GENETIC MANAGEMENT STRATEGIES AND POPULATION VIABILITY OF THE FLORIDA PANTHER

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GENETIC MANAGEMENT STRATEGIES

AND POPULATION VIABILITY

OF THE

FLORIDA PANTHER
(*Felis concolor coryi*)

Proceedings from Workshops:

National Zoological Park
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CAPTIVE BREEDING SPECIALIST GROUP
SSC/IUCN

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REPORT OF A WORKSHOP

Prepared by
U.S. Seal and the Workshop Participants

at

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Executive Summary

The Florida panther is one of the most critically endangered taxa in the United States. Population declines and associated inbreeding have resulted in significant losses in genetic variability and viability. Natural gene exchange existing historically between the Florida panther and other North American puma subspecies is no longer possible because of human induced isolation. Successful recovery of the Florida panther is doubtful without a reinstitution of lost gene flow.

The Florida Panther population was found to be at high risk of extinction over the next 25-40 years at the time of a population viability assessment in 1989 (Seal et al.). The analysis indicated that genetic heterozygosity would continue to be lost at the rate of about 6% per generation if the population was not increased in size. Substantial evidence for inbreeding depression was presented and led to the conclusion that the establishment and management of a captive population was the only way to preserve existing genetic viability. Delays, imposed by external events, prevented timely implementation of management actions to establish the captive population, rapidly expand the population, and secure full representation of the genetic diversity present in the population at that time.

Renewed concerns over reduced levels of genetic heterozygosity and the complete loss of historic gene flow patterns led to a workshop in 1991 to consider genetic augmentation as a tool in Florida panther recovery (Seal editor, 1991). General conditions and criteria for the use of genetic augmentation to ameliorate the adverse effects of inbreeding depression in a population were formulated in the workshop. These criteria were applied, at that workshop, to the Florida panther population with the determination that there was substantial evidence for inbreeding depression and that genetic augmentation should be undertaken.

The present Workshop was convened because of increasing evidence that the population displays multiple physiologic abnormalities that are likely a consequence of recent close inbreeding among the surviving individuals. Re-evaluation of the wild population viability indicates that it is continuing to decline genetically, remains at high risk of extinction, and that adverse effects of inbreeding are accumulating rapidly. It was the consensus of the Workshop participants that the reinstitution of historic gene flow between the Florida panther and adjoining Felis concolor subspecies, i.e., genetic augmentation, is needed to reverse these effects of inbreeding depression, that effective action needs to be taken quickly and that the amount of introgression required may reach 20%.

Eight alternative management scenarios for controlled introgression of genetic material from another population of Felis concolor were examined in terms of: accomplishing the biological objectives, evaluating population source of the individuals to be used for the intercrosses, accessibility, of Florida panther and intercross animal timing, availability of the requisite technology, and possible adverse effects on the individual animals. The scenarios given top priority were (1) direct introduction of animals into suitable unoccupied territories or potential territories, (2) AI of females brought into captivity for a brief period of time and then returned to the wild, and (3) breeding or AI of non-Florida animals in captivity with Florida panther males or their sperm. All 3 scenarios will need to be implemented in parallel given the time span required, the high incidence of abnormalities in the population, and the continued loss of animals from the wild population.

GENETIC MANAGEMENT STRATEGIES FOR THE FLORIDA PANTHER

Workshop Report

22 October 1992

Introduction

Historically, the Florida panther, *Felis concolor coryi*, ranged across much of the southeastern United States. Today, it is one of this nation's most critically endangered taxa. A single population in southern Florida, estimated to consist of 30 to 50 adults, is all that remains in the wild. Population declines and associated inbreeding have resulted in significant losses in genetic variability and viability. Natural gene exchange, existing historically with other populations of *F. c. coryi* and other *F. concolor* subspecies (Young and Goldman reported gene exchange between *F. c. coryi* and *F. c. cougar* to the north and to the west and northwest with *F. c. stanleyana* and *F. c. hippolestes*), is no longer possible because of isolation. Analysis of the present status of the endangered Florida panther (*Felis concolor coryi*) indicates that the population exhibits multiple physiologic abnormalities that are likely a consequence of recent close inbreeding among surviving individuals. To correct this serious and rapidly deteriorating situation, the consensus of the workshop was to immediately reinstate gene flow (or genetic augmentation) lost because of human caused isolation. The goal of the recommended genetic augmentation is to reverse the consequences of inbreeding as well as to reconstitute genetic variation that may have occurred naturally in Florida panthers when its former range and the ranges of adjacent subspecies were occupied.

The consequences of demographic contraction in the Florida panther are evident from a decade of field and biomedical monitoring. The Florida panther, reduced to less than 50 adult individuals in south Florida by human depredation and habitat depletion, display a remarkable array of genetic and physiologic impairments that pose a direct threat to survival. Relative to other puma subspecies the Florida panther has reduced genetic variation based upon mitochondrial DNA, allozyme and DNA fingerprint analyses, likely a result of genetic drift and close inbreeding caused by range and population contractions. Several cases of consanguineous matings have been documented directly in the surviving population.

Correlated with the genetic uniformity is the occurrence of several aberrant congenital defects including:

1) an average incidence of 95% morphologically-abnormal sperm per ejaculate (including a 41% incidence of malformed spermatozoal acrosomes), in contrast to

western or South American samples that have an incidence of 83% and 58% abnormal sperm respectively;

2) 71% cryptorchidism in living males (12 of 17) with 12% (2 of 17 living males) of these being bilateral and, thus, sterile;

3) emergence of fatal cardiac abnormalities.

Furthermore, the population has suffered from a score of pathological infectious agents that have been fatal in 8 panthers to date. These infections may be a consequence of a defective immune system compromised by inbreeding. Combined with the non-physiological perils that contribute to mortality (e.g. road kills, intra-specific aggression, mercury toxicity) and interacting stochastic effects that threaten the population (e.g. demographic fluctuations, genetic drift), the results indicate a precarious population at high risk for extinction. The cumulative results strongly indicate an imperative to manage more directly the residual genetic representation of the remaining population and support the reinstatement of gene flow with conspecific populations of puma.

In May 1991 a workshop was convened in Washington D. C. to consider "Genetic Management Considerations for Threatened Species with a Detailed Analysis of the Florida Panther". The report of that workshop (attached to this report) developed explicit criteria for considering genetic augmentation of an endangered population when inbreeding and associated consequences negatively affect population viability. These criteria were reexamined by the present group and applied to the available knowledge about the Florida panther. Our conclusion is that the Florida panther's status is sufficiently grave to recommend immediate implementation of a genetic augmentation program as outlined below.

A managed reinstitution of gene flow between *F. c. coryi* and a historic adjacent *F. concolor* subspecies would likely improve the genetic health and viability of the extant Florida panther gene pool. Although genetic augmentation is recommended and represents a management attempt to reconstitute the genetic variation that was once present in the ancestors of today's population, we emphasize that this measure does not address the need to identify and designate increased suitable panther habitat required to sustain a demographically viable population with a high confidence of persistence.

Sources and Levels of Genetic Augmentation

The primary goal of genetic augmentation for the Florida panther is the reduction in frequency of deleterious traits that can result from inbreeding by introducing genetic material from other *Felis concolor* populations. For such situations, the workshop on 'intercrossing' ("Genetic Management Considerations for Threatened Species with a

Detailed Consideration of the Florida Panther" 30-31 May 1991) recommended a small percentage of admixture of non-local genes in a single episode. The amount of genetic admixture should be sufficient for the target population to recover from the deleterious effects of inbreeding, but not so large as to swamp the local gene pool which may be adapted to local environmental conditions. A small admixture of non-local genetic material into a population suffering from inbreeding depression should permit natural selection to reduce the frequency of deleterious mutations over the course of several generations while maintaining local adaptations. The amount of admixture chosen for a given management program represents a tradeoff between rapid recovery from inbreeding effects and possible swamping of local adaptations. Because of the recent rapid increase in frequency of cryptorchidism and heart defects, and documented cases of close inbreeding in the wild Florida panther population, the panel members recommend immediate genetic augmentation of the population by introduction of 20% of the target population's genetic material from another puma population to rapidly reverse the deleterious effects of inbreeding within two or three generations.

A crucial part of the management strategy is that the initial genetic admixture should be monitored to track its spread through the Florida population to confirm recovery of intercrossed offspring from the proposed deleterious effects of past inbreeding, and to determine if and when further admixture is necessary.

A secondary, long-term goal of genetic augmentation is to maintain genetic variability in the Florida panther population at levels comparable to the historic panther population and to other *F. concolor* populations, to allow natural selection and adaption to environmental changes. After the initial augmentation, this can be accomplished by continued introduction from a non-Florida population of about one successful breeding individual every generation (generation time=6 yrs). Augmentation may also be considered or required for demographic purposes.

In a target population as small as the Florida panther, with 30 to 50 adult individuals, the primary goal can be met by introducing 6 to 10 unrelated, successful non-Florida individuals, or twice as many unrelated, successful F1 intercrosses between Florida stock and another population. One advantage of introducing a larger number of intercross individuals is that there is less risk that their genetic contribution will be completely lost from the population before integration by chance demographic events. Intercross individuals are also more likely to be adapted to local environmental conditions than non-Florida animals, if local adaptations exist.

To be genetically effective, individuals introduced into the target population must become part of the breeding pool. The behavior and social structure of panthers suggests that this may best be accomplished by introducing subadult intercross or non-Florida females (or as a second choice subadult males) into vacant territories. Another option is

artificial insemination of wild females by non-Florida or intercross males, if existing techniques can be refined.

There are six possible source populations for genetic augmentation of the Florida panther population:

1. Captive generic *F. concolor's* of known ancestry.

2. Piper captive stock (intercrosses between Florida, South American and possibly other *F. concolor's.*

3. "Everglades" panthers (intercrosses between early Piper stock and Florida panthers).

4. Wild Texas *F. concolor's.*

5. Wild Western (non-Texas) *F. concolor's.*

6. Central American or South American wild *F. concolor's.*

Of these six possible source populations, captive stocks 1 and 2 have the disadvantage that they may be partially adapted (behaviorally and/or genetically) to captivity, and less adapted to the Florida habitat than any wild population. "Everglades" panthers are already part of the present wild panther population, but because of their intercross ancestry do not, at this time, experience the high frequency of cryptorchidism or heart defects characterizing the remaining Florida panthers. However, they do have a similarly high incidence of sperm abnormalities that may or may not improve upon intercrossing with other Florida panthers. If the overall Florida panther population were demographically stable, natural intercrossing with "Everglades" individuals might eventually reverse most or all of the deleterious characters in the Florida panther. But the small numbers of "Everglades" panthers, the fact that they have a high proportion of Florida panther genes, and their peripheral location in relation to the main Florida population suggest that it would take several generations for their genetic contribution to naturally spread through the Florida population and perhaps still achieve slight improvement at best. All of these considerations dictate that genetic augmentation of the Florida panther population should be conducted with genetic material from a separate wild population.

Because of the small amount of genetic differentiation among puma populations throughout their range, and the absence of barriers to intercrossing between puma populations, it probably does not make much difference which wild population is used for

genetic augmentation. However, a single wild puma population should be chosen for genetic augmentation of the Florida panther, and when the source population is determined, genetic studies on it should be conducted in conjunction with introduction and intercross efforts to characterize the introduced gene pool for monitoring and follow up studies.

Of the existing sources, wild Texas pumas formerly constituted part of a continuous range across which genetic material was likely exchanged with the Florida subspecies. For restoring the genetic fitness and natural pattern of genetic variation in the Florida panther, wild Texas pumas therefore appear to be the best source for genetic admixture. However, Texas puma populations sampled have also exhibited low genetic variation.

Possible Scenarios for Florida Panther Management With and Without Introgression.

The following is a list of management options that involve varying degrees of introgression. The list is ordered from the most drastic introgression (elimination and replacement of native stock) to the least drastic intervention (no action). The options are not mutually exclusive and some can be used in combination. For each option we have described some of the conspicuous advantages and disadvantages. All of the options that involve introgression (1-5) have the advantage that they would help the wild Florida population recover from inbreeding depression (i.e., lower the incidence of undescended testes, incompetent sperm, etc.). Such recovery would be immediate in the sense that intercrossed animals will not express its recessive genetic defects. The options that do not involve introgression (6-9) will not correct the genetic problems of the south Florida panther population but could have beneficial demographic effects.

1. Eliminate native stock and replace with non-Florida stock.

The present Florida panther population is genetically unique. This population is currently surviving and reproducing with apparent natural mortality and natality rates but showing signs of inbreeding depression. Elimination of this population would mean the extinction of a subspecies. Introduction of non-Florida individual may be met with high mortality due to dispersal and environmental factors. Finally, it is not necessary to institute a drastic plan of replacement when the current problems with genetic vitality can be more simply addressed through genetic augmentation.

2. Artificial insemination of south Florida females with sperm from non-Florida males.

This scenario includes the use of artificial insemination (AI) of free-living females to produce genetically-enhanced offspring in situ. Using frozen sperm from non-Florida males, this approach would rapidly place new genetic material into the wild population without disturbing the social structure of the free-living animals.

The greatest challenge associated with this scenario is the difficulty of manipulating females under field (uncontrolled) conditions. Timing of hormonal stimulation for the induction of estrus is critical to AI success. For this reason, the technique may require that females be removed from the wild for 30 days for the administration of hormones and AI, and released back into the wild immediately following inseminating. The disadvantage of this approach would be the unknowns associated with potential behavioral and physiological stress related to short-term captivity.

The reproductive biology techniques can be developed in conjunction with the captive breeding program. If field related problems preclude AI being used in the field, the technology could be useful in the captive breeding program and for the release or reintroduction of inseminated wild-caught or intercross females.

3. Translocation of wild non-Florida subspecies into south Florida.

The most expedient route for introducing genetic material into the south Florida panther population is to relocate individuals from another appropriate wild *F. concolor* population. The recommended action would involve capture of young (12-18 month old) non-Florida females and release (following only quarantine as necessary to assure that no diseases are carried) into vacant female Florida panther home ranges. Because female panthers are generally philopatric (do not disperse) and subadults are unattached to a home range, this sex and age class would be most likely to stay where released. In addition, introduced subadult females would be unlikely to interact aggressively with local residents. While subadult non-Florida males could also be used, the low frequency of male recruitment would probably delay the genetic contribution of these individuals for several years. Furthermore, the propensity for long distance dispersal by males increases the chances for mortality or travel to less desirable areas of panther range.

Advantages of this approach include very low cost (individual could be kept in captivity for a minimal, mandatory quarantine period) and very low probability of disrupting the existing social organization of south Florida panthers.

Disadvantages include less knowledge of medical history (compared with captive reared individuals) and a relatively slow incorporation of genetic benefits (compared with AI).

Offspring produced from non-Florida x Florida panther matings should be evaluated and radio-instrumented before they disperse in order to score key traits (e.g., incidence of cryptorchidism, heart murmurs, etc.) and to monitor survival and recruitment. Tissue samples should be collected at the first opportunity so that the progeny of these animals can be recognized by DNA fingerprints.

4. Release of captive-raised non-Florida *F.concolor's* into south Florida.

This option differs from direct translocation of wild non-Florida subspecies (option 3) in that the source of animals will be captive-raised and conditioned non-Florida individuals. Variations using males versus females follow the same rationale suggested for Option 3.

An advantage of this approach is that the medical condition and history of released animals is better controlled and understood with little chance of introducing an unwanted pathogen into south Florida.

A possible disadvantage of this approach is that the adaptability of captive raised individuals to the wild is unknown and could influence success. The cost of raising (housing, feeding, etc.) individuals would be high.

5. Captive production and reintroduction of intercrossed panthers.

Florida panthers could be intercrossed with non-Florida individuals with the aim of reintroducing first-, second- or later- generation crosses to south Florida. Intercrosses may have a better chance of surviving and reproducing than non-Florida individuals. Furthermore, planned captive introgression may be more successful than simple propagation (option 6) in perpetuating south Florida panther genotypes. In particular, using non-Florida animals greatly expands the number of breeding options. For example, using non-Florida males as mates for Florida females circumvents the testicular and sperm problems that plague Florida males. Putting problems of breeding space and facilities aside, intercross production could also be used as an adjunct to captive propagation of Florida panthers. Reproductively competent male Florida panthers could be used to sire the litters of both Florida and non-Florida females. Under these or other scenarios involving intercrosses, a set of guidelines should be produced to steer the breeding program. Without guidelines and monitoring (e.g., with DNA fingerprints) a breeding program could produce animals of uncertain parentage.

6. Captive propagation of south Florida panthers without introgression.

The advantage of this option is that native genetic stock could be perpetuated for later reintroduction. Furthermore, the captive population could act as insurance against natural catastrophes or more gradual extinction of the wild population. Captive propagation does face problems. The overall health and vigor of Florida panthers has been compromised by genetic defects and pathogens. Compared with individuals from outbred populations, Florida panthers may prove to be difficult to breed in captivity. Secondly, the available stock in nature is currently limited to at most **two or three dozen** animals. Third, the panther that currently exists only partially resembles the historic population genetically and physiologically. Fourth, this option perpetuates the problem we have as a result of inbreeding depression. Finally, breeding facilities and space are currently limited and would have to be expanded. Optimistically, captivity may offer an opportunity to ameliorate the health problems of wild animals and to improve their breeding.

7. Shuffle animals between locations in south Florida.

Such a program could help equalize the reproductive success of individuals (particularly males) and so increase the effective population size and delay loss of genetic variation. For example, dominant male #12 is probably over-represented genetically in the Florida panther population and due to his relatedness with most of the females (daughters, grand-daughters, etc.) may be exacerbating the rate of inbreeding. Replacing this dominant male with a non-related peripheral male would provide an opportunity to introduce new genetic material and for a short time, decrease the rate of inbreeding. This dominant male and any other surplus males could be removed and used with non-Florida females to establish a new population in a reintroduction site. If the removed dominant male's home range is allowed to fill naturally, there is a high probability that he will be successfully replaced by a local, peripheral male. On the other hand, if a peripheral male is moved from Big Cypress National Preserve (e.g., #42) or Everglades National Park (e.g., #16) into the vacant range, there is a chance that the introduced male will home and not establish himself where he is needed. Also, the new male may be killed by intra-specific fighting, or he may kill other males or females. This option does less to correct genetic defects than introduced non-Florida genes. This option would allow demographic management but may not solve genetic problems.

8. Use surplus south Florida males and non-Florida females to found new populations Reintroduction sites.

Under this option at least some Florida genotypes are perpetuated but in an intercrossed background. Young male panthers have limited options for dispersal and establishment in south Florida and several have been killed in intra-specific fighting. This option involves the removal of surplus male panthers in the south Florida population for translocation and reintroduction. This options assumes that males can be identified for translocation to sites that are unoccupied by panthers. Female non-Florida individuals would be placed into the reintroduction area with the males. This option would allow the opportunity for young male panthers to establish territories, breed with non-Florida individuals and produce intercross offspring.

9. Leave the Florida panthers where they are, without introgression.

Inaction does not appear to be a viable option. Without some sort of intervention the current wild population of Florida panthers is expected to become extinct within 25-50 years (see updated Population Viability Analysis below).

Reassessment of the Viability of the Florida Panther Population in the Absence of Further Intervention.

A. Data for Parameters of Population Viability Analysis

The 1989 Population Viability Analysis workshop concluded that the present population of Florida panthers was vulnerable to extinction within the next few decades, due to the combined effects of multiple sources of mortality and stochastic demographic and genetic instability. Since the 1989 workshop, additional data on mortality and reproduction have become available from the radio-collaring studies. These newer data have been incorporated into revised viability analyses, as described below. A more complete description of the bases of the estimates of population parameters and description of the simulation model are presented in the original PVA report (Seal et al. 1989).

1. Mortality

Mortality of radio-collared panthers has been somewhat lower than reported during the 1989 PVA workshop. Over the entire course of the radio-collaring studies (since 1981), 28 collared animals died during 1,444 animal-months of observation. This yields an annual mortality estimate of 21% (monthly mortality = 28/1444 = .0194; monthly survival = .9806; annual survival = $(.9806)^{12}$ = .7906; annual mortality = .2094). Because mortality was higher and samples sizes smaller during the early years of collaring, mortality was also estimated from data collected since 1/1/85. Between 1/1/85 and 6/30/92, 23 deaths occurred during 1,257 animal-months of observation, yielding an annual mortality of 20%. This estimate was used in the revised analyses. Animals were assumed to become post-reproductive and, hence, no longer capable of contributing to the population at age 12.

2. Reproduction and juvenile mortality

Data presented in the Florida Game Fresh Water Fish Commission annual medical reports indicate that approximately half of the litters observed or inferred to have been produced died at early ages. Among surviving litters, the mean litter size observed has been 1.9. However, data from known litters within the core study area indicate that, except for litters rapidly replaced, perhaps as few as 20% of litters die. In the Everglades National Park, three of six known litters were lost.

For the modelling analysis, we assumed a mean litter size of 2, distributed as 25% litters of one, 50% litters of two, and 25% litters of three. First year mortality was initially assumed to be 50%. To test the sensitivity of results to this uncertain parameter, scenarios were also examined with an assumption of 20% mortality of juveniles, as would occur if litters died when and only when the dam died (as adult mortality is 20%).

3. Age and frequency of breeding

Although some panthers have been observed to produce offspring by 2 years of age, it is not known whether this early age of breeding is typical or unusual. Analyses were conducted with breeding commencing either at 2 or at 3 years of age. Females were assumed to produce, on average, a litter every 24 months.

4. Breeding system

The population was assumed to be polygamous, with all adult females and half of the adult males in the breeding pool. The computer model assumes that the males within the breeding pool are reselected randomly each year.

5. Population size and carrying capacity

It is estimated that there is presently habitat in south Florida for about 50 adult panthers. If this habitat is saturated, then about 50 adults and about 25 young may exist in the population (Maehr et al. 1991). Some panther biologists have expressed concern that numbers might be lower. Analyses were conducted with either 30 or 50 initial animals (censuses prior to the breeding season) and with a habitat carrying capacity of 50.

