracts were prepared by sonication and subjected to aqueous gel electrophoresis as previously described (14, 15). Histochemical stains for 50 feline isozyme systems were applied and evaluated by using the criteria for genetic variants listed by Newman *et al.* (15). Enzyme abbreviations are after the human convention (17).

RESULTS AND DISCUSSION

Semen was collected by electroejaculation (9, 10) from 10 male east African cheetahs, 8 free-ranging individuals in the Serengeti ecosystem in Tanzania, and 2 captive males from the Nairobi Animal Orphanage. The semen was analyzed for a variety of physiological traits summarized in Table 1. The mean spermatozoal concentration was very similar to the concentration previously observed in 70 south African cheetahs and much less than that seen in the domestic cat (9, 10). In addition, spermatozoa from the east African cheetahs had a high proportion of morphological abnormalities (78.6%), comparable to levels observed in the south African cheetah subspecies (70.5%). The distribution of specific pleiomorphic defects, which were classified according to primary (spermatogenesis dysfunction) or secondary (anomalous gamete transport through excurrent duct) deformities, was equivalent between the two subspecies. The extent of spermatozoal abnormalities of both subspecies was extreme, a level that in other species (humans, bulls, dogs) would almost invariably be associated with infertility (18–20).

We had suggested earlier that the high incidence of spermatozoal defects was a result of genetic factors (7–10); however, we could not exclude the possibility that chronic stress associated with captivity had adversely affected testicular function. This, in fact, may be the case in other specialized felids such as the east Asian clouded leopard, *Neofelis nebulosa*. This species in captivity not only produces high proportions of pleiomorphic spermatozoa but also expresses extraordinary levels of circulating glucocorticoid hormones (an index of stress), at concentrations 4-fold greater than in cheetahs (21–23). Free-ranging cheetahs in east Africa express levels and classes of abnormal spermatozoa comparable to those of captive cheetahs derived from both regions (Table 1). These results support the idea that the diverse morphological characteristics of cheetah spermatozoa are species specific and unrelated to the influences of captive breeding and rearing.

A previous electrophoretic survey of allelic isozyme (allozyme) variation in 55 south African cheetahs revealed an absence of genetic variation for each of 52 loci (7). This was unusual for felids and for most mammals, because the vast majority of over 250 species examined with similar techniques revealed appreciable genetic variation with frequencies of polymorphic loci ranging from 0.15 to 0.60 and average heterozygosity estimates from 0.0 to 0.26 (Fig. 1). Isozyme samples were derived from erythrocytes, lymphocytes, and plasma separated by centrifugation of heparin-treated blood from 30 east African cheetahs. The extracts were tested for each of 49 isozymes previously typed in south African cheetahs and in nine other feline species (7, 8, 15). Forty-three additional blood samples from captive animals originating from southern Africa (Namibia and Transvaal province), but maintained in U.S. zoological parks, were similarly examined.

In the east African populations, two polymorphic loci were discovered; a nonspecific esterase locus termed *ESD*, and the adenosine deaminase locus, *ADA*. The *ESD* locus had three alleles (*a*, *b*, and *c* with gene frequencies of $a = 0.5$, $b = 0.15$, $c = 0.35$) and was in genetic (Hardy–Weinberg) equilibrium. Of the 43 zoo-held south African cheetahs, two siblings from the Detroit Zoo (North American cheetah studbook nos. 105 and 106) (25) were heterozygous for ESD^a/ESD^b. All other south African-derived animals were ESD^a homozygotes. The polymorphism for *ADA* revealed a previously undiscovered allele, ADA^b, with a frequency of 0.15 in the east African population only. The other 47 loci tested (listed in ref. 7) were monomorphic and identical in both subspecies. As illustrated