6. Habitat loss

About 50% of the present panther habitat is on privately-owned lands, and an unknown fraction of that land is likely to be converted to uses that make it unsuitable for panthers in the coming few decades. Scenarios were assessed with no change in habitat, a 1% annual loss over 25 years (approximately 25% total loss), and a 2% annual loss over 25 years (approximately 50% total reduction in habitat).

7. Environmental variation

At this time, as in 1989, there are insufficient long-term data to allow estimating the annual fluctuations in birth and death rates that might be caused by variation in the environment. All modelling was conducted under the assumption that birth and death rates are subjected to no environmental variation. If environmental variation was substantial, it would further destabilize the population relative to the results presented here.

8. Effects of inbreeding

Although the deleterious effects of inbreeding appear to be impacting the panther population, it is not known, quantitatively, how severely inbreeding has reduced fitness, nor how it will further impact fitness if the panthers become even more inbred. Models were assessed under an assumption of no effect of further inbreeding, an assumption of 1 recessive lethal per present animal (a moderately low impact), or an assumption of 3 lethal equivalents (modelled as heterotic, rather than recessive, effects) per animal, as is typical of many other species of mammals.

B. Population Viability Analysis Results

Each scenario described above was examined by simulating 250 populations with the population viability analysis program VORTEX (Lacy, in press). The following tables present the population fates projected 25 years, 50 years, 100 years, and 200 years into the future. The input parameters that varied among scenarios are described at the top of the tables and in the first four columns. The six tables show the results for three levels of inbreeding depression with either 50% or 20% juvenile mortality. Inbreeding depression (Inbreeding depression) was set at 0, at 1 recessive lethal per individual, or at 3 lethal equivalents. Initial population size (N_0) was set at 30 or 50 (just prior to the breeding season). Carrying capacity (K) was assumed to remain stable, decrease at 1% annually, or decrease at 2% annually for 25 years. First breeding by both sexes was assumed to occur at 2 years or at 3 years of age.

The mean and standard deviation of the exponential growth rate (r) observed in the simulated populations, prior to any carrying capacity truncation each year are given in the next two columns of each table. The probability of extinction (PE), mean size (N_t) and standard deviation in size of those simulated populations not yet extinct, and the mean proportion still remaining of the initial expected heterozygosity (H) or gene diversity are presented at 25 year, 50 year, 100 year, and 200 year intervals. The median time to

extinction (TE) for those scenarios in which at least 50% of the simulated population went extinct is given in the last column of each table. Because of rounding, scenario results occasionally show a mean size and heterozygosity of remaining populations when the (rounded off) probability of extinction is displayed as 100%. These are cases in which the probability of extinction was 99.6% (249 out of 250 simulations).

The expected deterministic population growth rate (r) for the scenarios, calculated from standard life table analysis of birth and death rates, assuming no fluctuations in annual rates, is given at the bottom of each table.

C. Discussion

The revised population viability analyses project that the population is both demographically and genetically unstable, and is likely to become extinct within about 24 to 63 years if juvenile mortality is 50%. The effects of higher survival, but lower litter size, estimated from current data relative to the earlier analyses, are approximately offsetting, and the results presented here do not lead to substantially different conclusions than those that arose from the 1989 population viability analysis. Population biology parameters estimated from field research do not ensure a self-sustaining population. The time and certainty of extinction varies under the various scenarios analyzed, but all suggest that the population will be highly vulnerable to extinction if genetic, habitat, and demographic conditions cannot be improved.

If juvenile mortality is as low as is adult mortality (20%), the population would show, on average, positive population growth, but it is still subjected to inbreeding depression and random demographic effects that can cause extinctions. In particular, if the effects of inbreeding on juvenile mortality are similar to the median effects seen in other mammalian species (Ralls et al., 1988), the joint and synergistic effects of demographic fluctuations and inbreeding virtually always drive the simulated populations to extinction.

Although there is a possibility of population survival under the scenarios that assume weak or no impact of inbreeding and 20% juvenile mortality, the population was projected to become highly inbred (beyond the inbreeding that has already occurred) within a few generations. Those simulated populations which did survive contained little genetic variation, generally at levels expected after about four to nine generations of brother-sister or parent-offspring matings (about 32% and 8% of present genetic variation, respectively). However, the much greater and longer stability of the simulated populations when there is low juvenile mortality and no effects of inbreeding indicates that if genetic problems can be avoided through managed introduction of genetically divergent stock and continued movement of genetic material, and if juvenile mortality can

be kept low, the south Florida population of panthers can be a demographic "source" population as a component of a metapopulation of panthers, rather than the "sink" that is projected if inbreeding effects accumulate or if juvenile mortality is about 50%.

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Florida panther population viability without further intervention: No inbreeding effects, 50% juvenile mortality

Inbr. depr.	N_0	Change in K: 25 yrs	1st breed	Growth:r		25 yr results				50 yr results				100 yr results				200 yr results				THE
				mean	SD	PE	N_e	SD	H	PE	N_e	SD	H	PE	N_e	SD	H	PE	N_e	SD	H	
None	30	-2%/yr	2	-.042	.204	14	17	7	76	55	12	7	51	92	12	7	22	100	4	0	0	47
			3	-.082	.223	34	10	6	72	92	9	6	53	100	--	--	--	100	--	--	--	30
		-1%/yr	2	-.039	.199	11	19	10	76	48	18	10	58	85	16	11	37	98	22	5	30	53
			3	-.081	.226	34	11	7	70	87	9	7	50	100	5	0	18	100	--	--	--	31
		None	2	-.040	.193	16	22	12	78	46	18	12	60	85	20	13	35	99	15	3	4	53
			3	-.083	.225	38	11	6	72	87	9	6	55	100	--	--	--	100	--	--	--	29
	50	-2%/yr	2	-.040	.200	5	18	7	81	46	13	7	56	86	13	7	25	100	--	--	--	53
			3	-.083	.201	22	12	7	79	84	9	6	53	100	--	--	--	100	--	--	--	35
		-1%/yr	2	-.039	.186	6	22	10	82	44	17	10	59	82	19	10	31	97	17	9	17	56
			3	-.080	.210	18	13	8	78	81	10	7	57	99	6	1	22	100	--	--	--	35
		None	2	-.035	.178	3	26	13	83	31	21	13	67	76	21	12	41	95	23	18	21	63
			3	-.079	.208	18	14	8	79	80	9	6	58	100	--	--	--	100	--	--	--	38

Note: deterministic r (from life table) = -.018 with breeding at 2, r = -.056 with breeding at 3.

Florida panther population viability without further intervention: 3 lethal equivalents, 50% juvenile mortality

Inbr. depr.	N_0	Change in K: 25 yrs	1st breed	Growth:r		25 yr results				50 yr results				100 yr results				200 yr results				TE
				mean	SD	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	
3 H	30	-2%/yr	2	-.077	.207	25	13	7	76	92	5	3	54	100	--	--	--	100	--	--	--	33
			3	-.108	.227	53	8	5	72	99	5	2	35	100	--	--	--	100	--	--	--	24
		-1%/yr	2	-.080	.213	26	13	8	76	91	7	4	56	100	--	--	--	100	--	--	--	32
			3	-.104	.225	53	8	5	72	98	3	1	33	100	--	--	--	100	--	--	--	25
		None	2	-.079	.215	19	15	11	75	86	8	6	54	100	--	--	--	100	--	--	--	35
			3	-.102	.227	51	8	5	73	100	2	0	38	100	--	--	--	100	--	--	--	25
	50	-2%/yr	2	-.073	.199	8	15	7	80	82	7	4	59	100	--	--	--	100	--	--	--	39
			3	-.102	.213	28	10	6	77	98	4	3	51	100	--	--	--	100	--	--	--	31
		-1%/yr	2	-.069	.194	3	19	9	82	71	9	6	58	100	--	--	--	100	--	--	--	44
			3	-.100	.209	27	11	6	78	98	5	6	56	100	--	--	--	100	--	--	--	31
		None	2	-.073	.194	7	20	12	82	75	10	7	62	100	--	--	--	100	--	--	--	40
			3	-.100	.216	26	11	6	77	95	5	2	46	100	--	--	--	100	--	--	--	31

Note: deterministic r (from life table) = -.018 with breeding at 2, r = -.056 with breeding at 3.

Florida panther population viability without further intervention: 1 recessive lethal, 50% juvenile mortality

Inbr. depr.	N_0	Change in K: 25 yrs	1st breed	Growth:r		25 yr results				50 yr results				100 yr results				200 yr results				TE
				mean	SD	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	
1 L	30	-2%/yr	2	-.050	.204	14	15	7	77	61	12	6	54	96	12	7	7	100	--	--	--	42
			3	-.085	.223	38	9	6	73	93	8	7	55	100	--	--	--	100	--	--	--	28
		-1%/yr	2	-.049	.201	20	18	10	78	62	15	9	56	92	16	10	32	100	5	0	0	42
			3	-.091	.226	44	9	6	70	93	8	5	52	100	4	0	22	100	--	--	--	27
		None	2	-.047	.198	18	19	11	78	56	16	12	56	89	17	11	36	99	18	14	22	46
			3	-.091	.226	42	10	6	73	93	11	8	47	100	--	--	--	100	--	--	--	28
	50	-2%/yr	2	-.046	.196	7	17	7	82	55	13	7	61	94	13	7	29	100	25	0	0	47
			3	-.087	.207	22	12	6	79	88	7	4	53	100	--	--	--	100	--	--	--	34
		-1%/yr	2	-.049	.194	5	21	10	83	50	15	9	62	93	15	9	31	100	--	--	--	50
			3	-.085	.208	17	13	8	80	85	9	7	60	100	3	0	44	100	--	--	--	35
		None	2	-.044	.185	5	24	13	84	43	20	13	68	87	20	14	42	99	13	6	0	58
			3	-.088	.213	20	12	8	79	82	8	5	55	100	--	--	--	100	--	--	--	34

Note: deterministic r (from life table) = -.018 with breeding at 2, r = -.056 with breeding at 3.

Florida panther population viability without further intervention: No inbreeding effects, 20% juvenile mortality

Inbr. depr.	N_0	Change in K: 25 yrs	1st breed	Growth:r		25 yr results				50 yr results				100 yr results				200 yr results				TE
				mean	SD	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	
None	30	-2%/yr	2	.063	.163	0	25	3	82	3	22	5	61	14	21	5	35	32	22	5	9	--
			3	.001	.189	4	23	5	81	18	17	7	62	58	18	7	34	87	17	7	14	85
		-1%/yr	2	.069	.128	0	36	4	84	1	34	5	70	2	34	5	48	5	34	5	21	--
			3	.006	.160	2	31	8	84	12	28	9	69	31	26	10	49	63	26	10	24	149
		None	2	.072	.109	0	48	5	85	0	47	5	75	0	48	4	57	1	48	5	34	--
			3	.011	.136	2	38	12	84	8	39	12	74	20	38	12	55	41	39	11	35	--
	50	-2%/yr	2	.062	.161	0	25	3	82	1	22	5	63	12	23	5	36	28	22	5	13	--
			3	.005	.185	1	23	4	84	19	18	7	65	52	17	7	39	84	17	7	14	98
		-1%/yr	2	.068	.129	0	36	4	85	1	35	5	71	4	34	5	51	8	34	5	25	--
			3	.008	.155	1	31	8	86	9	28	9	72	30	27	9	47	58	27	10	21	173
		None	2	.072	.108	0	48	4	87	0	48	5	77	0	48	5	59	1	47	6	38	--
			3	.014	.131	0	40	10	88	3	37	13	76	15	39	12	58	29	39	12	32	--

Note: deterministic r (from life table) = .081 with breeding at 2, r = .023 with breeding at 3.

Florida panther population viability without further intervention: 3 lethal equivalents, 20% juvenile mortality

Inbr. depr.	N_0	Change in K: 25 yrs	1st breed	Growth:r		25 yr results				50 yr results				100 yr results				200 yr results				TE
				mean	SD	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	
3 H	30	-2%/yr	2	-.010	.191	0	24	4	83	18	14	7	59	98	7	5	38	100	--	--	--	65
			3	-.037	.199	4	20	6	82	48	11	6	60	99	3	0	47	100	--	--	--	51
		-1%/yr	2	-.009	.177	0	34	6	85	6	26	9	70	79	10	8	39	100	--	--	--	85
			3	-.040	.192	6	26	10	83	37	15	9	66	98	5	1	14	100	--	--	--	56
		None	2	-.008	.166	1	45	8	86	4	37	12	75	54	18	12	53	100	--	--	--	98
			3	-.039	.189	2	30	14	83	32	22	13	71	94	8	3	52	100	--	--	--	64
	50	-2%/yr	2	-.005	.192	0	24	4	83	18	15	7	64	98	6	5	30	100	--	--	--	66
			3	-.036	.196	0	21	5	85	41	11	6	61	100	2	0	38	100	--	--	--	53
		-1%/yr	2	-.008	.175	0	34	6	86	4	24	10	72	80	10	8	45	100	--	--	--	83
			3	-.037	.186	0	28	9	86	22	17	10	70	98	5	3	46	100	--	--	--	63
		None	2	-.006	.162	0	46	6	88	1	41	10	77	42	21	13	53	100	12	0	22	108
			3	-.035	.175	1	36	12	88	14	23	13	74	91	8	5	48	100	--	--	--	71

Note: deterministic r (from life table) = .081 with breeding at 2, r = .023 with breeding at 3.

Florida panther population viability without further intervention: 1 Lethal recessive, 20% juvenile mortality

Inbr. depr.	N_0	Change in K: 25 yrs	1st breed	Growth:r		25 yr results				50 yr results				100 yr results				200 yr results				TE
				mean	SD	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	PE	N_t	SD	H	
1 L	30	-2%/yr	2	.054	.166	0	24	4	83	5	22	5	63	20	21	6	28	40	22	5	8	--
			3	-.003	.192	2	21	6	83	22	17	7	63	58	16	7	28	89	18	6	9	87
		-1%/yr	2	.065	.130	0	36	5	85	1	34	6	71	3	34	5	41	8	35	5	16	--
			3	-.001	.170	2	29	10	84	17	25	10	71	46	24	11	42	75	24	11	11	105
		None	2	.068	.110	0	47	7	87	0	47	5	76	0	47	7	55	1	48	5	25	--
			3	.006	.145	4	36	13	85	14	35	13	74	34	35	13	52	52	42	10	23	190
	50	-2%/yr	2	.056	.167	0	25	3	84	9	21	5	63	24	21	5	33	44	21	5	9	--
			3	-.006	.190	0	22	5	85	19	15	7	62	69	17	7	36	91	20	6	13	71
		-1%/yr	2	.066	.128	0	36	4	86	0	33	6	72	3	34	6	46	7	34	5	18	--
			3	.003	.161	0	31	8	87	10	25	9	73	35	25	10	42	65	27	9	15	141
		None	2	.068	.108	0	48	5	88	0	47	5	78	1	47	5	55	2	48	5	29	--
			3	.006	.139	0	38	12	88	8	36	13	77	27	36	13	55	46	36	14	25	--

Note: deterministic r (from life table) = .081 with breeding at 2, r = .023 with breeding at 3.

Appendices

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2. Possible Reproductive Strategies

Artificial insemination might be used in an introcross protection program. The feasibility of using AI in this connection rests on two immediate needs:

a) the production of hybrid offspring for basic research into the effects of hybridization on health and reproductive fitness and release into the wild for genetic augmentation purposes, and

b) the continued development and refinement of assisted reproduction including (i) improved ovulation induction (estrus control) therapy and (ii) enhanced artificial insemination (AI) technology using fresh and frozen sperm.

A systematic research plan is needed that focuses upon the following AI combinations: non-Florida male X Florida females; Florida male X non-Florida female; Florida male X Florida female; non-Florida male X non-Florida female. The highest priority should be testing the viability of non-Florida sperm in Florida females, because this combination has the highest likelihood of success (due to compromised sperm quality in Florida panther males). However, a simultaneous, comparative evaluation of all 4 AI strategies will provide the first definitive data on the true biological vitality of Florida panther sperm. Another advantage is that these studies can be initiated immediately; (1) both Florida and non-Florida cougars already are in captivity (4 coryi males, 2 coryi females, 2 stanleyan males, 5 stanleyana females) and are maintained in state-of-the-art research facilities, (2) a substantial database already exist for this cougar population and (3) preliminary data suggest that AI can be used to produce living young. Needs include (1) increasing the captive population with at least 2 additional Florida females, 1 additional non-Florida male and 4 additional non-Florida females, and (2) providing facilities and research support.

3. References (for Introduction)

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GENETIC MANAGEMENT CONSIDERATIONS

FOR THREATENED SPECIES

WITH A

DETAILED ANALYSIS OF THE FLORIDA PANTHER

(*Felis concolor coryi*).

A report by the participants in a genetic augmentation workshop sponsored by the U. S. Fish and Wildlife Service in cooperation with the Captive Breeding Specialist Group SSC/IUCN.

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GENETIC MANAGEMENT CONSIDERATIONS FOR THREATENED SPECIES

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DETAILED ANALYSIS OF THE FLORIDA PANTHER

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Introduction And General Position Statement

Biodiversity is maintained and enhanced by natural, geographic structure in the environment. To take a large scale example, different continents contain distinctive floras and faunas such that overall global species diversity is much higher than would otherwise be expected. Human-mediated introductions of exotic animals and plants have resulted in reduced global species diversity and are increasingly recognized as highly undesirable in terms of ecological effects on recipient biotas. Numerous examples exist in which extinction of native species was attributable to the introduction of exotic taxa. Recent experience in North America with exotics such as the zebra mussel and grass carp exemplify additional and sometimes disastrous ecological problems that can attend species introductions.

Perhaps less well appreciated is that geographic translocation of conspecifics within the range of a species can also have strong negative consequences. In the last two decades, the evidence from molecular genetics has confirmed and extended earlier suspicions based on morphological comparisons that geographic populations within many species are genetically differentiated to varying but often substantial degrees. Geographic differentiation within a species may have both historical and adaptive components. The following are some of the likely consequences of ill-conceived translocations of individuals and genetic material from one population to another:

1. Homogenization of the genetic composition of populations through decay of between-population differences;

2. Blurring or irretrievable loss of genetic information on the intraspecific evolutionary histories of populations;

3. Placement in jeopardy or outright destruction of local adaptations, through introduction of foreign genetic material, breakup of coadapted gene complexes, or genetic swamping;

4. Creation of reproductive difficulties when transplanted individuals differ from recipients in karyotype or other genetic characteristics that may decrease fitness of intercross progeny or their descendants;

5. Disruption, in some species, of the social structure and population stability of the recipient population;

6. Subsequent spread of introduced forms into unintended areas;

7. Unintentional introduction or spread of parasites or disease vectors;

8. Creation of a false sense of management accomplishment (and a masking of underlying environmental difficulties) in situations where repeated translocations from a demographically strong source population are absorbed lost in a recipient population that is not self-sustaining and represents a demographic sink.

Human-mediated translocation of plants and animals is fraught with dangers and should be strongly discouraged as stated in the IUCN Policy on Translocation (IUCN, Gland, 1990). However, in some special circumstances, translocation (managed gene flow and population augmentation) may be warranted and desirable to maintain small populations that are isolated because of human-induced fragmentation of the environment. The burden of proof in any proposed translocation program should rest squarely on the advocates rather than on the opponents of this management option. The purpose of this document is to outline the necessary procedure for considering or initiating a translocation program.

Exceptions To The General Position On Translocation Of Plants And Animals:

Identifying Candidate Species for Genetic Management and Population Augmentation.

Translocation (managed gene flow and population augmentation) may be necessary when a population is small and artificially isolated due to human-induced habitat fragmentation. The guidelines outlined here apply to the augmentation and genetic management of existing populations. They do not apply to introduction of exotic species for game, food or amusement, reintroductions of species into formerly occupied areas, introductions for biological control or environmental remediation (e.g. release of natural or genetically engineered organisms to metabolize or sequester pollutants).

Two types of threats to continued existence of a population could lead to categorizing it as a candidate for population augmentation:

1. Demographic threats. Current or past rates of population decline, current or anticipated achievement of a critical small size, and skewed sex ratios or age structure that would threaten the existence of a population.

2. Genetic threats. Current or anticipated loss of genetic variability that is currently or potentially adaptive, and inbreeding depression.

Demographic Threats

Current trends in population size should be assessed in the context of historical demographic

information. Data on the life history and age structure, the temporal and spatial structure of the population, and its behavioral/social system including territoriality and cultural transmission are especially important.

The possibility of a critical threshold size or density of a population necessary to its survival should be investigated. Such a threshold could result from the difficulty of finding a mate in a sparsely distributed population, cooperative hunting or group defense behavior, dispersal from limited areas of suitable habitat into unsuitable habitat, or the dynamics of local extinction and colonization in a fragmented habitat (Lande, R. 1988. Genetics and demography in biological conservation. Science 241:1455-1460.).

The first course of action in response to a perceived demographic threat should be to remove the cause of the threat, and to allow the population to increase by itself. If the demographic threat cannot be removed in time to allow natural recovery, then temporary augmentation of the population from the same or closely similar genetic stock should be considered. Use of a genetically differentiated stock for purposes of demographic augmentation should be avoided if possible. In the absence of genetic threats, local amplification of the population, e.g. by captive breeding, is preferred.

Genetic Threats

The main criteria for genetic threats are small population size and (geographic) isolation caused by human action, e.g habitat destruction. Often, if not usually, genetic threats will be manifested only after demographic threats are apparent; that is, genetic threats become important at smaller population sizes. Loss of genetic variability in all types of characters becomes a significant concern for populations below an effective size of a few hundred individuals. However, some characters such as disease resistance may be based on genetic variants that are usually rare and found only in very large populations. It is conceivable that a specific genetic threat, such as lack of resistance to a particular disease, could be met by introduction of a specific resistant allele or genotype into a population, rather than random gene flow.

Desirability of preserving a population in a given area can be based on a number of considerations, including the degree of genetic differentiation from other populations of the same species as indicated by morphological, molecular and reproductive traits. The risks from demographic and genetic factors have to be weighted against the risk of diluting or swamping local genetic differences or adaptations by artificial gene flow or introduction of genetic incompatibilities such as major chromosomal rearrangements. A level of gene flow much less than the local selective advantage of a character is unlikely to result in swamping of that character by gene flow, although other less adaptive characters may be significantly diluted or swamped. The adaptive value of a character can be inferred from behavioral or ecological observations. However, its adaptive value can be directly demonstrated in terms of fitness effects only by measurements of natural selection in the natural environment. This requires studying

individual variation within a population or transplantation experiments among populations in different environments, which may not be feasible in many species.

Different manifestations of inbreeding depression should be distinguished, along with the types of evidence for their occurrence. Within a population, decrease of the mean of a character such as body size upon inbreeding can be estimated from pedigree data or breeding experiments. Fitness components including reproductive rates and offspring viability are often subject to substantial inbreeding depression upon matings between close relatives in historically large outcrossing populations. Inbreeding depression affecting an entire population, e.g. due to the fixation of a deleterious recessive gene, can be documented by transplantation experiments among populations (which again may be impractical for many species), or implicated by extensive comparative data among populations.

Inbreeding depression usually is manifested only upon matings between close relatives, or continued random mating in small population of effective size at or below a few dozen individuals.

Before augmenting a population to reduce current or future inbreeding depression, ideally it should be verified by experimental intercrossing, e.g., in a captive stock initiated as part of an augmentation program, that inbreeding depression does indeed exist and can be ameliorated by artificial gene flow. Augmentation should be based on the most similar genetic stock available, even another, small, isolated population that may be suffering from genetic problems provided that these are not identical in detail to those of the target population (i.e. the source population could show inbreeding depression in morphological traits different from those in the target population). Augmentation through a captive population to a wild population allows control of the rate and amount of genetic material to be introduced.

Time scales for action should be evaluated by balancing the relative risks of extinction or genetic damage to the population versus the risks associated with artificial gene flow.

Levels of Gene Flow

The level of artificial augmentation should be commensurate with the demographic or genetic risks faced by the population. Demographic augmentation should counteract (artificial) causes of population decline (including interaction with exotic introduced species), until these can be ameliorated or removed.

Current genetic problems, especially inbreeding depression, require enough gene flow to solve the problem. This may initially be greater than the level of original gene flow to prevent anticipated genetic risks from small population size and geographic isolation caused by human action. However, the cumulative genetic augmentation necessary to mitigate current inbreeding depression generally should not require addition (or substitution) of more than several percent

(2-5%) of the total genetic material in the target population.

After currently existing genetic problems have been solved by genetic augmentation, if the only apparent genetic risk is in the future, the management goal should be to achieve a natural level (that which occurred before isolation of the population) of gene flow. This can be assessed from historical and current observations of dispersal and geographic distribution, and/or (with caution) from molecular genetic studies (e.g. estimates of number of migrants per generation among population subdivisions [Slatkin, M. 1985. Gene flow in natural populations. Annual Review of Ecology and Systematics 16:393-430; Slatkin, M. and N.H. Barton. 1989. A comparison of three indirect methods for estimating average levels of gene flow. Evolution 43:1349-1368.). The level of artificial gene flow should be lower than the estimated natural level if the only remaining source is more genetically differentiated than the historical source(s).

Procedural Overview For Population Augmentation Program

I. Overall Procedural Issues - Advanced Planning & External Review of Plan.

A. The decision trees and sequence of steps below should be documented in advance and sent out for external peer review.

B. Suitable reviewers, in addition to other agencies and academic reviewers, should include 3 Specialist Groups of the IUCN - World Conservation Union (who offer expertise based in part on assembly of experience and mistakes made by others worldwide):

1. Reintroduction Specialist Group

2. Captive Breeding Specialist Group

3. Relevant Taxon Specialist Group (e.g., Cat Specialist Group)

II. Source of Stock

A. Demographic supplementation of an existing local population should generally be accomplished with stocks known to be very similar genetically, and evaluated in advance for risk of disease transmission.

1. Stock from another historically nearby (i.e. contiguous) population which formerly exchanged significant numbers of dispersing individuals with the

threatened population and is genetically very similar, is suitable.

2. Stock withdrawn directly from the population at risk and amplified by captive breeding or other means, is suitable.

3. When a population has been completely extirpated, the preferred source of stock for re-establishment may be a more complex issue which warrants further analysis.

B. Genetic supplementation of an existing local population should generally be accomplished with stocks known to be very similar but not identical genetically, and evaluated in advance for risk of genetic incompatibilities and disease transmission. See the following section of this document, where this issue is developed in more detail.

III. Method of Introduction - the emphasis here is on practical means of lowest disease risk which can be monitored for success and effect - see Follow-up section.

A. Introduction of early life stage material offers the advantage of natural integration and cultural transmission. Possibilities include:

1. Artificial insemination where techniques exist.

2. Embryo transfer where techniques exist.

3. Egg-swapping

4. Youngster swapping

B. Introduction of adults with relevant wild experience

C. Introduction of captive-bred individuals trained for release.

IV. Follow-up

A. Sound follow-up study design (in advance) is critical:

1. Managers need to know outcomes.

2. Techniques can only be improved if their success and failure is measurable.

3. Success must be recognizable so the effort can stop when success has been assured or if it becomes apparent that the chosen strategy will not succeed.

4. Poor follow-up has made many release programs a wasted effort from which little is learned.

B. Follow-up is part of the necessary ongoing monitoring and evaluation needed for a population at risk. This should consist of at least:

1. Creation of studbook data sets for the wild population and any existing captive populations.

2. Analytical evaluation of age- and sex-specific fecundities, mortalities, age structure, and population growth or decline rates.

3. Before/after evaluation of genetic composition of population, changes in fitness traits.

4. Public reactions before, during, and after the translocation.

5. Evaluation (of a surviving population) should begin with a 3-5 year baseline study prior to treatment (concurrently with capture studies) and continue for 3-5 years following treatment. Reevaluation of the program should occur at least every 3-5 years.

Criteria For Assessing Appropriateness Of Population Augmentation Program

The following steps should be examined prior to any genetic augmentation of a natural population. The urgency of preventing imminent extinction might necessitate action based on an assessment of partial information before each step can be addressed fully, but adequate attention to the concerns below should not be needlessly postponed until a crisis demands sudden action on behalf of a population. Translocation of organisms involves considerable risks not only to that population, but to all components of the natural communities affected (see introductory section). Concern for the natural environment and biodiversity demands that artificial intercrossing (see definitions) be undertaken only after careful deliberation, after all reasonable precautions have been taken, and after alternatives have been examined. Possible benefits of augmentation must be weighed against costs and risks of artificial translocations, and lack of knowledge concerning any points below must be viewed as contributing substantially to the risks.

Verify that the problems facing the population include genetic loss.

Possible indicators of genetic loss include (temporal trends or traits relative to other populations): (1) Projection of a high rate of genetic loss in the past and/or in the future based on population size and/or structure; (2) Low genetic variation observed in the population; (3) High rate of observed close inbreeding; (4) High prevalence of morphological abnormalities; (5) Health problems; (6) Compromised reproductive status (e.g., poor sperm count or viability, lack of regular cycling of females); (7) Low reproductive output; (8) Poor survival. For the last four possible indicators of genetic problems, attempts should be made to assess whether non-genetic causes (e.g., poor nutrition, social stress, shrinking habitat, or disease) might be responsible for poor performance.

Confirm that genetic problems can be ameliorated by intercrossing.

Experimentally verify potential reversal of genetic problems by intercrossing. This would likely be in a captive setting, in which non-genetic factors could be controlled and data easily collected while not placing the wild population at risk. Such studies could be concurrent with the genetic and demographic studies of the wild population.

Evaluate habitat availability, occupancy, quality, and trends to demonstrate the existence of sufficient habitat to allow the population to benefit from the introduction of additional genes.

There is rarely value in augmenting a population in already saturated habitat, or if continued habitat deterioration is likely to preclude population recovery. Restocking should not be used to continually replenish areas that are functionally population "sinks", and it would commonly be difficult for translocated animals to become established in a resident population that fully occupies available habitat. However, there may be situations in which genetic problems could be remedied while steps are taken to recover habitat quality or to prepare alternative habitat. The purpose would not be to bolster numbers of animals (genetic augmentation might take the form of demographic exchange rather than addition), but rather to improve the genetic health of a population in order to increase resiliency to perturbations and to allow for population expansion when habitat becomes available or to increase viability within existing habitat with lessening of human related pressures.

Demonstrate lack of negative effects of intercrossing (before gene pools are irreversibly mixed).

Serious problems are much less likely to arise if the source and recipient populations had exchanged migrants prior to human-caused habitat or population fragmentation. Notwithstanding the perceived similarity of the populations, it would be prudent to test experimentally, or otherwise under controlled circumstances, the viability, fecundity, and morphological continuity of first and second generation intercrosses.

Confirm availability and appropriateness of potential source population(s).

To minimize negative impacts (foreseen and otherwise) while achieving desired goals of restoring genetic and demographic viability to a small, isolated population, the following ranked list of criteria is suggested for choosing source population(s):

1. Use source population(s) historically in closest geographic proximity, preferably one(s) formerly in contact with the remnant (recipient) population and not formerly separated by geographic barriers to natural dispersal. The goal of the translocation is to restore, to the extent possible, processes that augmented genetic variation prior to human disruption of natural gene flow.

2. Use source population(s) demonstrated to be genetically similar to the recipient population. Karyotypic differences between populations are often indicative of difficulties in intercrossing (often not apparent until the second generation), and information on karyotypic similarity can often be obtained relatively quickly. The diversity of molecular (allozyme, immunological, DNA) techniques available allow quantification of the degree of genetic divergence over a very wide range, from relationships among higher order taxa down to familial relationships within a local pedigree. Study of genetically based morphological variation can be important in revealing adaptive divergence among populations.

3. Use source population(s) from similar habitats. The goal is to allow restoration of potentially adaptive genetic variants into a population that is so small as to be subjected to considerable non-adaptive drift (loss of alleles adapted to components of the habitat and fixation of deleterious alleles).

Establish ability (and plan) to monitor impacts of translocations for intercrossing.

Expected outcomes should be specified. Potential dangers must be identified. Methods need to be designed for determining if anticipated benefits are achieved without serious negative impacts. Contingency plans should be made for changing, halting or, if possible, reversing a management plan that fails to meet pre-defined acceptable levels of performance. Data collection throughout is essential to evaluate success and to help guide future efforts at recovery of endangered populations.

The evidence from each of the above considerations must be evaluated relative to each other and to the perceived urgency of action. The required level of assurance of benefit and minimization of risk could be less if the population is unlikely to persist for long in the absence of action. If risks are judged to be low (e.g., source and recipient populations are known to have regularly exchanged migrants until recently), modest benefits (e.g., sustenance of historic levels

of variation) may lead to a decision to proceed with intercrossing. Higher risks should be accepted only if benefits are large and highly likely to be obtained and if the cost of not taking the action is judged to outweigh the risks.

Definitions

1. Problems With The Word "Hybrid": Mixing Species, Subspecies, And Populations

Words such as "hybrid", "intergrade", "mongrel", and "interbreed" tend to carry strong but varying connotations to most people. For example, many taxonomists confine the use of "hybrid" to the products of crosses between distinct species, whereas geneticists commonly use the word in reference to products of crosses between any organisms that carry different genetic markers. "Intergrade" usually refers to progeny from crosses between "subspecies", but definitions and criteria for identification of subspecies vary. Furthermore, many traditionally-recognized named subspecies (i.e., populations within a species assigned a Latin trinomial) are often highly suspect as guides to the major evolutionary or genetic partitions within species, having originally been described from very limited assessments of morphological or other attributes with unknown genetic basis (See next section on Subspecies).

To provide a general and neutral word to be used in initial discussion regarding progeny resulting from parents of different sources, we suggest "intercross". This word should be used in a generic sense to encompass what is normally meant by more conceptually loaded terms such as hybrid or intergrade. Additional refinement of what is meant by the term in particular instances must be achieved by reference to additional sources of information, such as the magnitude and pattern of genetic divergence and reproductive relationships of the populations or taxa involved. A schematic representation of the term is as follows:

INTERCROSS

"Hybrid"	"Intergrade"	"cross",interbreed"
(Species level)	(subspecies level)	(geographic populations)

<<<<<<<<<<<< Continuum of Possibilities >>>>>>>>>>>>>>>>>>>>

(requiring additional knowledge on such factors as magnitude of genetic differentiation, reproductive relationships, geographic partition, etc).

2. Problems of Using Named Subspecies As Units For Conservation, Management, And Recovery Decisions

The use of named subspecies as units in conservation, management, and recovery decisions is plagued with a series of problems. These problems are highlighted by the history of using subspecies nomenclature in systematics. For several decades the prevailing practice among systematists has been to avoid naming subspecies. The move away from naming subspecies was spurred by the observation that different traits often show different patterns of geographic variation within a species (discordant geographic variation (Wilson, E.O. and W.L. Brown. 1953. The subspecies concept and its taxonomic application. Syst. Zool. 2:97-111.). In this common situation, the naming of subspecies depends on which traits or characters are being considered. Because of this arbitrary aspect of subspecies designation, most systematists stopped naming subspecies in the 1960's or earlier. Nevertheless, subspecies names persist in the literature.

Increasing application of molecular techniques in the past 30 years has further weakened the case for using named subspecies as units for conservation. The molecular techniques now enable us to estimate the phylogenetic relationships of populations within a species (Avise, J.C. and R. Martin. 1990. Principles of genealogical concordance in species concepts and biological taxonomy. Oxford Surveys in Evolutionary Biology). Sometimes the new molecular results show geographic or phylogenetic patterns that coincide with the old subspecies names. Frequently, however, the new results conflict with the old nomenclature. The conflicts may reflect: (a) discordance between molecular and phenotypic patterns of geographic variation, (b) molecular resolution of units not represented by named subspecies (e.g., units within subspecies), (c) failure of named subspecies to reflect phylogenetic relationships. Thus, valid units commonly exist within species, but often these are not reflected by the named subspecies.

Valid units within a species could be diagnosed by searching for geographic concordance between different sets of traits. The issue in making a conservation decision is whether new molecular information shows geographic concordance with older subspecies names based on analysis of phenotypic traits or geographic separation. Taking analysis a step further, one can ask whether the phylogenetic relationships of populations are concordant across traits (Ball, R.M. and J.E. Nigel, J.C. Avise. 1990. Gene genealogies within the organismal pedigrees of random mating populations. Evolution in press.). Using this more detailed analysis requires data on multiple, genetically-based traits. For example, when multiple genetic differences concordantly distinguish populations, those populations might be considered a candidate unit for conservation, regardless of whether they reside in the same or different named subspecies.

Application Of Decision Criteria To Florida Panther Genetic Management:

Augmenting The Florida Panther Population By Intercrossing With *FELIS CONCOLOR* From Other Populations

1. Does the Florida panther meet requirements to be a candidate as an exception to the general guideline that proscribes augmentation with genetically divergent stock?

The Florida panther was formerly widespread throughout the southeastern United States and was contiguous with other populations (subspecies) of *Felis concolor*. Due to human destruction of habitat and direct persecution of animals, the subspecies has been reduced over the past few centuries to a remnant population existing only in south Florida. The south Florida population is very small, numbering no more than 30-50 adult panthers. The number of breeding animals may be no more than 20-30. Extensive surveys of possible habitat and investigations of reported sightings has demonstrated that the only remaining viable, breeding population of *Felis concolor coryi* is the south Florida population under intensive study and management. Isolated animals elsewhere, if they exist, could not be part of the breeding population. The remnant population of Florida panther is well-separated from the next closest population of *F. concolor*, in western Texas, and the two cannot exchange migrants.

Thus, the Florida panther population meets the criteria of being very small and totally isolated from all conspecifics, due to human-induced fragmentation and destruction of habitat and animals.

2. Is the Florida panther population at substantial risk of extinction?

The Population Viability Analysis conducted on the Florida panther projects, under existing demographic and genetic conditions, the extinction of the population within 25-40 years. The population size is well below criteria that have been suggested for numbers needed to assure viability (see above; O.H. Frankel and M.E. Soule. 1981. Conservation and Evolution. Cambridge University press; Franklin, I.R. 1980. Evolutionary change in small populations. In: Soule, M.E. and R.A. Wilcox (eds.). Conservation Biology. Sunderland, MA, Sinauer. Pp. 135-150.). The habitat available to the south Florida population is not sufficient to allow for expansion of the population to a size that would assure self-sustaining capabilities. Recovery of the population, whether or not it includes genetic augmentation, will require habitat preservation and management, and the identification and/or development of additional suitable habitat within the historic range of the subspecies.

3. Do the problems facing the Florida panther include genetic loss with adverse effects?

The Florida panther PVA projected a loss of 3% to 7% of genetic diversity (heterozygosity) per generation under current conditions of population size and structure. This loss is expected to accelerate unless aggressive management reverses habitat contraction and population decline. During the past decade (1981-1991), mortality of founder animals (those containing genes not known to be contained elsewhere among the living panthers) has been 49% per 24 months (M. Roelke, pers. comm., FL GFWFC). Of the 5 populations of *Felis concolor* that have been investigated by molecular genetic methods, the Florida population has the least genetic variation (7.5% polymorphic loci, 0.028 mean heterozygosity). Much of the genetic variation that does exist in the Florida panther population is contained in those animals believed to be intergrades between *F. concolor coryi* and as yet unidentified subspecies from Central or South America. Assuming that the ancestral population of Florida panthers contained as much genetic variation as do other populations of the species, approximately 50% of the genetic variation that once characterized the subspecies has already been lost.

The pedigree available information demonstrate that close inbreeding (matings between parents and offspring) has been documented in at least 3 breeding events. Second generation inbreeding is probable but undocumented.

There are a number of indicators that inbreeding and losses of genetic diversity are having damaging effects on the population. Male Florida panthers average more than 93% abnormal sperm, more than any of 5 other felid species examined to date. Of male panthers examined since 1985, 44% are cryptorchid (having only one descended testicle), and the rate of cryptorchidism has been increasing markedly since then. As of 1991, 90% of living male Florida panthers are cryptorchid (M. Roelke, FL GFWFC). Vaginal fibropapillomas were observed in at least six female panthers. These papillomas are thought perhaps to impede penile penetration during copulation and or impede transport of sperm through the female tract. Two of the females did not breed during 6.5 years of observation even though they were in regular contact with breeding males (1990 FP Report, FL GFWFC).

Recently, heart murmurs have been detected in Florida panther young adults and kittens. It is not known whether this condition is genetic in cause or whether it will change with age. However, 2 panthers have died since 1988 due to complications associated with congenital atrial septal defects.

Several unusual morphological traits that have traditionally been used to help characterize the subspecies are likely non-adaptive genetic traits that have become common in the small population by chance. Prior to 1990, all panthers thought to be historic *F. c. coryi* have a kink in the end of the tail, while this abnormality is rare among those panthers with some South American ancestry. Likewise, the majority of the historic *F. c. coryi* have a cowlick on the back. The cowlick shows up in museum specimens, and may have been common in the Florida

panther population for at least 100 years. Differences in skull morphology distinguishing Florida panthers from other subspecies are probably indicative of genetic divergence among subspecies, perhaps representing adaptive differentiation. These differences would not be taken as indications of deleterious effects of inbreeding.

Inbreeding is known to cause increased juvenile mortality and decreased reproduction in many populations (Falconer, D.S. 1990. Introduction to Quantitative Genetics. 3rd Ed. Longman, New York; Ralls, K. and J. Ballou. 1983. Extinction: lessons from zoos. Pages 164-184 in C.H. Schonewald-Cox, S.M. Chambers, B. MacBryde, L. Thomas eds. Genetics and Conservation. Menlo Park, CA: Benjamin/Cummings; Ralls, K., J.D. Ballou, and A.R. Templeton. 1988. Estimates of lethal equivalents and the cost of inbreeding in mammals. Conservation Biology 2:185-193; Wildt, D.E., M. Bush, K.L. Goodrowe, C. Packer, A.E. Pusey, J.L. Brown, P. Joslin, and S.J. O'Brien. 1987. Reproductive and genetic consequences of founding isolated lion populations. Nature 329:328-331.). Juvenile mortality has not been noted to be elevated in Florida panthers but it has not yet been well-quantified. Similarly, there is not yet evidence of poor reproductive performance by those panthers that have been breeding. Of those female panthers that have not been breeding, non-genetic causes (e.g., poor nutrition, lack of available males) have been implicated.

The above observations together strongly indicate that loss of genetic variation has been and continues to be substantial in the Florida panther population and that inbreeding and genetic loss has increasing impacts on the panthers. The lack of demonstrated loss of fitness (survival and reproduction) attributable to inbreeding may suggest that genetic losses have not so damaged the population as to preclude recovery of the population as it exists genetically at this time.

4. Are the perceived genetic problems correctable via intercrossing?

It is possible that managed translocation of animals already within the south Florida population could ameliorate immediate effects of close inbreeding. (Known pedigrees are not sufficiently deep to provide detailed knowledge of the genealogical relationships between animals in the ENP and in the Big Cypress subpopulations.) The PVA for the Florida panther and the consequent management decisions outline courses of action designed to manage the existing gene pool to recover the population. If the existing genetic variation can be captured and the founder base expanded rapidly, it is hoped that the population can be recovered without intercrossing to other subspecies. If deleterious genetical traits persist in spite of aggressive management of the existing gene pool, it may be possible and desirable, and perhaps necessary for population survival, to augment the genetic variability of the Florida panther with genetic material from other subspecies. The history of intercrossing of Florida panthers in captivity and in the Everglades National Park suggest that the reproduction and health concerns identified above may be reversible. Neither the ENP sub-population nor the Piper captive stock (both thought to be composed of mixtures between Florida panthers and South American panthers) show

cryptorchidism and kinked tails occur only rarely.

The histories of neither the Piper stock nor the ENP animals (thought to be partly derived from the Piper stock) are well documented. Controlled, experimental crosses among populations would be needed to confirm that deleterious traits could be prevented by genetic augmentation via intercrossing. Animals produced by experimental inter-populational crosses could be examined for sperm quality, presence or absence of health problems (e.g., heart murmur, vaginal fibropapillomas), and morphological traits (e.g., cryptorchidism, kinked tails).