Table 1. Seminal traits in east African and south African cheetahs

Population	No. of individuals	Motile spermatozoa × 10^{-6} per ejaculate	Spermatozoal morphological abnormalities, % per ejaculate		
			Primary defects*	Secondary defects†	Total
East African cheetah					
Captive	2	0.9 ± 0.9	57.0 ± 7.0	32.0 ± 8.0	89.0 ± 1.1
Free-ranging	8	25.3 ± 9.9	39.0 ± 3.9	36.9 ± 2.9	75.9 ± 4.4
Combined	10	20.4 ± 8.4	42.3 ± 4.0	36.0 ± 2.7	78.6 ± 3.6
South African cheetah					
Captive	70	21.7 ± 4.3	30.3 ± 3.8	40.5 ± 2.4	70.8 ± 2.2
Domestic cat	16	147.0 ± 39.5‡	5.8 ± 0.3	23.3 ± 1.1	29.1 ± 3.7

Values are means ± SEM. Semen was collected from animals subjected to a standardized electroejaculation technique while under a surgical plane of anesthesia (ref. 9).

*Structural deformity originating as a result of spermatogenic dysfunction, including head, acrosome, or midpiece abnormalities or a tightly coiled flagellum.

†Structural deformity originating as a result of gamete transport through the excurrent duct system, including bent neck/flagellum or presence of cytoplasmic droplet.

‡Spermatozoa × 10^{-6} per ml.

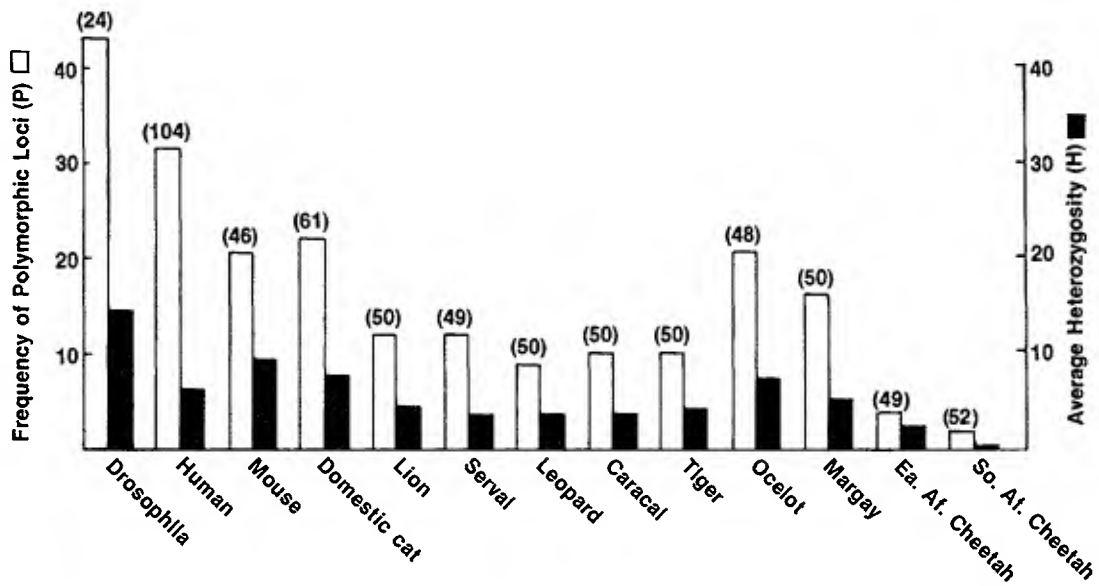

FIG. 1. Estimates of the extent of genetic variation based on allozyme electrophoretic surveys in the east African cheetah (*A. jubatus raineyi*), the south African cheetah (*A. jubatus jubatus*), eight additional feline species (8, 15), and three nonfelid species. The number in parentheses indicates the number of genetic loci that were considered in the estimate. For a review of over 250 such electrophoretic surveys see ref. 24. Enzymes typed and procedures used are as previously described (7, 8, 15).

in Fig. 1, the east African sample had a frequency of polymorphic loci (P) of 2/49 (4%) and an average heterozygosity (the average frequency of heterozygous loci in a single individual, H) estimate of 0.014. The rare *ESD* polymorphism in the south African population, now consisting of 98 individuals, provides a recalculated frequency of polymorphism ($P = 0.02$) and average heterozygosity ($H = 0.0004$).