5. Would there be negative effects of intercrossing?

The amount of genetic divergence between the Florida panther and other *F. concolor* subspecies appears to be slight (O'Brien data, 1990 FL GFWFC Report), indicating a recent and shallow evolutionary separation of populations that formerly would have been connected by gene flow. The weak inter-populational differentiation is consistent with observations that pumas are capable of long-distance dispersal. Based on the success of crossing between much more divergence populations of other carnivores, and the apparent success in crossing between Florida panthers and a South American stock (among the populations most genetically divergent from the Florida panther) likely during the creation of the Piper stock and the intergradation into the ENP population, it seems very unlikely that crosses between Florida panthers and similar subspecies from elsewhere in North America would display any negative effects in the first or later generations. The crossing experiments proposed above to verify the benefits of intercrossing also would provide an opportunity to confirm the lack of deleterious effects of intercrossing. Any evidence of "hybrid breakdown" in health, viability, or reproduction should be examined carefully in experimental crosses through at least 2 generations.

6. Are appropriate source populations available for intercrossing with Florida panthers?

The closest extant geographic population to the Florida panther is in south and west Texas. Animals from this source have already been used for experimental releases in northern Florida, and those animals appeared to adapt well to that habitat. Molecular evidence indicates that this population is genetically similar to the Florida panther, although not necessarily the most similar of the extant subspecies. Given the apparent close genetic relationships among all the North American populations, any other population could probably be used to augment the Florida panther population.

Further genetic research should be done to quantify more precisely the relationship of the Florida panther to other populations. Although the Texas population seems suitable for intercrossing experiments, other candidate populations may be found to show much closer genetic

affinities, more genetic variation, or more similar habitat use. In particular, nothing is yet known of the relationships of Central American populations of *F. concolor* to Florida, other North American, or South American populations. Central American populations inhabiting approximately comparable environments may be found to have close genetic affinities to the Florida panther. Another group of animals of interest and deserving of more extensive genetic analysis, is the Piper stock. Although it is inbred and exhibits hip problems, this captive stock has some Florida panther ancestry, and may contain Florida panther genes no longer present in the wild.

It should be noted that an option to utilize the most genetically divergent population of *F. concolor* available for intercrossing was considered. Such a strategy could maximize the input of new genetic material into the Florida panther population. If the desire were to replace the Florida panther with a healthy population of the species (but not necessarily most closely related to the animal that formerly inhabited the SE US and that still inhabits south Florida), then use of a source population or a mixture of multiple source populations to maximize genetic variation could be appropriate. At this time, however, it is still hoped that the Florida panther can be saved from extinction with as little genetic alteration as possible. The attempt should be to preserve and restore a population that resembles the ancestral populations of the subspecies as closely as possible, augmenting the gene pool of the population as much as is necessary to assure continued viability of the population.

7. Strategy for incorporating intercrossing into the recovery of the Florida panther

As stated in the Recovery Plan, in the Population Viability Analysis, and above, it is believed that the Florida panther can be recovered to viable populations with aggressive management of the existing animals. Recovery will require management and restoration of habitat combined with measures to increase productivity and survival in the wild and in a captive population. The survival and continued adaptive evolution of the Florida panther is far from assured, however, and many uncertainties in our data on the current demographic and genetic status of the population, concerning the future changes in the environment, and in our understanding of basic population processes are recognized in the various recovery documents. The extent of genetic deterioration of the Florida panther and the impact that past and ongoing genetic losses will have on the viability of individual animals and the population remains one of the areas of greatest uncertainty and concern.

The recovery of the Florida panther should proceed through three levels of increasingly interventive management. First, ongoing attempts to secure and enhance the wild population must continue. Second, the newly established captive population has been identified as an important demographic and genetic back-up for the existing wild population and as the probable only source of sufficient Florida panthers for translocation to re-establish populations in other parts of the former range. Third, having identified (above) the evidence that genetic problems

are likely contributing to the vulnerability of the Florida panther, and that opportunity probably exists to utilize other populations of the species to genetically augment the Florida panther population, it would be recommended that panther management and recovery should include intercrossing experiments. Given the urgency of action to protect the panther (PVA projects extinction in 25-40 years if current genetic and demographic trends continue), and the necessarily long development time to implement captive propagation and intercrossing wisely, it is important that the three components of panther management proceed simultaneously. Sequential implementation of the three phases, rather than overlapping implementation, would leave the population highly vulnerable to extinction between phases if one were found to be insufficient and then next phase became the primary focus of recovery efforts. Time exists now to cautiously and wisely investigate options while not jeopardizing complementary components of an overall program and while animals exist with which to undertake such actions.

The PVA projects the captive breeding program to be implemented over a 20 period. During that time, concurrent experiments on intercrossing Florida panthers with other populations can proceed. Given the potential for serious deleterious consequences of unwise and poorly planned intercrossing of populations (see opening section), and the necessity for producing two generations of intergrades to confirm the presumed benefits and the lack of dangers in intercrossing, it would be prudent to begin investigations of intercrossing as soon as is possible. It is fortunate that experimental verification of the assumptions of a program of intercrossing can be done before such a program becomes the only and last hope for preserving Florida panthers. By crossing male *F. c. coryi* to females from other populations (perhaps by artificial insemination), investigations of intercrossing could proceed without harm to the wild population in Florida nor to the captive breeding program designed to propagate Florida panthers. Panthers from Texas or elsewhere could be used immediately. Knowledge gained from such crosses would be valuable even if future genetic investigations reveal better source populations for augmentation of the Florida panther. If intercrossed panthers are never needed because efforts to protect the Florida panther with its existing gene pool are successful, any such animals produced would be good subjects for planned trial releases into candidate reintroduction sites. If it becomes desirable or necessary to augment the Florida panther population by intercrossing, the animals produced experimentally could be used as the initial stock for such augmentation.

For the purpose of restoring genetic health to the Florida panther population it should not be necessary to introduce many animals from (an)other population(s). Demographic recovery and stability of the Florida panther population (and considerable genetic stability) can be afforded by the captive breeding program with Florida panthers bred solely from the genetic stock already existing in south Florida. However, intercrossing would be necessary to accelerate improvement of the genetic health and variation of the existing population and with relatively few animals should be sufficient to restore genetic variation, should that course be determined to become necessary. In that case, further analyses will be needed to determine the optimal amount and rate of genetic augmentation.

Any efforts to genetically augment Florida panthers by intercrossing must be closely monitored. Releases of panthers produced in captivity, especially if by intercrossing, should be made in areas where the resident population (if any) is well monitored and, consequently, social interactions between resident and translocated animals can be documented. The social structure of the recipient population should be evaluated and prepared for the introduction of individuals from captivity to reduce the likelihood of social disruption and death of important individuals in the population. All released or translocated panthers should be monitored by radio-collars, in order to track dispersal, habitat use, and social interactions with other panthers, and to indicate quickly death or serious injury. If possible, unique genetic markers for each translocated panther should be identified, permitting later verification of which animals successfully enter the breeding population in the wild. The ongoing data collection that serves now to provide understanding of the population status and structure will become the baseline data for comparison to similar data taken following translocations or other manipulations of the population.

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The consequences of demographic reduction and genetic depletion in the endangered Florida panther

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The Florida panther has recently suffered severe range and demographic contraction, leaving a remarkably low level of genetic diversity. This exerts a severe fitness cost, manifested by spermatozoal defects, cryptorchidism, cardiac abnormalities and infectious diseases that threaten the survival of the subspecies.

Introduction

The recent expansive development and spread of human populations has precipitated the highest rate of species extinction since the demise of the dinosaurs [1–4]. Recognizing these events, national and international conventions have been established to identify and conserve endangered species [5–8]. Dwindling populations of endangered species have been suspected to suffer primarily from stochastic demographic factors (such as altered sex ratios and accidental mortalities), reproductive decline and disease outbreaks [9–13]. In addition, small populations often undergo inbreeding, despite instinctive avoidance of matings with close relatives. This can result in the expression of rare, normally cryptic deleterious alleles that contribute to developmental, reproductive and immunological impairments [14–22]. Close observation of the extinction process in lost species has been rare, and evidence for the various causal components has been inferential or correlative, so that conclusions as to the causes of species extinction could only be tentative. In this review we summarize previously published and new data about the population genetic variability, reproductive function and physiological fitness of populations of the Florida panther (*Felis concolor coryi*), a subspecies in imminent danger of extinction, and argue for the relevance of these observations to attempts to conserve the subspecies.

The Florida panther (Fig. 1) exists as a small relict population of approximately 30 individuals that resides in southern Florida, primarily in the Big Cypress Swamp (BCS) and adjoining Everglades National Park (ENP) ecosystems [23–30]. Before the immigration of European settlers, the panther's range included the entire southeastern portion of the United States, while populations of other *F. concolor* subspecies — called panthers, pumas, cougars or mountain lions — were spread throughout North and South America

Fig. 1. The Florida panther, *Felis concolor coryi*.

(Fig. 2). Human depredation, spurred principally by fear, legends of ferociousness toward livestock and humankind, and imposition of bounties, reduced the subspecies' range to hardwood swamps and cypress prairies of south and central Florida by the 1920s

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[23,24]. The US Department of Interior recognized the Florida panther as an endangered puma subspecies in 1967, and the Endangered Species Act of 1973 [7,8] established authority for legal protection. In 1976, field studies of the remaining free-ranging panthers were initiated, including tracking, evaluations of available prey, habitat use and causes of mortality [28]. In addition, basic studies were conducted on the remaining Florida panthers: their genetic variation and reproductive function was analysed; the contamination of their habitats was assessed; and individuals were subjected to clinical veterinary tests, including tests for exposure to infectious diseases [31–33]. The results of these analyses, summarized here, provide a telling glimpse of the genetic, physiological and environmental pressures on a large specialized carnivore on the verge of extinction in spite of heroic efforts to reverse the process.

Modern Florida panthers have been subjected to a number of risk factors that threaten their survival. For example, since 1973, some 62% of mortalities in the wild have been associated with human interaction, such as road kills, illegal hunting or injuries. Depletion of white tailed deer, their primary prey, on public lands has led to increased consumption of raccoons that carry inordinately high levels of toxic mercury; at least one mortality was the result of mercury poisoning [25,31,34]. Free-ranging panthers display physiological and endocrine impairments that could influence reproductive success [32]. Demographic modelling on

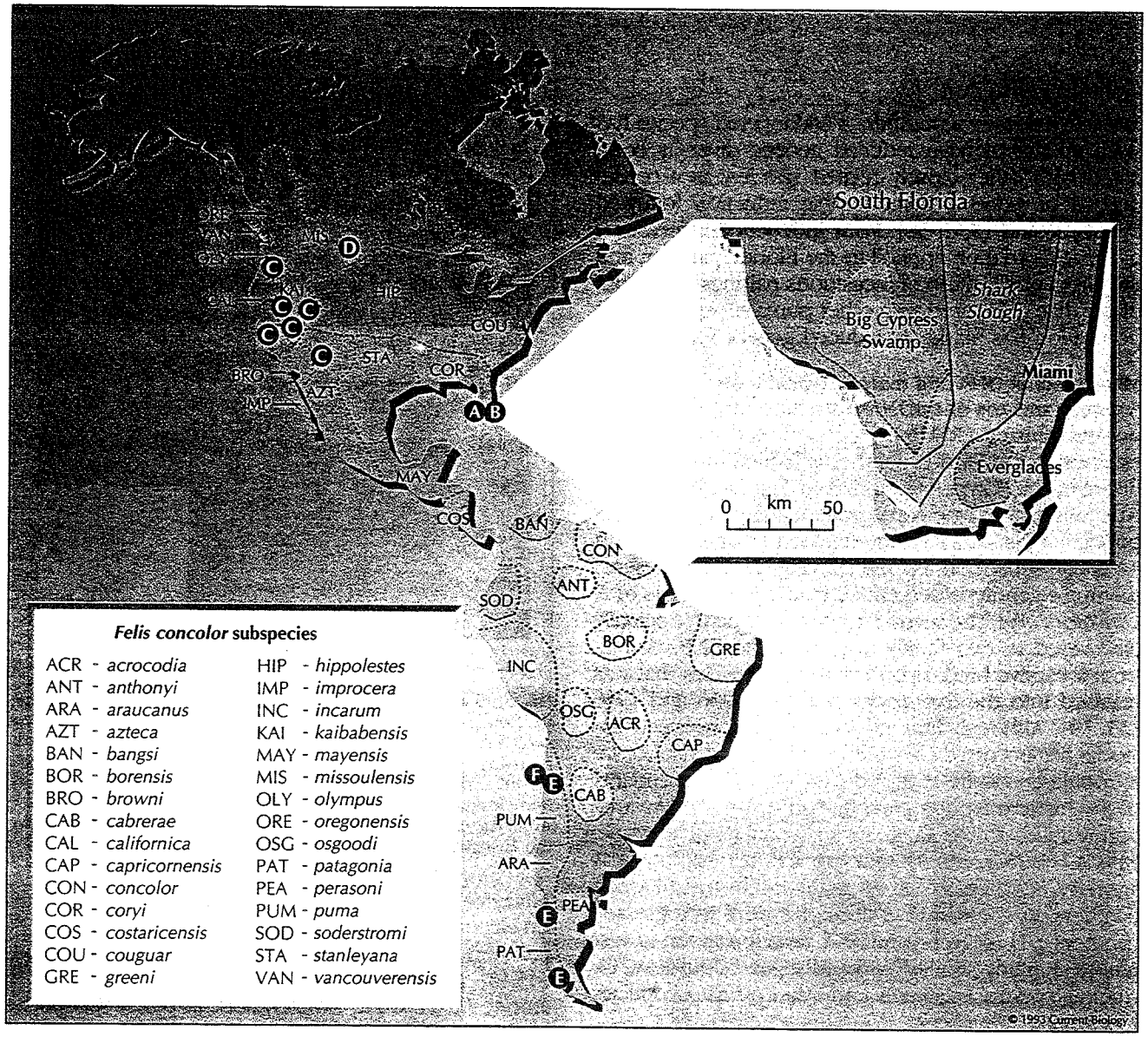

Fig. 2. Distribution of *Felis concolor* subspecies, including the historic and present (inset) ranges of *F. concolor coryi* [76]. Solid circles indicate the locales of specimens collected for this analysis; the letters inside the circles represent the mtDNA RFLP haplotypes defined by restriction enzyme typing of 109 restriction sites [33]. Haplotypes A, C and D differ from each other by one or two restriction sites.

the basis of actual age, breeding, social structure and birth rates of the present population predicts that, left alone, the subspecies is likely to become extinct in 25–40 years [29]. The historic record of drastic reduction of the subspecies, the occurrence of aberrant morphological features and the presence of only a single mitochondrial DNA (mtDNA) haplotype in survivors of historic Florida panthers are all consistent with the occurrence during the Florida panther's recent history of one or more population bottlenecks, followed by periods of inbreeding [23,27,33]. Here we shall review data that extend these preliminary observations and reveal a remarkable reduction in genetic diversity correlated with skeletal, reproductive and congenital abnormalities that severely threaten the survival of the Florida panther.

Genetic reduction

In the last decade a number of endangered species has been shown to have reduced genetic diversity compared with other closely related species, as a consequence of extreme demographic reduction followed by inbreeding [3,35–38]. Four independent measures of genetic variation applied to the Florida panther, and the direct observation of incestuous pairings *in situ*, indicate that, compared with other puma subspecies, this population has markedly reduced genetic variability.

Mitochondrial DNA and morphological traits

A recent analysis of mtDNA restriction fragment length polymorphisms (RFLPs) [33] indicated that genetic variation in the Florida panther is limited to two highly divergent haplotypes (A and B in Fig. 2). One haplotype, designated type A and confined largely to the BCS population, is similar to the haplotypes of other North American subspecies (C and D in Fig. 2) and represents the single genotype descended from the ancestors of *F. concolor coryi*. The second haplotype, type B, originally discovered in two family groups in the ENP, derives from a captive-bred, hybrid puma subspecies that was released into the Everglades between 1956 and 1966 [33, 39]. Although limited interbreeding between the stocks has occurred (see below), it is likely that before introduction of the Everglades stock, the descendants of the historic Florida panther had their mtDNA variation reduced to the single invariant mtDNA haplotype, type A. (BCS panthers that are mtDNA haplotype A are referred to below as 'authentic' or 'historic' Florida panthers, in contrast to ENP haplotype B introgressed panthers.) This level of mtDNA variation is the lowest reported in any similarly studied feline population, including leopards, cheetahs and other puma subspecies [33,40,41].

The original type description of *F. concolor coryi* was based on geographic distribution and cranial morphology, specifically the occurrence of a broad, flat frontal region of the skull with broad, highly arched nasals [42,43]. Belden [24] noted a high frequency of two morphological characters, a cowlick and a kinked tail (Fig. 3), that are common among authentic Florida panthers (their prevalence is 94% and 88%, respectively) but are seen only rarely in pumas outside of Florida. Both the kinked tail and the cowlick occur at low frequencies (9% and 27%, respectively) in the ENP panthers, supporting the traits' genetic basis and the occurrence of subspecies introgression in the history of the ENP animals [33,40]. The high degree of concordance between mtDNA haplotype A and the kinked tail and cowlick phenotypes in the BCS panthers indicates that hybridization with the ENP introgressed animals was very recent and perhaps restricted by localized mating in the limited habitat [33].

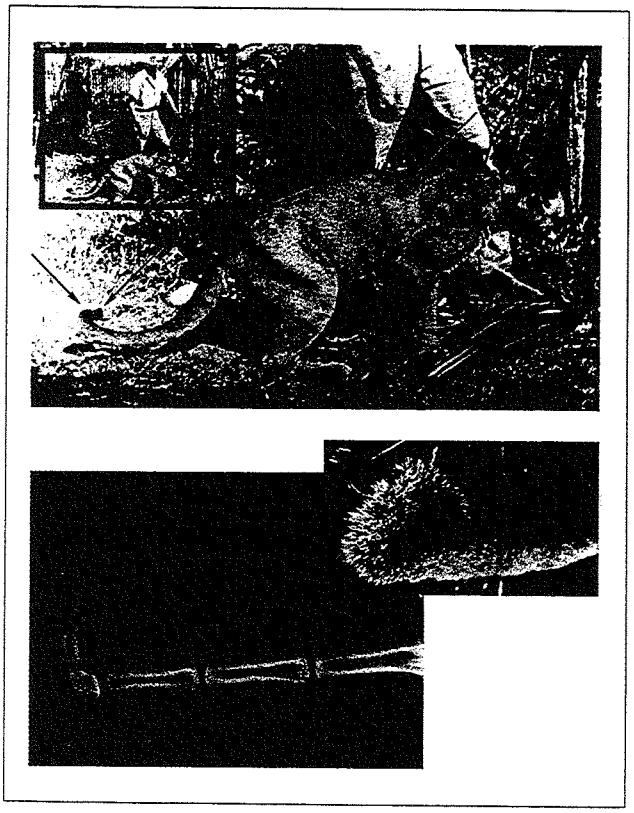

Fig. 3. Diagnostic cowlick and kinked tail found in authentic Florida panthers [23,24,44]. The top photograph was taken *circa* 1945, when the original Piper stock [33] was established; note the kink (shown in greater detail in the bottom photograph) and cowlick in the captive panther. This stock became introgressed in the 1940s and 1950s, with pumas from South American subspecies, and seven descendents were released in the Everglades between 1957 and 1967.

Allozyme polymorphism

An electrophoretic survey of 41 allozyme loci in Florida panthers was conducted using established methods [45,46] and compared with nuclear genetic variation among six separate North American puma subspecies (Table 1). Outbred pumas have relatively abundant allozyme diversity, including a frequency of polymorphic loci (P) of 27% and an estimated average heterozygosity (H) of 1.8–6.7%. The authentic Florida panther (excluding ENP panthers) has P and H values

of 4.9% and 1.8%, respectively. This is less variation than any other puma subspecies (for which the ranges in P and H are 7.3–17.1 and 2.0–6.7%, respectively) or other feline species, and is nearly as low as the level of allozyme variation reported in the two cheetah (*Acinonyx jubatus*) subspecies (Table 1). Combined with the mtDNA and morphological data, the allozyme study supports the view that there has been an appreciable reduction in the genetic variation of Florida panther populations, which is likely to be a consequence of close inbreeding during range contraction.

DNA fingerprinting

The discovery of hypervariable minisatellite genetic loci (also called variable number tandem repeats, VNTR) in human DNA, and their widespread occurrence in all vertebrate genomes, has provided a powerful new method, DNA fingerprinting, for estimating overall genomic diversity in natural populations. Because minisatellite alleles change at a rate 100–1000 times faster than conventional allelic variation, their population variation reflects rather recent historic events, such as founder effects, bottlenecks and assortive mating [40,47–50]. We used molecular clones of two feline-specific minisatellite families [49,50] to assess minisatellite diversity in the Florida panther, and to compare it with the diversity in western pumas and other feline species. The results are illustrated in Figure 4 and tabulated in Table 2.

Minisatellite diversity was quantified by estimating the average percent difference (APD) in DNA fragments shared in pairwise comparisons of individual fingerprints run on a single gel. APD is a phenotypic estimator of variation that is highly correlated with average heterozygosity ($r = 0.986$) and with other measures of overall genomic variation [49–52]. The Florida panther (BCS) has a remarkably low level of minisatellite

Table 1. Allozyme polymorphisms in puma (*F. concolor*) and other felid species.

Locale	Sub-species*	No. individuals	Polymorphic loci (%P)	Average heterozygosity (%H)	Polymorphic allozyme loci†
Florida-BCS‡	*coryi*	17	4.9	1.8	PGD, PP
Florida-ENP‡	*coryi*	7	7.3	1.8	PGD, PP, APRT§
Oregon	*oregonesis*	5	7.3	2.8	PP, ESA, ES1
California	*californica*	4	7.3	3.4	PGD, PP, ES1
Texas	*stanleyana*	12	9.8	4.1	PP, ADA, ES1, GOT
Arizona	*azteca*	6	17.1	6.7	PGD, PP, ADA, ES1 GPI, ACP1, GOT2
Colorado	*hipolestis*	3	4.9	2.0	ES1, GOT2
Utah	*kaibabensis*	2	7.3	2.8	PGD, ESA, ACP1
Captive pumas‖	mixed	25	21.9	6.1	PGD, PP, APRT§, ADA, CAT, ES1, GOT2, GPI, DIAI
Eastern Africa	*Acinonyx jubatus raineyi*¶	30	4	1.4	ESD, ADA
Southern Africa	*A.j. jubatus*¶	98	2.0	0.04	ESD
	Other Felids#	6–56	8–21	3–8	[35,75]

*For location, see Figure 2.

†Allozyme loci that were invariant in all puma specimens included: ACP2, AKI, CPKB, G6PD, GLOI, GOT1, GSR, IDHI, IDH2, LDHA, LDHB, MDH1 MEI, MPI, PEPB, PEPC, PEPD, PGAM, PGM1, PGM2, PGM3, PK1, HEXA, HK1, MDH2, NP, SOD1, TF, TPI, HBB.

‡BCS-Florida panthers (mtDNA Type A) are authentic descendents of historic *F. c. coryi*; ENP (mtDNA Type B) are introgressed hybrids between subspecies introduced into the Everglades National Park between 1956 and 1966, see text.

§APRT polymorphism is not observed elsewhere in North America but is observed in South American subspecies, supporting the introgression hypothesis for the origin of ENP stock [33].

‖Captive pumas including the Piper stock; see text.

¶Cheetahs from two African subspecies [35,77].

#Other felid species include *Felis catus, Leptailurus serval, Panthera leo, P. tigris, P. pardus, Caracal caracal, Leopardus pardalus, L. weidi* [35].

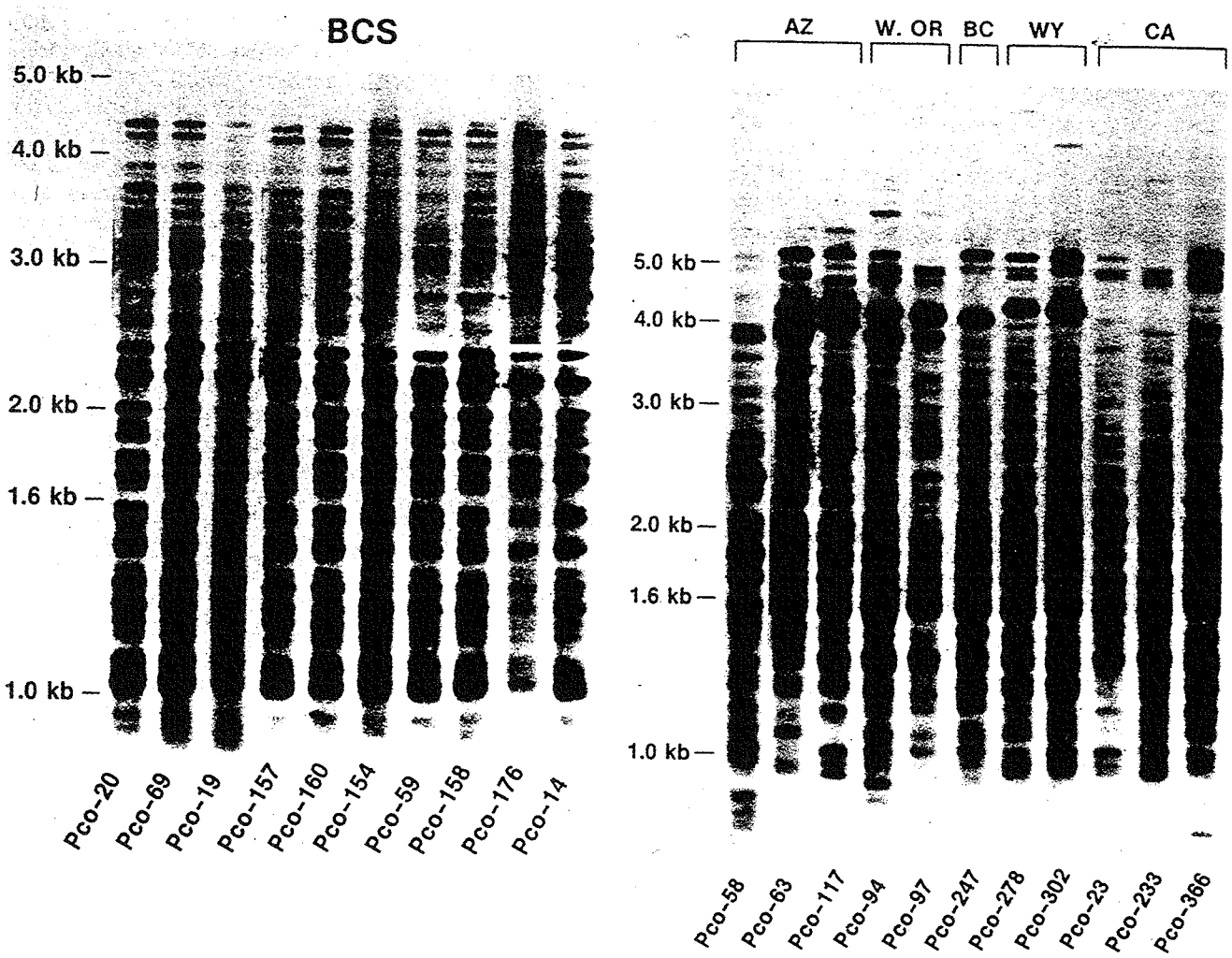

Fig. 4. DNA fingerprints of Florida panthers (left) and western cougars (right) from the indicated locales: AZ, Arizona; W. OR, western Oregon; BC, British Columbia; WY, Wyoming; CA, California. To obtain these fingerprints, DNA was digested with *Hae*III and probed with FCZ8. The DNA was isolated from leukocytes or established tissue cell lines according to Sambrook *et al.* [88]. To determine minisatellite DNA diversity, total genomic DNA (6–8 μg) was digested with *Hin*fI, *Hae*III or *Msp*I. Digestion products were electrophoresed on 1% agarose gels in 40 mM Tris acetate/ 1 mM EDTA (pH 8.0) and capillary blotted to Biotrace RP (Gelman) or HyBond N+ (Amersham) nylon membranes overnight, with either 0.4 M NaOH/ 1 M NaCl or 10 x SSC. Membranes were prehybridized overnight at 65°C in Church and Gilbert solution (0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM disodium EDTA, 1% bovine serum albumin). Hybridizations were carried out in the Church and Gilbert solution with the addition of 1.3–1.6 x 10^6 cpm of a ^{32}P-labelled feline-specific minisatellite probe, FCZ8 or FCZ9 [49]. Washes of membranes, autoradiography and scoring of fingerprints were as in [49].

variation (mean APD = 9.5%), which is 85% less variation than was observed in western pumas and 65% less than was seen in the sympatric ENP panthers or in the Piper captive stock from which ENP panthers are probably derived (Table 2). The variation seen in the Florida panther is nearly as low as the extremely compromised genetic variation in Asiatic lions from the Gir Forest Sanctuary in India [38,49]. The dramatically low level of DNA fingerprint variation observed in the Florida panthers supports the inference from the mtDNA and allozyme findings that the surviving authentic Florida panthers have experienced substantial inbreeding and concomitant loss of genetic diversity in their recent history.

The cost of genetic uniformity

Spermatozoal traits

Semen quality and endocrine and reproductive functions have been shown to be adversely affected in several inbred species, including mice, cats, lions and cheetahs [14,15,35,38,53]. Comparative reproductive analyses of seminal traits in five feline species (puma, cheetah, leopard, tiger and domestic cat) by Wildt and his collaborators [32,53–56], revealed that Florida panther males display some of the poorest seminal quality traits ever recorded for any felid species or subspecies (Fig. 5). A particularly important finding was that the total motile sperm per ejaculate in the Florida panther

Table 2. Levels of DNA fingerprint variation in Florida panthers relative to other pumas and felid species.

Species	Population	No. individuals	Probe	Restriction enzyme	APD±SE (%)	Average heterozygosity (%)
F. concolor coryi	Florida BCS	13	FCZ8	*HinfI*	9.7±1.4	9.2
	"	13	FCZ8	*HaeIII*	11.0±3.1	12.0
	"	10	FCZ9	*HinfI*	4.2±1.8	5.6
	"	13	FCZ9	*HaeIII*	12.9±3.1	14.2
				Average:	9.5	
	Florida ENP	7	FCZ8	*HinfI*	32.6±15.7	31.7
	"	7	FCZ8	*HaeIII*	30.1±12.6	35.4
	"	7	FCZ9	*HinfI*	30.3±3.4	33.2
	"	7	FCZ9	*HaeIII*	17.8±8.4	18.3
				Average:	27.7	
	Florida-Piper	6	FCZ8	*HinfI*	34.2±9.6	29.4
	"	6	FCZ8	*HaeIII*	27.7±8.2	25.7
	"	6	FCZ9	*HinfI*	34.7±11.3	38.5
	"	6	FCZ9	*HaeIII*	30.8±8.2	34.0
				Average:	31.9	
	Western US subspecies	4	FCZ8	*HinfI*	59.7±8.9	51.5
	"	11	FCZ8	*HaeIII*	46.7±8.6	53.6
	"	4	FCZ9	*HinfI*	56.4±6.8	43.7
	"	4	FCZ9	*HaeIII*	47.2±4.5	38.9
				Average:	57.9	
Domestic Cat	Eastern US	17	FCZ8	*MspI*	47.3±3.6	46.0
	Eastern US	15	FCZ9	*MspI*	44.5±3.6	41.8
Lion	Serengeti, Africa	76	FCZ8	*MspI*	49.0±3.3	48.1
	Serengeti, Africa	17	FCZ9	*MspI*	44.5±3.0	50.0
Lion	Gir Forest, India	16	FCZ8	*MspI*	3.8±0.2	2.8
	Gir Forest, India	16	FCZ8	*RsaI*	2.6±0.7	2.9

The percent difference (PD) in comparisons of individuals a and b was determined as the number of unshared bands (F_{ab}) divided by the total number of bands ($F_a + F_b$) x 100. An average of the PD of all pairwise comparisons in a population is the average percent difference (APD), a measure of the genetic diversity within or between subspecies. Fragments with a size range of 1000–10000 base pairs were scored; fragments ≤ 1000 base pairs run off the gel but do not affect estimated APD [79]. All individuals that were compared in estimating minisatellite diversity were unrelated, and APDs were calculated only from comparisons made from a single autoradiograph. Average locus heterozygosities (H) of the multiple hypervariable loci probed by the feline-specific minisatellite were estimated according to Stephens *et al.* [51]. Domestic cat and lion data are from [49].

Genomic DNAs from puma populations were analyzed using two feline-specific minisatellite probes, FCZ8 and FCZ9, originally isolated from domestic cat [49]. These probes detect different minisatellite families based upon a distinct core sequence and different fingerprint patterns produced in the same individuals in several species including pumas [40,49,50]. Minisatellite DNA fragments resolved for each probe were assumed to represent independent alleles at chromosomally dispersed loci, based on a test for fragment independence of restriction fragments in test populations [76]. Two restriction enzymes were tested for each probe as a technical redundancy for each gene family and should not be considered as independent estimators of genetic diversity, but rather as verification of each gene family's (FCZ8 and FCZ9) genetic variation.

is 18–38 times lower than in other puma subspecies, 30–270 times lower than in other felids and 30 times lower than in the cheetah. Although pumas and other large felids tend to produce high proportions of morphologically abnormal sperm, the Florida panther has a significantly greater frequency of malformed spermatozoa (average 94.3% per ejaculate) than any other puma subspecies; particularly noteworthy was a 40% incidence of acrosomal defects, a trait that renders sperm deficient in fertilization potential [54,55]. Similar defects have been observed in inbred mice, livestock, and wild populations with a history of genetic impoverishment, and are almost invariably associated with infertility [54–59].

Cryptorchidism

The Florida panther population displays an unusually high incidence of cryptorchidism (56% of males examined since 1978). In affected males, one or two undescended testicles are located within, or adjacent to, the inguinal canal, in an atrophied state; undescended testicles are associated with progressive loss of spermatogenesis, possibly due to the higher temperature than in the scrotum. The developmental defect is heritable and is suspected to result from a sex-limited recessive (or possibly dominant) autosomal gene in several domestic species: dog, sheep, swine and cat [60–64]. In miniature Schnauzer dogs, for example, intentional inbreeding resulted in an incidence of 67% cryptorchidism [65].

Cryptorchidism has not been reported previously in non-domestic cats of any species. Further, it has not been observed in medical examinations of over 40 free-ranging pumas captured in Texas, Colorado, British Columbia, or Chile, and was observed in only two of more than 50 captive males, one in Chile and one in a US zoo ([32] and MER, unpublished observations). Circulating testosterone concentrations were lower in

Table 3. Infectious disease agents observed in Florida panthers.*†

Agent	Disease	Prevalence
Viruses:		
*Rabies Virus	Rabies	26%
*Feline Panleukopenia Virus	Distemper	78%
*Feline Calicivirus	Pneumonia	56%
Puma Lentivirus	Feline AIDS	34%
*Feline Syncytia-forming Virus	Fibroblast cytopathology only	71%
*Feline Coronavirus (FIP)	Subclinical or feline infectious peritonitis	19%
*Feline Rhinotracheitis Virus	Respiratory disease	Sympatric in bobcats
Pseudorabies Virus	Fatal neuropathology	Sympatric in feral hogs
Parasites:		
Ancylostoma pluridentatum	Neonatal mortality Intestinal hookworm	>50%
Cytauxzoon Felis	Blood parasite fatal in cats	53%
Toxoplasma gondii	Multi-systemic illness	80%
Other helminths, protozoa, and arachnids	*Dirofilaria striata, Giardia* sp., *Trichinella spirilla, Sarcocystis* sp., *Alaria marcianae, Notoedres cati,* and seven species of tick	
Bacterial Agents:		
Enteric Pathogens	*Salmonella* sp., *Campylobacter* sp., *Edwardsiella tarda,* and *Plesimonas shigelloides enteritis*	
Escherichia coli	Fatal septicemia	2 panther deaths
Streptococcus equisimilus/ equi	Fatal pyothorax and septicemia	1 panther death
Clostridium sp.	Neuropathology	2 panther deaths
Pseudomonas aeruginosa	Pneumonia, bronchitis	1 panther death
Miscellaneous		
Vaginal papilloma	Unknown	93% females

*Florida panthers are now vaccinated against these agents.
†Data collected from [31,81–89] and from C.M. Glass, M.E. Roelke, unpublished observations.

male Florida panthers with only one descended testicle than in those with two, whereas testosterone levels in Florida panthers with two normally descended testicles were no different from males in other puma populations [32]. In addition, cryptorchid male Florida panthers tend to produce fewer motile sperm per ejaculate than normal males: 0.54×10^6 compared with 2.19×10^6 [32].

The incidence of cryptorchidism has increased dramatically in the last two decades and is associated with documented consanguineous matings. In Figure 6, the frequency of cryptorchid males is shown to increase from very low (0% of pre-1975 births) to very high (80% of the males born after 1989), leading to a 90% incidence among living males, with 22% (2 of 9) bilateral and sterile. During the same period, two male offspring of a consanguineous mating of BCS parents were both cryptorchid, while all males from ENP parents ($n=7$), including one consanguineous mating, were normal in testicle descent (Figs 6 and 7). These observations document the rapid rise to genetic fixation of a maladaptive genetic trait in a small inbred population sustained by incestuous matings.

Emergence of cardiac defects

Complete necropsies were performed on 49 panthers that died from 1978 to 1992. In 1990, a life-threatening atrial septal defect (ASD), termed '*patent foramen ovale*', in which the opening between two atria fails to close normally during fetal development, was observed and deemed the cause of death in two panthers (ages 2 and 5 years). A third ASD case was diagnosed during a routine physical examination of a juvenile male that had an extreme heart murmur. This panther, the offspring of a backcross of the sire with his daughter (number 43 in Figure 7), displayed marked cardiomegaly and pronounced tricuspid valve dysplasia; he died in October 1992 of complications after reconstructive heart surgery.

ASD has been observed in several species including man (ASD is the most common congenital heart defect in human adults [66,67]), but has not previously been observed in pumas and only rarely in domestic cats [68]. The etiology of ASD is not well understood, but certain cases in man suggest an autosomal dominant mode of inheritance [69,70]. Coincident with the appearance of ASD has been the detection of a high frequency of heart murmurs in Florida panthers (about 80%) compared with other puma subspecies in which heart murmurs are very rare ($\leq 4\%$). The relationship between ASD and heart murmurs is not clear, nor is the etiology of either character. The high incidence of cardiac abnormalities [34] may be due to

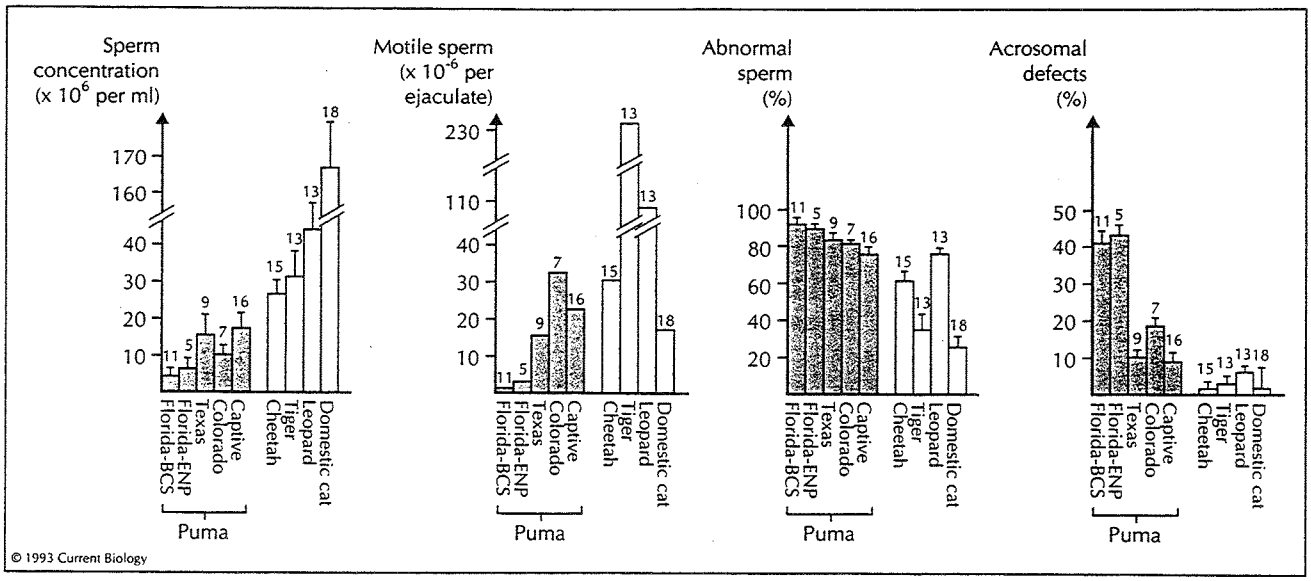

Fig. 5. Spermatozoal characteristics of authentic Florida panthers (BCS), introgressed ENP panthers, Texas cougars, Colorado cougars and a mixture of captive pumas of mixed subspecies backgrounds. Mean values ±SE are compared with similar measurements for four felid species: *Acinonyx jubatus* (cheetah), *Panthera tigris* (tiger), *Panthera pardus* (leopard) and *Felis catus* (domestic cat). The numbers of individuals tested for 2–3 ejaculates are shown above the bars on each histogram. Complete descriptions of the data and results are in [32,54–56].

high levels of toxic mercury in the natural habitat, or it may also have a genetic explanation.

Infectious disease

One of the most critical ecological and selective pressures on free-ranging populations is the periodic outbreak of infectious pathological disease [12,13,20,71]. The importance of population genetic diversity for loci involved in immune responses has been empirically demonstrated in several species, particularly with respect to the major histocompatibility complex. Natural examples of the increased vulnerability of several genetically compromised populations to infectious agents have been described [13,15,20,35,72,73] and the reason for avoiding inbreeding in managed populations is to provide protection against both inbreeding depression and increased susceptibility to infectious diseases.

We present in Table 3 a list of infectious agents that have been identified in Florida panthers or in sympatric species within their habitat. The pathogen–parasite load is relatively high in Florida panthers, and some agents (such as *Pseudomonas aeruginosa*) are unexpected except in hosts with disarmed immune systems [74]. Several of these agents have caused mortalities, others are clear threats, and some have an uncertain pathological prognosis. At least eight panther mortalities have been attributed to infectious pathogens (Table 3), although no single agent has caused widespread mortality during the study period. Nevertheless, the accumulation of diverse pathological agents is substantial and likely to be abated by vaccination of all captured panthers against known fatal pathogens.

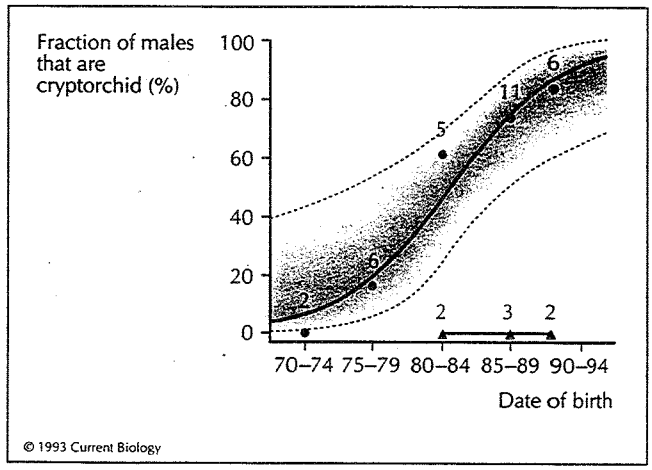

Fig. 6. The frequency of cryptorchid males born among authentic Florida panthers (circles) versus ENP males (triangles) during four year intervals from 1970 to 1992. The numbers of males born in each time period are indicated above the symbols. The shaded area shows the 95% confidence interval for the mean prevalence. A total of 28 males were examined by capture or at necropsy from 1981 to 1992. Date of birth for adults was estimated from tooth wear [26,80] for older individuals and was known accurately for kittens. Because survivorship of cryptorchid kittens tracked since 1984 does not appear to be different from normal males, the calculation assumes that pumas born before 1984 were representative.