Although two loci were discovered to be polymorphic in the east African population, the species still is lacking in genetic variation. In comparing the different cat species tested to date, the cheetah clearly has the least detectable genetic variation (Fig. 1). In fact, one of the most striking aspects of our analysis is the similarity between the two cheetah subspecies. The Nei genetic distance (D) (26, 27) between eastern and southern populations was computed, using 49 loci, to be equal to 0.004. This distance is 1/80th of the distance between chimpanzees and humans ($D = 0.310$) (28), 1/8th of the distance among human racial groups ($D = 0.03$) (29, 30), and 1/10th of the distance among four inbred mouse strains, each simultaneously derived from the same fully outbred Swiss mouse population ($D = 0.045$–0.067) (31).

How do these results influence our estimates for the time of occurrence of the population bottleneck responsible for the evolution of the nearly monomorphic cheetah in south Africa and the only slightly more variable cheetah in east Africa? The simplest interpretation may be that the major bottleneck (or series of bottlenecks) was rather ancient and preceded the subdivision of the modern zoogeographic subspecies. Because of the extremes of inbreeding required to produce a strain as homozygous as the cheetah (32), it is likely that several population bottlenecks may have been operative in leading to modern populations. The ancient bottlenecks may have occurred over time, over space, or both, with small populations being founded and surviving, while the larger parent populations died out. The space consideration is not at variance with the paleontological record, which shows the cheetah's range to be worldwide as recently as 10,000 years ago (33, 34). The present results seem consistent with an interpretation of one or more major genetic purges a long time ago, possibly at the end of the late Pleistocene some 10,000 years ago, when large numbers of mammals became extinct (35, 36). Subsequent to the geographic separation of the *raineyi* and *jubatus* subspecies, perhaps within the last 100 years, a second bottleneck or a chance-driven founder effect may have caused the south African population to lose its *ADA* alleles and change its *ESD* allelic frequency. This final bottleneck may have resulted from any number of ecological consequences, including perturbations in prey and competitor-predator numbers or by excessive overhunting by cattle and sheep farmers in south Africa at the turn of the century or by poaching for the skin trade today. The latter factors have been documented to have reduced cheetah populations considerably in that region (13).

Although the future prospects for the cheetah's survival may appear precarious, there are aspects of our results that are encouraging. If the primary bottleneck did occur as long ago as the late Pleistocene (10,000–12,000 years ago), then continued propagation for that many generations would surely have eliminated the most deleterious genes early in the process by natural selection. Some of the breeding and rearing problems observed in captive populations may be artifacts of captivity, where cheetahs are pampered by defined diets and live to be 10–15 years old, considerably longer than their estimated longevity in the wild (T.M.C., unpublished data). Also, in certain places (e.g., Namibia) cheetah populations are stable and possibly on the rise. If our hypothesis is correct, that is, an ancient bottleneck reduced the majority of genetic variation and then a more recent bottleneck occurred in the history of the south African subspecies, then some improvement may be achieved by introducing east African and south African animals into a composite captive breeding program. At least one such effort already is in progress at the Zoological Society of London's Whipsnade Park (25). There is certainly no genetic reason to keep these so-called "subspecies" apart, since the results presented here place their common ancestors as 5–10 times more recent than the ancestors of related inbred mouse strains or human racial groups. Nonetheless, our ignorance concerning the potential for disease complications, behavioral inability to adjust to new ecosystems (for example, to hunt new prey species), and other local adaptations contingent upon genetic loci not examined here, preclude similar recommendations for the remaining wild populations.

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