Conclusions

The observations presented here provide a graphic picture of a severely threatened population waging a struggle for survival. Human depredation for nearly a century has reduced the Florida panther's range and number to fewer than 50 individuals. Ecological and biomedical assessments of the population reveal a collection of interacting ecological, demographic and genetic factors that threaten the survival of this fragile population. The

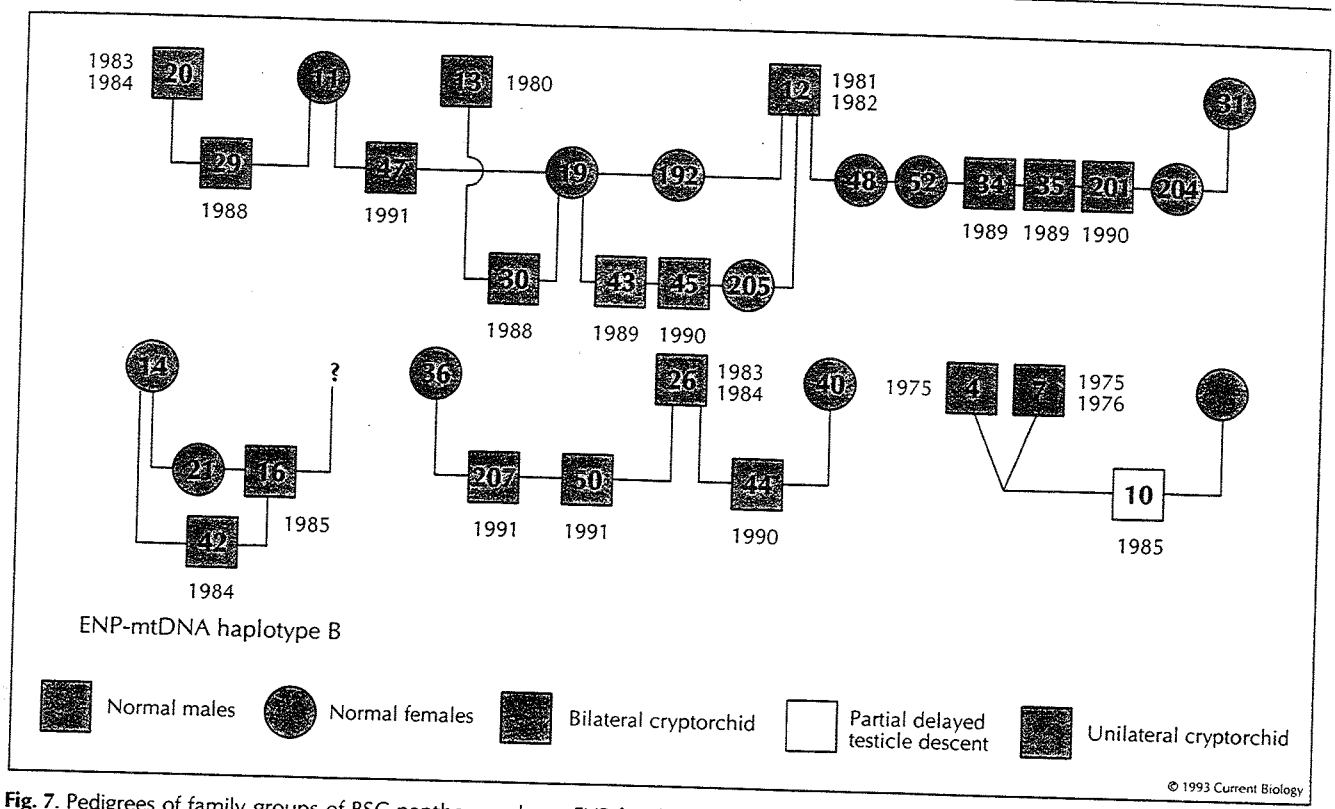

Fig. 7. Pedigrees of family groups of BSC panthers and one ENP family, with panther identification numbers inside the symbols and their years of birth below the symbols. The cryptorchid phenotype of the males is indicated by the key. Two males captured in 1992, numbers 47 and 207, were bilateral cryptorchid and sterile.

overall genetic diversity of the panthers is markedly reduced relative to other puma subspecies, as assessed by the fixation of rare morphological traits, reduced allozyme diversity, and diminished DNA variation at minisatellite loci and in mtDNA. In concert with these genetic measures, the population displays several physiological impairments, notably the most malformed spermatozoa of any population yet described, a recent increase in the incidence of cryptorchidism (up to 90% of remaining males), and the abrupt appearance of a lethal congenital heart defect and heart murmurs. In addition, a score of infectious pathogens (Table 3) has been observed to threaten the population; even in the face of an aggressive vaccination program, there is little room for optimism.

Although it is difficult to quantify the relative contributions of differing factors in the decline of the Florida panther, the accumulation of various threats is alarming. The Florida panther provides a dramatic example of the process of human-caused population decline. Reduction to small numbers facilitates inbreeding and its consequences and amplifies the effects of demographic stochasticity. Genetic impoverishment lowers fitness and reduces both the potential for reproductive recovery and the abundant immune defenses accumulated over millions of generations by epidemic episodes. Reversal of the apparently perilous course being followed by the Florida panther poses a formidable challenge. First, the numbers must be increased before demographic factors finish the species. Once the numbers have increased, the genetic diversity of the population must be improved, perhaps by genetic augmentation of closely related subspecies, as occurred serendipitously by the release of seven non-Florida pumas into the ecosystem 30 years ago [33].

The most recent workshop on Florida panther conservation (Yulee, Florida, October 1992; United States Fish and Wildlife Service, in press) recommended immediate augmentation of the population with Texas pumas (*F. concolor stanleyana*), to reverse both demographic and genetic depletions. The potential disadvantages of managed subspecies hybridization are likely to be less than the expected consequences of taking no action ([29,33,75] and Yulee Florida, October 1992, USFWS, in press). Managed introgression would represent an attempt to reconstitute the genetic diversity present in the ancestors of *F. concolor coryi*, whose range was contiguous with the Texas puma *F. c. stanleyana* (Fig. 2). Finally, a sufficiently large suitable habitat must be provided with enforced protection to permit the opportunity for genetic and demographic factors to be ameliorated and for a stable population to be achieved.

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Worldwide Prevalence of Lentivirus Infection in Wild Feline Species: Epidemiologic and Phylogenetic Aspects

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The natural occurrence of lentiviruses closely related to feline immunodeficiency virus (FIV) in nondomestic felid species is shown here to be worldwide. Cross-reactive antibodies to FIV were common in several free-ranging populations of large cats, including East African lions and cheetahs of the Serengeti ecosystem and in puma (also called cougar or mountain lion) populations throughout North America. Infectious puma lentivirus (PLV) was isolated from several Florida panthers, a severely endangered relict puma subspecies inhabiting the Big Cypress Swamp and Everglades ecosystems in southern Florida. Phylogenetic analysis of PLV genomic sequences from disparate geographic isolates revealed appreciable divergence from domestic cat FIV sequences as well as between PLV sequences found in different North American locales. The level of sequence divergence between PLV and FIV was greater than the level of divergence between human and certain simian immunodeficiency viruses, suggesting that the transmission of FIV between feline species is infrequent and parallels in time the emergence of HIV from simian ancestors.

The rapid emergence of AIDS during the past decade has brought about extensive efforts to determine the origin and natural history of human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), the etiologic agents of the disease. Lentiviruses that have gene sequence homology with HIVs have been found in several mammalian species (including sheep, goats, horses, cattle, cats, and several Old World monkey species), indicating that humans are merely the latest unfortunate species to have become infected via another mammalian species (7, 9, 10, 16, 17, 26, 38). The evolutionary relationship between various viral groups has been approached by using genome organization, tissue tropism, pathological sequelae, and viral gene sequence similarity as characters upon which phylogenetic inference is based (7, 9, 16, 17, 26).

The closest relatives of HIV have been isolated from several species of African and Asian primates. The African primates (notably sooty mangabeys, mandrills, green monkeys, grivets, and Sykes' monkeys) appear to serve as natural reservoirs for simian immunodeficiency virus (SIV), although infected native African species do not develop clinical symptoms (25, 35). In contrast, Asian macaques manifest an AIDS-like illness when infected with certain strains of SIV in captive settings but do not appear to have been exposed to the virus in their natural habitat (19, 25, 27, 35). These observations, combined with findings of extensive genetic variation within SIV isolates, have prompted the hypothesis that the human species acquired HIV rather recently by a transspecies infection from African primates that had harbored the ancestral lentiviruses for a long period, perhaps before the radiation of the African primate species (3, 7, 19, 21, 25, 27, 35).

Interpretations of natural histories of retroviral origins are contingent upon virus isolation properly reflecting geographic origins of host species (9, 17, 26). However, most SIV isolates have been recovered from captive monkeys, raising the possibility that the virus was acquired in an unnatural setting where exposure via other captive species can occur (we think that macaques acquired SIV in this way in primate research centers). For example, a recent isolate of simian immunodeficiency virus (SIV-CPZ) from two captive chimpanzees in Gabon is the closest relative to HIV-1 yet described (20, 39), thereby making SIV-CPZ a strong candidate for a precursor of HIV-1. However, the virus is extremely rare in captive chimpanzees (infecting ≤1 of 250 animals), and there is no evidence for its occurrence in nature. Thus, it is possible that the SIV-CPZ was transmitted from humans to chimpanzees and not the other way around. Clearly, to interpret these data in a historical context, we need more information on the prevalence of lentiviruses in nature, specifically in other wild animal species.

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Our laboratories have been studying the molecular characterization of feline immunodeficiency virus (FIV) as well as the genetic statuses of several populations of nondomestic cat species. FIV is a recently discovered lentivirus isolated from domestic cats (*Felis catus*) that is distantly related to the primate lentiviruses. Like HIV, FIV is T-cell tropic and associated with immunodeficiency syndrome in infected hosts (38, 47). FIV is widely prevalent in domestic cats and displays abundant inter se genetic diversity, suggesting that it has infected domestic cats for a long period. Recent serological studies have also detected antibodies against FIV in isolated individuals of nondomestic felid species, primarily from zoo collections (5, 24). We extend these studies by a survey of serum samples from 12 species of the family Felidae, including serum samples from free-ranging populations of lions, cheetahs, and pumas collected in their native habitats. We report a high prevalence of cross-reactive antibodies to an FIV-like lentivirus in free-ranging African lions and cheetahs as well as in North American pumas. Multiple infectious isolates were obtained from Florida panthers (*Felis concolor coryi*, a puma subspecies). Genomic sequences of viral *pol* genes permit phylogenetic analysis of puma lentivirus (PLV) genomes that are interpreted in a phylogeographic context. The wide prevalence of lentiviral infections in natural populations of large felids provides a rare opportunity to track retrospectively the pattern and consequences of an ongoing epizootic.

MATERIALS AND METHODS

Western blots (immunoblots) for antibodies against FIV. Plasma samples were incubated with immunoblot strips prepared with FIV-Petaluma antigens, and Western blot assays were performed as previously described (36, 47).

Virus isolation. Peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation of heparinized whole blood from seropositive Florida panthers within 24 h following peripheral venous bleeding. PBMCs (10^7 cells) from seropositive animals were cocultured with donor PBMCs (10^7) from seronegative specific-pathogen-free domestic cats or from seronegative pumas. Cultures were maintained in RPMI 1640 containing 10% fetal calf serum and 10% interleukin-2 after mitogen stimulation (concanavalin A; 5 μg/ml for 72 h). The presence of replicating virus was monitored by measuring Mg^{2+}-dependent reverse transcriptase (RT) activity in the culture supernatant fluids. At the peak of RT activity, cells were processed for electron microscopy.

The Maryland isolate of FIV, FIV-MD, was obtained from a Mt. Airy, Md., domestic male cat, approximately 2 years old, that was suffering from respiratory tract infection, gingivitis, and weight loss. This cat was FIV positive and feline leukemia virus negative as determined by enzyme-linked immunosorbent assay (CITE Combo test kit; Agritech Systems, Portland, Maine). The virus was isolated by cell coculture as described above.

Samples were collected in full compliance with specific federal fish and wildlife permits (CITES: Endangered and Threatened Species; Captive Bred) issued to the National Cancer Institute, principal officer S. J. O'Brien, by the U.S. Fish and Wildlife Service of the Department of the Interior.

Amplification and sequence determination of PLV *pol* gene in virus-infected cells. Genomic DNAs containing the PLV or FIV proviral sequences were isolated from RT-positive PBMC cocultures (isolate PLV-14, PLV-16, PLV-21, PLV-42, or FIV-MD), or from uncultured PBMCs isolated from whole blood (isolate PLV-8, PLV-18, PLV-64, or PLV-80). The RT region was amplified by polymerase chain reaction (PCR) from 0.5 μg of genomic DNA during 30 heating-cooling-extension cycles (94°C for 1 min, 37 or 45°C for 1.5 min, and 72°C for 1 min). Reaction volumes, reagents, equipment, and oligonucleotide syntheses were as previously described (21). A second round of 30 cycles using 10 μl from the first-round reaction products and a pair of oligonucleotides located within the boundaries of the first pair was necessary for the amplification of RT sequences of PLV-8, PLV-18, PLV-64, and PLV-80. PBMC DNA from seronegative pumas and no template controls were used routinely to detect PCR contamination. The amplified products were gel purified, prepared for blunt-end ligation, and cloned into a plasmid vector by conventional methods. Plasmid clones were isolated and sequenced by the chain termination method with T7 DNA polymerase (United States Biochemical). Oligonucleotide primer sequences and the nucleotide positions of the FIV-14 proviral sequence (37) (in parentheses) were as follows: 669F (2403), 5'CAATGGCCATTAACAAATG3'; 1217R (3118), 5'CCTGCTAATTTTTGCAACTCATT3'; 1258F (2430), 5'GAAGCATTAACAGAAATAGTAG3'; 1260R (3007), 5'GGTTCTTGTTGTAATTTATCTTC3'; 1259F (2466), 5'GAAGGAAAGGTAAAAAGAGCAGATC3'; 1261R (2990), 5'ATCTTCAGGAGTTTCAAATCCCCA3'; 1152F (2544), 5'TGGAGAATGCTCATAGATTTTAGAGAATT3'; 1314R (2905), 5'GATCCTATATATATATCATCCATATATTG3'; 1086F (2559), 5'GATTTTAGAGAATTAAACAA3'; and 1068R (2902), 5'CCTATATAAATGTCATCCAT3'. Primers 669F and 1217R were used for amplification of the PLV-14 and FIV-MD sequences; 1258F and 1260R were used for PLV-16, PLV-21, and PLV-42. A second round of 30 cycles using 10 μl from the first-round reaction products and a pair of oligonucleotides located within the boundaries of the first pair was necessary for the amplification of RT sequences of PLV-8, PLV-18, PLV-64, and PLV-80: 1258F and 1260R (outer pair) and 1259F and 1261R (inner pair) were used for PLV-18; 1152F and 1314R (outer pair) and 1086F and 1068R (inner pair) were used for PLV-8, PLV-64, and PLV-80. Primers 669F, 1217R, 1152F, 1086F, and 1068R are FIV-14 sequence specific and were derived from conserved regions detected in alignments of several lentiviral *pol* gene sequences with FIV (data not shown). Primers 1258F, 1259F, 1260R, 1261R, and 1314R are PLV-14 sequence specific and represent conserved regions shared by PLV-14 and FIV-14 RT sequences.

Phylogenetic analyses. All sequences except PLV-8, PLV-18, PLV-64, and PLV-80 were subjected to phylogenetic analysis by using a total of 576 bp of nucleotide sequence representing 192 amino acid residues upon translation. Shorter sequences (318 bp, 106 amino acids) were also analyzed for all PLV isolates and all control lentiviruses. Each full-length sequence was separately aligned with every other sequence by the GAP program of the Genetics Computer Group (University of Wisconsin) computer software package (8), which uses the algorithm of Needleman and Wunsch (28). Distances are expressed on the basis of percent difference in amino acid sequence identity; gaps are given a weight of a single residue substitution regardless of their length (41).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this article for PLV-8, PLV-14, PLV-16, PLV-18, PLV-21, PLV-42, PLV-64, PLV-80, and FIV-MD have been deposited in the GenBank sequence library under accession numbers M95475, M95471, M95477,

TABLE 1. Worldwide prevalence of feline lentivirus exposure in nondomestic felids[a]

Felid species	Source(s)	No. positive	No. tested	% Positive
Free-ranging				
Lion (*Panthera leo*)	East Africa (Tanzania and Kenya)	84	106	79
	South Africa (Kruger National Park)	10	12	83
	Southwest Africa (Namibia)	0	22	0
	Total	94	140	67
Cheetah (*Acinonyx jubatus*)	East Africa (Tanzania and Kenya)	10	46	22
Puma (*Felis concolor*)	Florida (Big Cypress Swamp)	9	37	24
	Florida (Everglades National Park)	4	9	44
	Arizona	8	10	80
	California	9	16	56
	Colorado	6	9	67
	New Mexico	1	2	50
	Oregon	1	11	9
	Texas	6	18	33
	Utah	1	2	50
	Wyoming (Yellowstone National Park)	5	25	20
	Idaho	0	3	0
	Canada and Alaska	3	7	43
	South America (Chile)	0	2	0
	Total	53	151	35
Bobcat (*Lynx rufus*)	Florida	2	23	9
Captive				
Lion				
Unknown subspecies	U.S. zoos	0	29	0
	Circuses	2	3	67
	Johannesburg Zoo, Johannesburg, South Africa	6	9[b]	67
Asiatic	U.S. zoos	23	35	66
	Sakkarbaug Zoo, Sakkarbaug, India	0	28[c]	0
	Negara Zoo, Kuala Lumpur, Malaysia	0	8	0
	Total	31	112	28
Cheetah	Zoos (United States, Australia, England)	1	64	1.6
	De Wildt Breeding Center, Pretoria, South Africa	0	45	0
	Total	1	109	1
Puma	Canada	2[d]	8	25
	United States	0	58[e]	0
	Central and South America (Belize, Chile, and Brazil)	0	16[f]	0
	Total	2	82	2
Other	U.S. European and South African zoos			
Tiger (*Panthera tigris*)		0	20	0
Snow leopard (*P. uncia*)		0	11	0
Jaguar (*P. onca*)		0	7	0
Leopard (*P. pardus*)		0	11	0
Serval (*Leptailurus serval*)		0	5	0
Sand cat (*F. margarita*)		0	4	0
Marbled cat (*Pardofelis marmorata*)		0	1	0
Bobcat		0	1	0
Flat-headed cat (*Ictailurus planiceps*)		1	3	33
	Total	1	63	1.6

[a] All serum and plasma samples (1:100 dilution) were tested by immunoblot assay for antibodies to FIV-Petaluma antigens.
[b] All were probably born in the wild.
[c] Four of these lions were born in the wild and had been in captivity for 1 to 12 years before sampling.
[d] Both positive animals were born in the wild; they came into captivity at 6 months and 2 years of age.
[e] Eight of these pumas were born in the wild and came into captivity as juveniles or young adults.
[f] At least 10 of these pumas were born in the wild and came into captivity as juveniles or young adults.

M95476, M95478, M95470, M95473, M95474, and M95472, respectively.

RESULTS

Prevalence of FIV antibodies in wild felid species. A total of 726 serum or plasma samples, representing 12 nondomestic felid species, were screened for antibodies that recognized FIV; 360 of the specimens were from free-ranging cats, and the rest were from captive-held animals. The samples were collected between 1978 and 1991, but the earliest specimens from free-ranging animals were collected in 1983 from Serengeti lions and cheetahs. The sera were typed by Western immunoblotting (Fig. 1), and the results of all the typings are presented in Table 1.

Several important observations are revealed by these data. First, there has been widespread exposure to lentiviruses related to FIV in sampled free-ranging populations of four species (lions, pumas, cheetahs, and bobcats). The incidence of seropositive lions was 79% in the Serengeti (Tanzania and Kenya) and 83% in Kruger National Park in South Africa; 22% of the Serengeti cheetahs sampled were

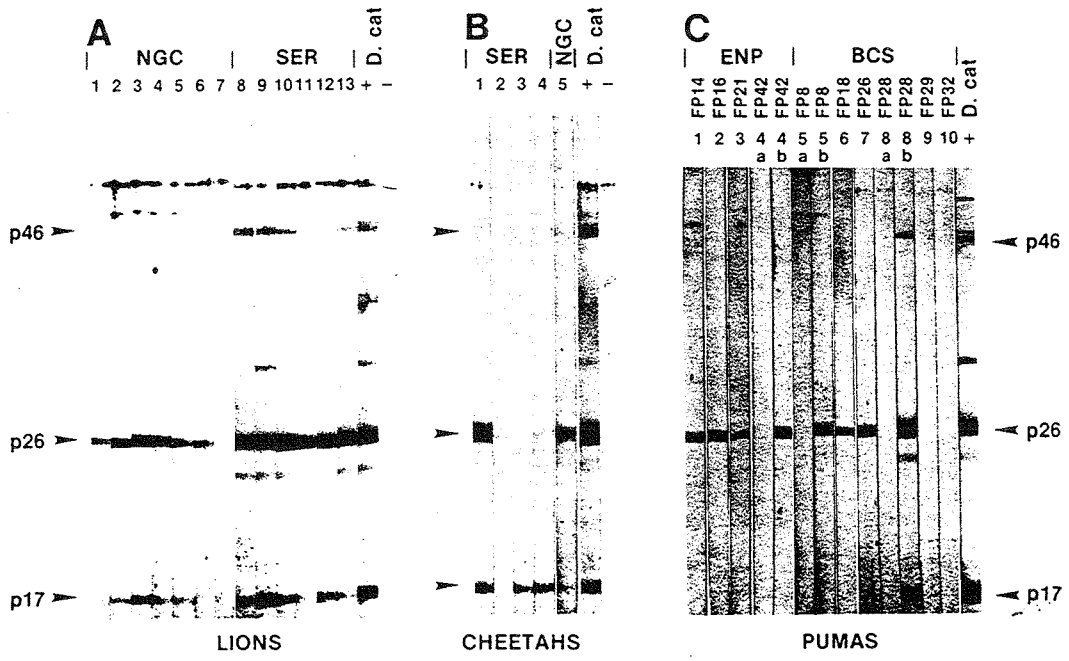
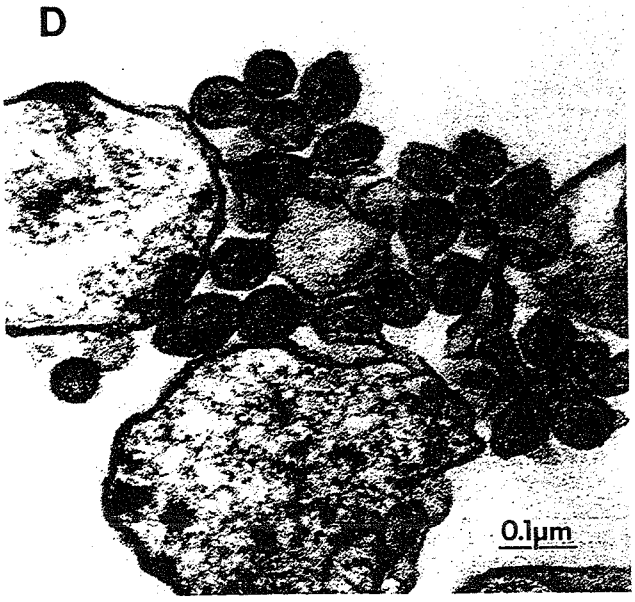


FIG. 1. Western blot analysis of selected serum and plasma samples from free-ranging East African lions and cheetahs and Florida panthers. (A) Samples from East African lions (*P. leo*) from Ngorongoro Crater (NGC) and Serengeti National Park (SER), Tanzania. Lanes 1 to 7, lions Ple-314 to Ple-320 (Ple-320 was seronegative), respectively; lanes 8 to 13, lions Ple-331 to Ple-336, respectively; lanes + and −, positive (FIV-infected) and negative control domestic cat sera. (B) Samples from East African cheetahs (*Acinonyx jubatus*). Lanes 1 to 5, cheetahs Aju-201, Aju-202 (seronegative), Aju-203, Aju-204, and Aju-213, respectively. (C) Samples from Florida panthers (*F. concolor coryi*) inhabiting the Everglades National Park (ENP) and Big Cypress Swamp (BCS) ecosystems. Panther designations are given above the lanes. Lanes 4a and b, samples obtained in March 1990 and May 1991, respectively; lanes 5a and b, samples obtained in March 1984 and January 1986, respectively; lanes 8a and b, samples obtained in March 1989 and January 1991, respectively; lanes 9 and 10, samples from seronegative panthers; lane +, FIV-infected domestic cat serum. (D) Electron micrograph of mature lentivirus particles in cultured PBMCs from Florida panther FP-16.



positive. In North America, 11 of 12 wild puma populations sampled had exposed individuals. Second, animals from certain locales were clearly negative, notably African lions from Namibia in southwestern Africa and Asiatic lions from the Sakkarburg Zoo in India that are derived from the Gir Forest population in western India (30). Third, with the exception of the Asiatic lions in U.S. zoos and Johannesburg Zoo lions, other captive animals were nearly always seronegative. The Johannesburg Zoo lions are derived from Kruger National Park, where they likely acquired infection. The U.S. Asiatic lions are a group of captive-bred lions descended from five founder animals, three authentic Asiatic lions (*Panthera leo persica*) derived from the Gir Forest sanctuary and two African lions (*P. leo leo*) (30). It is possible that the captive lion population was infected by exposure during captivity or via their African founders or both.

The immunoblots in Fig. 1 illustrate the seroreactivity patterns of infected lions, cheetahs, and pumas compared with those of domestic cat positive controls. Sera from two cheetahs recognized the FIV p17^{gagMA} core protein but not the p26^{gagCA} polypeptide seen in other animals. There was also a difference in reactivity patterns between two populations of seropositive lions from East Africa; i.e., the Ngorongoro Crater and Serengeti National Park populations (e.g., see p46^{gag} bands in Fig. 1). These pattern differences may reflect reactivities to distinct viruses in the two locales that differ in immunological epitopes shared with FIV. These immunologic differences were an early suggestion that FIV-related viruses have notable genetic divergences in different locations.

Isolation of PLV from Florida panthers. Our next objective was to isolate the suspected lentivirus(es) from one or more seropositive free-ranging animals. We concentrated on the small free-ranging Florida panther population (<50 individ-

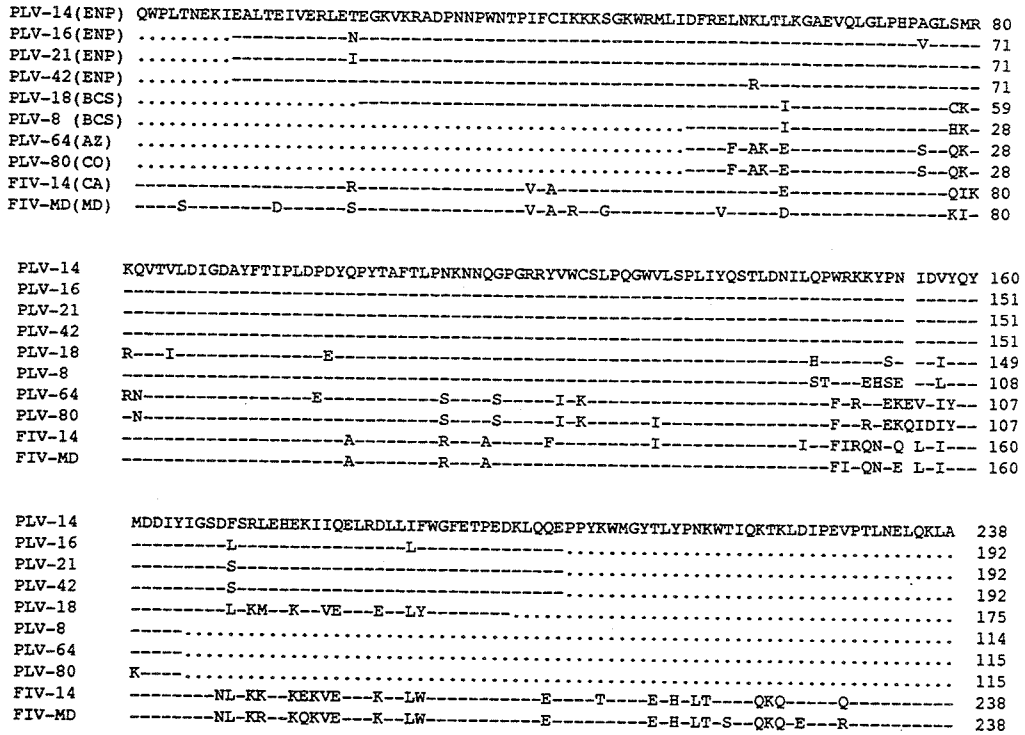


FIG. 2. Alignment of the predicted amino acid sequences of a conserved RT domain in the PLV and FIV *pol* genes (8, 28). Dashes below the PLV-14 sequence indicate identical amino acids. The open space is a gap introduced to maximize the alignment. Dots indicate sequences not obtainable with the available oligonucleotides used for PCR amplification. The geographical locations of virus isolation are indicated in parentheses. ENP, Everglades National Park; BCS, Big Cypress Swamp.

uals remaining) in the Everglades National Park and Big Cypress Swamp ecosystems in southern Florida (31, 33). The Florida panther is an endangered subspecies of *F. concolor* (other common names are puma, cougar, and mountain lion). Through efforts of the Florida Game and Freshwater Fish Commission, the Florida Panther Recovery Program was initiated in the early 1980s to try to prevent the extinction of this puma subspecies (13). Thus, a well-defined and closely monitored free-ranging population was available for both serological and virological studies.

A representative immunoblot analysis of plasma samples from 10 Florida panthers is shown in Fig. 1 (also see Table 1). Twenty-eight percent of the samples contained antibodies to FIV, extending the results presented in an earlier preliminary report (5). Analysis of sequential samples from three different animals revealed the development of cross-reactive antibodies to FIV over time. Notably, Florida panther FP-42, an offspring of FP-14 and FP-16, tested negative at 10 months of age in March 1990 but had seroconverted by 14 months later.

PBMCs collected from four seropositive Florida panthers (FP-14, FP-16, FP-21, and FP-42) were placed in culture and monitored for magnesium-dependent RT activity. RT activity was detected in each culture within 17 to 22 days after coculture. At the peak of RT activity, electron microscopic examination of the FP-16 PBMC coculture demonstrated the presence of virion particles with typical lentivirus morphology (Fig. 1D). The particles appeared to be similar in size to FIV particles but were slightly smaller than primate lentiviruses (38). We designated these isolates PLV.

Primary cell cultures of fresh PBMCs from a seronegative puma were readily infected with cell-free culture fluids from all four RT-positive cultures, thus demonstrating the infective capability of the lentiviruses. PLV isolates displayed a notable preference for growth in fresh puma PBMCs compared with fresh domestic cat PBMCs (data not shown). Transfusion of mitogen-stimulated PBMCs from FP-14 into an uninfected specific-pathogen-free domestic cat resulted in the establishment of a persistent infection. This cat developed humoral antibodies cross-reactive with FIV within 7 weeks posttransfusion, and virus isolation has been successful at each attempt during the first year postinfection. Genomic sequence analysis of the virus isolated from the chronically infected specific-pathogen-free cat demonstrated that it was identical to PLV-14 obtained in the original cocultures (see below).

Phylogenetic analysis of PLV *pol* gene sequences. We determined the nucleic acid sequence of a 714-bp amino-terminal segment of the *pol* gene by direct amplification of PLV-14-infected cellular DNA by the PCR. The PLV genome is 9,165 bp long. This *pol* region of the reverse transcriptase-encoding gene is the most slowly evolving portion of retroviral genomes and, as such, is particularly useful in reconstructing distinct ancestral relationships (3, 9, 21). For sequence analysis, we examined viral *pol* genes from PBMC genomic DNA of six PLV-infected Florida panthers (PLV-14, PLV-16, PLV-21, and PLV-42 from Everglades National Park and PLV-8 and PLV-18 from Big Cypress Swamp) and two western cougars (PLV-64 from Arizona and PLV-80 from Colorado). The nucleotide and translated amino acid sequences were aligned with each other and with 11 other lentivirus sequences (including those of three FIV isolates). The aligned amino acid sequences of the PLVs and FIVs are presented in Fig. 2. A matrix of pairwise sequence identities

TABLE 2. Sequence comparisons of conserved RT domains from *pol* genes of eight PLV isolates, FIV, and other lentiviruses

Virus[a]	% Identity with indicated virus[b]																		
	Everglades National Park				Big Cypress Swamp		PLV-64 (Arizona)	PLV-80 (Colorado)	California		FIV-MD (Maryland)	EIAV	Visna virus	HIV-1	SIV-CPZ	HIV-2	SIV-SM	BIV	BLV
	PLV-14	PLV-21	PLV-42	PLV-16	PLV-18	PLV-8			FIV-PET	FIV-SD									
PLV-14		100	99	99	92	91	82	83	84	83	88	63	61	61	61	57	61	52	33
PLV-21	100		99	99	92	91	82	83	84	83	88	63	61	61	61	57	61	52	33
PLV-42	98	98		98	91	90	82	83	83	83	87	62	60	61	61	56	60	51	33
PLV-16	99	99	98		91	90	82	83	83	83	87	63	61	61	61	56	60	52	33
PLV-18	88	88	88	87		91	84	81	81	79	84	61	60	60	60	56	61	50	33
PLV-8	88	88	88	88	87		80	81	82	80	86	62	61	61	60	57	61	53	34
PLV-64	76	76	75	75	75	76		93	78	77	79	64	57	57	58	55	63	47	33
PLV-80	79	79	79	79	75	78	86		80	78	79	61	58	60	59	56	60	46	33
FIV-PET	79	79	78	78	77	79	75	75		97	92	59	61	61	61	56	59	52	35
FIV-SD	79	79	79	78	77	79	74	75	96		90	58	59	61	60	56	59	51	35
FIV-MD	79	79	78	78	77	78	73	74	85	84		61	59	63	61	57	61	52	34
EIAV	70	70	69	70	67	70	64	66	66	67	68		54	58	59	55	58	54	35
Visna virus	69	69	68	68	66	68	64	64	65	64	64	61		57	57	57	56	50	35
HIV-1	65	65	65	65	64	65	62	63	66	65	65	60	64		94	72	74	52	30
SIV-CPZ	64	64	65	63	63	65	60	63	64	64	63	61	63	81		69	72	49	29
HIV-2	62	62	62	61	61	61	60	59	61	60	60	61	65	72	69		90	51	37
SIV-SM	64	64	63	63	62	63	61	63	63	63	62	61	63	70	69	83		50	37
BIV	63	63	62	62	61	62	58	61	59	58	59	65	59	56	56	57	58		35
BLV	43	43	43	43	43	43	41	41	45	44	42	47	42	40	39	46	42	45	

[a] Virus abbreviations: BLV, bovine leukemia virus; BIV, bovine immunodeficiency virus, EIAV, equine infectious anemia virus; FIV-SD (40) and FIV-PET (37), FIV isolates from San Diego and Petaluma, Calif., respectively.

[b] Percent amino acid (above diagonal) and nucleotide (below diagonal) sequence identities were determined by pairwise alignments of minimum overlaps of all feline lentivirus *pol* sequences shown in Fig. 2 and homologous nonfeline lentivirus *pol* sequences from GenBank. Geographic origins of feline virus isolates are indicated.

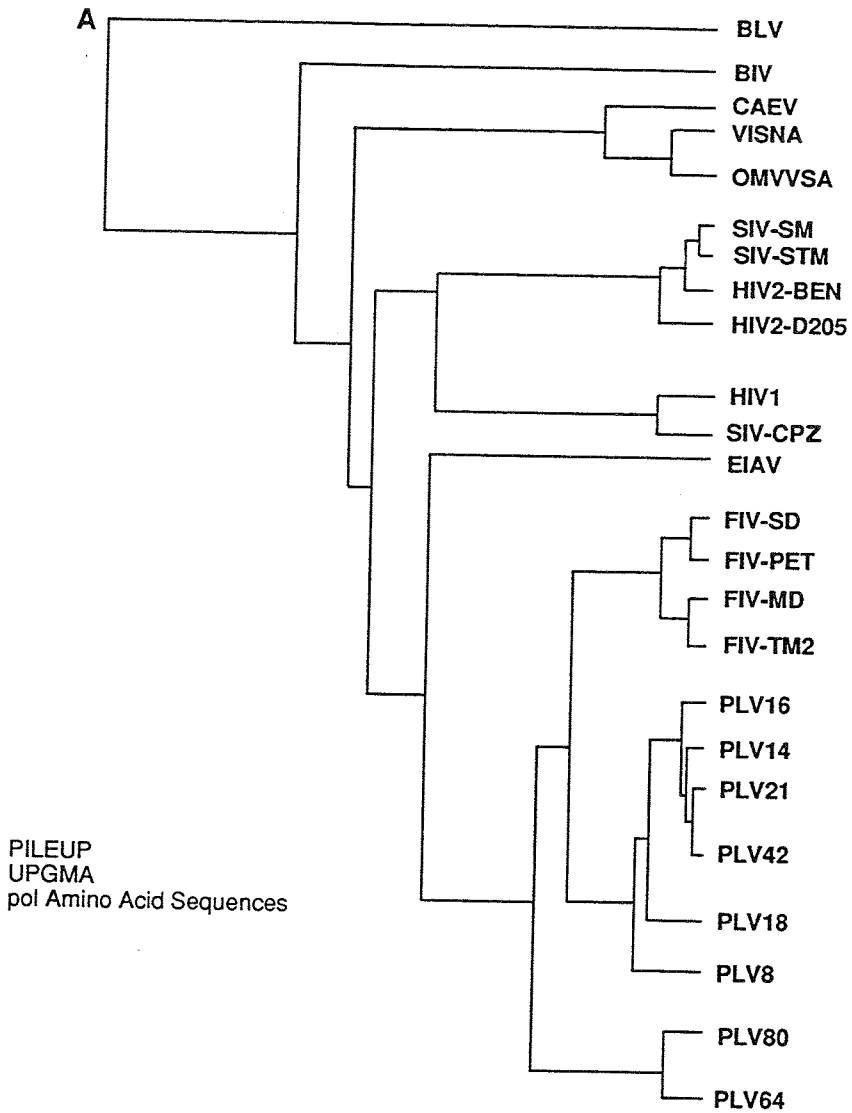


FIG. 3. Evolutionary trees developed from phenetic (A and B) and phylogenetic (C) analysis of *pol* gene sequences from the indicated lentiviruses. Nucleotide sequences were converted to translated amino acid sequences. Each full-length sequence was separately aligned with every other sequence by using the PILEUP program of the Genetics Computer Group software package (8), which uses the algorithm of Needleman and Wunsch (28). Distances are expressed on the basis of percent difference in amino acid sequence; gaps are given a weight of one residue difference (41). (A) UPGMA (Unpaired Group Method Analysis) tree derived by the PILEUP program (8, 44). This program employed a single alignment of all sequences. (B) Phenetic tree derived from amino acid sequence match frequency (Table 2) by using the Fitch-Margoliash algorithm (12), specifically, the KITSCH subroutine of the PHYLIP (Phylogenetic Inference Package) program, version 3.4 (11). This program computes a midpoint-rooted topology based on the least-squares method and the assumption of an evolutionary clock rendering all terminal species as contemporaneous. The numbered leg lengths are the number of amino acid substitution differences of an unrooted tree generated by the FITCH algorithm in the absence of these assumptions. The scale is based on the fraction of substitution differences between species sequences. (C) Phylogenetic tree derived by the PAUP (Phylogenetic Analysis Using Parsimony) program, version 2.4 (45). A strict consensus tree based on midpoint rooting is presented. Topologically equivalent trees were produced when BIV and BLV were designated as an outgroup for rooting. The scale and leg lengths are in amino acid substitutions (aa subs.). The tree shown has a length of 305 changes and an overall consistence index of 0.79, indicating a 21% convergence level. Virus abbreviations not introduced in the text are given in Table 2, footnote *a*, except for CAEV (caprine arthritis-encephalitis virus) and OMVVSA (ovine maedi-visna virus isolate SA).

between each *pol* region sequence is presented in Table 2.

The extent and character of sequence divergence (Fig. 2) allowed the construction of both phenetic (distance matrix-based) and phylogenetic (parsimony based on minimum length) evolutionary trees. We used three different phylogenetic methods to increase the reliability of the derived topologies, since tree-building algorithms depend on different assumptions. The derived trees and a description of the analytical methods are presented in Fig. 3 and the legend thereto.

The phylogenetic analysis of the PLV sequences (Table 2 and Fig. 3) revealed several important relationships between PLV, FIV, and other lentiviruses. First, in almost all cases the *pol* sequence-based trees were topologically equivalent,

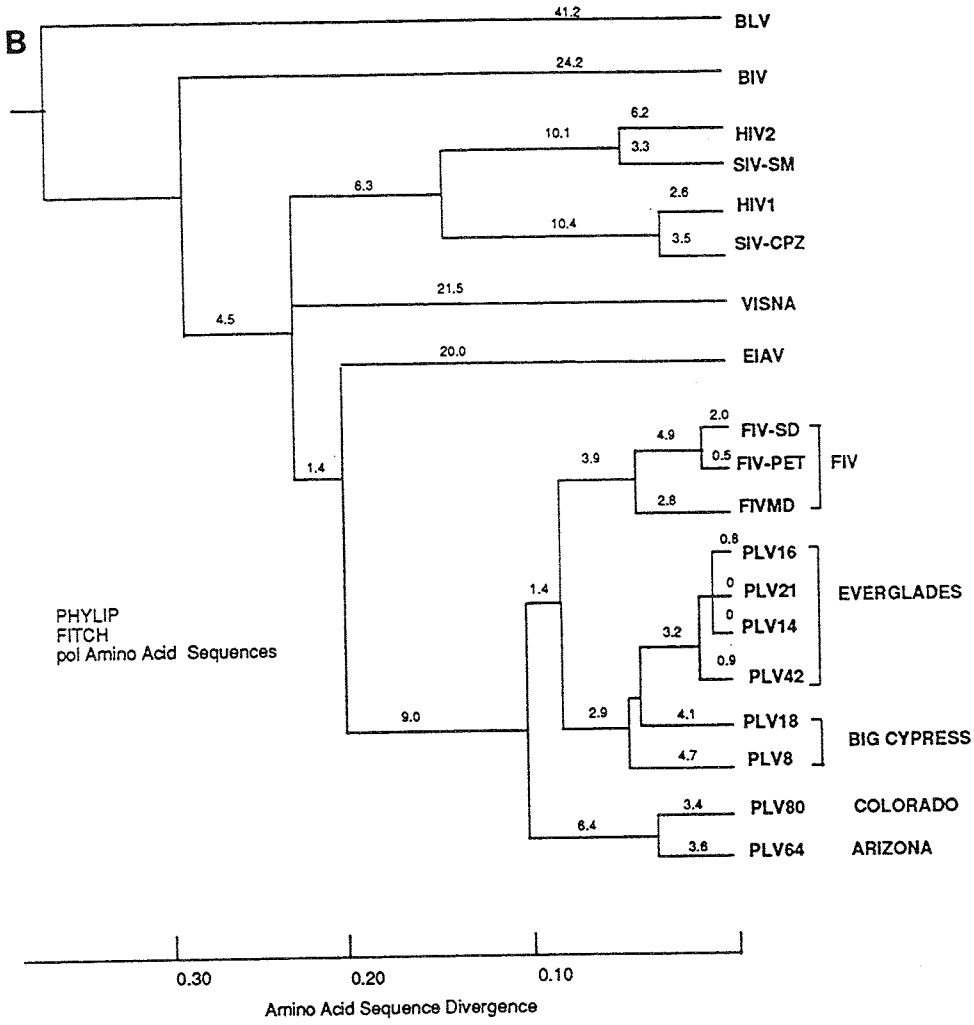


FIG. 3—*Continued.*

with the only uncertainty being the divergence positions of visna virus and equine infectious anemia virus relative to primate and feline lentiviruses. Second, the derived trees recapitulated the conclusions of previous phylogenetic studies of lentivirus evolution (7, 9, 16, 17, 20, 21, 26). Bovine leukemia virus and bovine immunodeficiency virus are more distant outgroups of four major evolutionary lineages, namely, the visna virus, equine infectious anemia virus, SIV-HIV, and feline lentivirus groups. Within the feline lentivirus group, the FIV isolates formed a monophyletic cluster, i.e., each FIV sequence was more closely related to other FIV sequences than they were to any other lentivirus type. As might be predicted from the immunological relatedness, the PLV isolates were more closely related to FIV than to other lentiviruses.

As a group, the PLV isolates assorted according to their geographic origins (Fig. 3A). Thus, the most similar isolates, PLV-14, PLV-16, PLV-21, and PLV-42 (98 to 100% amino acid sequence identity) were from a family group (mother PLV-14 and three offspring) living in Everglades National Park. PLV-8 and PLV-18, derived from animals that reside in the adjacent Big Cypress Swamp, showed 90 to 92% sequence identity to the Everglades group. Two PLV sequences from the western United States, PLV-64 and PLV-80, were as divergent from the Florida PLV sequences (80 to 84% identity) as any PLV isolates were from FIV (77 to 88% identity).

DISCUSSION

The serological survey and the genetic characterization of isolated PLV reported here demonstrate the widespread natural lentivirus prevalence in four genera of the cat family, Felidae. Feline lentiviruses appear to be endemic in lions of the Serengeti and Ngorongoro Crater in eastern Africa and in Kruger National Park in southern Africa, with infection rates surpassing the rates reported for SIV infection in African green monkeys (3, 7, 14, 15, 19, 21, 27). In contrast, free-ranging lions from Namibia (southwestern Africa) appear free of infection, at least by the limits of our sampling and assay criteria. Similarly, East African cheetahs were infected (22% incidence), while captive cheetahs in South Africa, despite being descended from cheetahs caught in the wild (Kruger National Park and Namibia) in the 1970s (32, 34), were negative. These observations suggest that FIV-like infection may be restricted geographically between African locales.

When the pattern of genomic variation among the feline

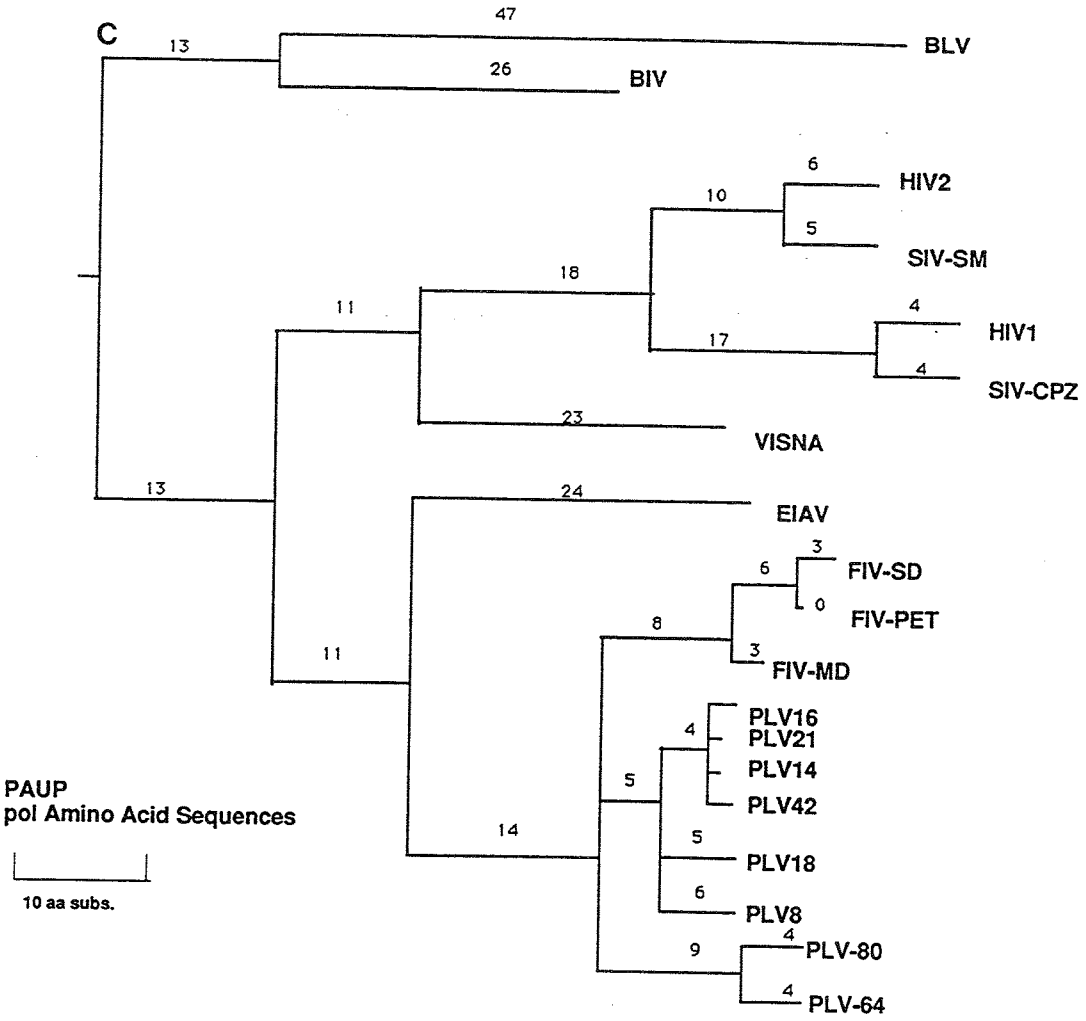


FIG. 3—*Continued.*

lentiviruses is compared with HIV-SIV divergence in primates, some important parallels become apparent. The closest simian virus to HIV-2 is SIV-SM, which was recently isolated from sooty mangabeys and is likely responsible for infection of Asian macaque species in U.S. primate research centers (15, 19, 27). The divergence between HIV-2 isolates and SIV-SM is greater than the amount of variation observed between most HIV-2 variants (19). The closest simian isolate to HIV-1 is SIV-CPZ, which was isolated from two chimpanzees at a primate facility in Gabon (20, 39). The genetic distance between HIV-1 isolates and SIV-CPZ is similarly outside the range of variation observed between most HIV-1 variants, making SIV-CPZ an attractive candidate for a recent ancestor of HIV-1. HIV-1 apparently diverged from HIV-2 much earlier than either split from the simian counterpart (Fig. 3), leading to speculation that HIV-1 and HIV-2 evolved separately in simian ancestors, probably species of the genus *Cercopithecus* (guenons and African green monkeys) (3, 10, 14, 21). Because *Cercopithecus* species display a relatively high degree of SIV sequence variation, their common simian ancestors were likely hosts of the primordial HIVs that have diverged into HIV-1 and HIV-2.

The divergence between PLV and FIV reported here (12 to 33% for amino acid sequences and 21 to 27% for nucleotide sequences [Table 2]) is greater than the divergence in the homologous *pol* region between HIV-2 and SIV-SM (10% amino acid sequence and 17% nucleotide sequence divergence) or between HIV-1 and SIV-CPZ (6% amino acid sequence and 19% nucleotide sequence divergence). Furthermore, the most extreme divergence observed between PLV isolates (25% nucleotide sequence difference [Table 2]) approaches the difference observed between HIV-1 and HIV-2 (28% nucleotide sequence difference). If these conserved *pol* sequences are changing at about the same rate in felids as in primates, it is likely that PLV and FIV are rather old felid viruses whose genomic divergence has proceeded primarily within separate species with interspecies exchange being rare. It is even conceivable that FIV and PLV have been isolated from each other since the species divergence estimated to have occurred 3 million to 6 million years ago (6, 23, 43, 46). Although it is not possible with available data to determine the direction in which an ancient FIV transfer between species occurred, the pattern of genetic divergence indicates that the FIV-PLV split occurred long ago and cannot be considered a frequent event.

To date, there have been no apparent immunological or pathological symptoms observed in infected free-ranging large cats. Since T-lymphocyte depletion has been observed

in FIV-infected domestic cats (2), it seems important to monitor certain infected free-living populations for possible disease or T-cell subset depletions by using newly available felid-specific monoclonal antibody reagents (1, 22, 42). Long-term clinical tracking may prove particularly informative in establishing either pathological symptoms or virus-to-host synergism that may have developed during the recent natural history of the populations. Ongoing field studies with lions, cheetahs, and pumas led by authors of this report (C.P., T.M.C., and M.E.R.) offer a rare opportunity to track virus and/or disease progression in a natural setting. The apparent asymptomatic character of SIV infection in African green monkeys may also parallel the feline situation, in which historic selective episodes may have led to genomic adaptations of both the virus and the host, leading to a modern symbiosis. The critical role of the dynamic balance between pathogen and host genomes in epidemics has been discussed in detail elsewhere (4, 18, 29). The natural history of feline lentivirus infection and disease processes in free-ranging felids would provide a new model system for empirically describing this still poorly understood natural process.

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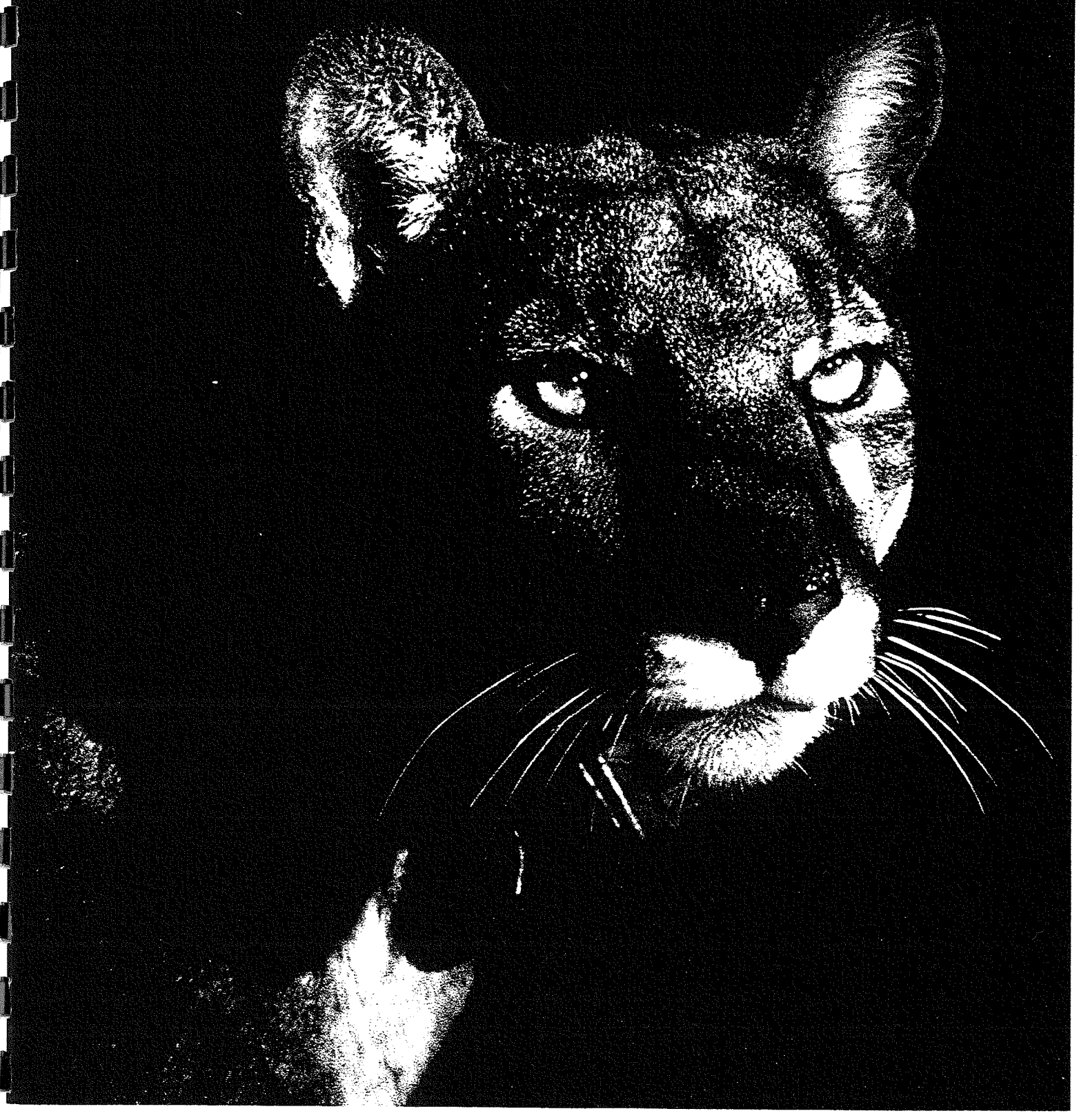

Bureaucratic Mischief: Recognizing Endangered Species and Subspecies

STEPHEN J. O'BRIEN AND ERNST MAYR

THE U.S. ENDANGERED SPECIES ACT OF 1973 WAS DESIGNED to identify and protect plant and animal species whose number and habitat had become sufficiently depleted to critically threaten their survival. The Act as amended specifically affords protection to three categories of biological taxa: species, subspecies, and populations. The operational definition of these terms, inadequate taxonomy, and the periodic occurrence of hybridization between species and subspecies have led to confusion, conflict, and, we believe, certain misinterpretations of the Act by well-intentioned government officials.

The listing of certain species as endangered has encouraged an increase in investigation of these taxa, notably in molecular genetics and field ecology (*1*). In some cases the molecular genetic results contradicted previous ideas about species integrity or taxonomic distinctions that were based on phenotypic (morphological) descriptions. Unfortunately these traditional taxonomic designations have been and continue to be the bases for management and eligibility for protection. This is a significant problem because the Endangered Species Act not only protects listed taxa from hunting, habitat exploitation, and other perils associated with human coexistence, but also provides significant financial resources for the effort to protect these species and to stabilize their populations. To illustrate the problem we summarize the interpretive difficulties posed by molecular results for four endangered groups.

The Florida panther. This is a small population of mountain lion (also called cougar or puma) that descended from the *Felis concolor coryi* subspecies that ranged throughout the southern United States in the 19th century (*2*). The few remaining panthers ($\leq$50) living in southern Florida show significant physiological and reproductive impairments that are likely the consequence of inbreeding depression. A recent allozyme and mitochondrial DNA (mtDNA) analysis of the population revealed that two very distinct genetic stocks were living in Florida (*2*), one that resembled other North American pumas and another that was more closely related to a puma subspecies that had evolved in South America. Apparently seven animals from a captive stock (that later turned out to be a mixture of authentic *F. concolor coryi* and South American founders) were released into the Everglades between 1957 and 1967 and promptly forgotten. Today the founder ecosystem contains a mixture of two subspecies.

The genetic advantages of introducing some additional genetic material into a population suffering from inbreeding would have been comforting except for one detail. Three opinions from the Solicitor's Office of the Department of the Interior (which is the counsel of the U.S. Fish and Wildlife Service) have ruled with the force of precedent that hybrids between endangered species, subspecies, or populations cannot be protected. Their opinions, referred to here as the Hybrid Policy, concluded that protection of hybrids would not serve to recover listed species and would likely jeopardize that species' continued existence. The current status of the Florida panther as endangered could be challenged or even revoked under a strict interpretation of the Hybrid Policy.

The gray wolf (*Canis lupus*). This wolf has suffered severe demographic contractions in North America owing to habitat depletion associated with the spread of agriculture. An mtDNA survey of wolves and coyotes across the northern United States (*5*) and Canada (*3*) revealed evidence for the presence of coyote mtDNA in wolf populations, but not vice versa, in a restricted region ranging from northern Minnesota to southern Quebec. Anecdotal accounts of wolf-coyote hybridization, the recent mtDNA results, and knowledge of the Hybrid Policy have prompted a formal petition from the Farm Bureaus of Wyoming, Montana, and Idaho to the U.S. Department of Interior that *C. lupus* be removed from Endangered and Threatened Lists. Similar logic has also been used to prevent reintroduction of gray wolves into Yellowstone National Park.

The red wolf (*Canis rufus*). The taxonomic status of the red wolf has been disputed for some time, with certain experts calling it a species and others suggesting that it be considered a subspecies of gray wolf (*4*). Extensive morphological studies plus recent molecular genetic analyses of captive red wolves and museum specimens (*3, 4*) raised the possibility that the red wolf group represented a hybrid between gray wolf subspecies and coyotes. Therefore, protection of the red wolf would be imperiled by strict enforcement of the Hybrid Policy.

The dusky seaside sparrow (*Ammodramus maritimus nigrescens*). This is a melanistic coastal subspecies that until recently inhabited the eastern coast of Florida (*5*). The population dropped until 1980, when five of these were brought into captivity and crossed with a morphologically similar subspecies from the gulf coast of Florida, Scott's seaside sparrow, *A. m. peninsulae*. The opinion of the Solicitor's Office in 1981 stated that the production of hybrids between the two subspecies (or any others) would not be in the interest of the Endangered Species Act. The dusky seaside sparrow became extinct in 1987.

These four examples emphasize the critical role that taxonomy plays in the enforcement of the Endangered Species Act, and the potential power of molecular genetic data in resolving taxonomic relationships and the unfavorable (and unnatural) consequences of the Hybrid Policy. To aid this process, we offer definitions for species and subspecies that can be applied to threatened fauna.

The Biological Species Concept. In 1940, Mayr (*6*) proposed the Biological Species Concept (BSC) that defined a species as "groups of actually or potentially interbreeding populations that are reproductively isolated from other such groups." Reproductive isolation, the primary component of the BSC, refers to the heritable tendency of distinct species to avoid gene flow or interbreeding even when they are brought into physical contact in nature. In clarifying this concept Mayr (*6*) noted that most species occupy distinct ecological niches and that this ecological distinctiveness is the keystone of evolution. Although various alternative species concepts and criticisms (*7, 8*) have appeared, the BSC has emerged as a biological paradigm with its major components affirmed (*8*).

A major strength of the BSC is that it reflects the occurrence in natural situations of the irreversible process of speciation. It emphasizes reproductive isolation as the sole discriminator of species as whole entities, but acknowledges the occasional production of hybrid individuals or even hybrid zones (*9, 10*). Further, the BSC acknowledges the existence of appreciable genetic diversity within species that is often partitioned geographically (or temporally) by population subdivision into subspecies, ordinarily under conditions of allopatry (reproductive barriers are geographic). The distinction however is that natural occurrences of hybrid individuals or hybrid

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zones between recognizable species do not disintegrate the genetic integrity of the species, while hybridizations between subspecies produce gene flow and genetic mixing. Reproductive isolation in nature provides an effective protective device against genetic disintegration of the species genotype (*6, 8*).

The subspecies category has been defined as "a geographically defined aggregate of local populations which differ taxonomically from other subdivisions of the species" (*6*). A valuable recent modification (*11*) urged that the evidence for BSC subspecies designation should come from the concordant distribution of multiple, independent, genetically based traits. In an attempt to provide formal criteria for subspecies classification we offer the following guidelines: Members of a subspecies share a unique geographic range or habitat, a group of phylogenetically concordant phenotypic characters, and a unique natural history relative to other subdivisions of the species. Because they are below the species level, different subspecies are reproductively compatible. They will normally be allopatric and they will exhibit recognizable phylogenetic partitioning, because of the time-dependent accumulation of genetic difference in the absence of gene flow. Most subspecies will be monophyletic, however they may also derive from ancestral subspecies hybridization (*12*).

In our view an allopatric subspecies has four possible fates; it may: (i) go extinct; (ii) exchange genes with another subspecies and become a new "mixed" subspecies; (iii) by genetic drift, selection, subdivision, or other demographic processes change its genetic character over time to become one or more new subspecies; and (iv) if effectively isolated, become a new species by acquiring genetic isolating mechanisms. It it not possible to know which subspecies will become new species, but they all have this potential. Moreover, as the time of allopatry increases, the probability of genetic differentiation increases, and included within these differentiative changes are ecologically relevant adaptations. The possibility that a subspecies carries such adaptations coupled with the potential to become a unique new species are compelling reasons for affording them protection against extinction.

The Hybrid Policy of the Endangered Species Act. The understanding of the BSC species, of subspecies, and of different categories of hybridization now leads to a recommendation for the Hybrid Policy with respect to endangered species. The Hybrid Policy that discourages production of hybrids between species seems appropriate and should be affirmed. The Hybrid Policy, however, should not imperil the listing or protection of species with sympatric hybrid zones as long as the existence of the zones does not disintegrate the genetic organization of the species in contact. Preclusion of protection for interspecies hybrids would correctly discourage capricious interbreeding between species in captivity as well as the facilitated introduction of species into natural habitats that are occupied by closely related but distinct species (*13*). For subspecies and threatened populations, the Hybrid Policy should be dropped. Subspecies can and do interbreed as a natural process whenever they are in contact; that is why they are not species. Subspecies that are defined by genealogical concordance and geographic partitioning can be protected because of their potential and their acquisition of unique characteristics; they represent important components of biological diversity. Occasional introgression or interbreeding should not be viewed as inconsistent with subspecies status; they simply change the phylogenetic description. Because subspecies do acquire ecological adaptations, the managed facilitation of subspecies mixing would generally be discouraged although, in certain extreme cases, it may be justified.

Under application of BSC principles a recommended easement of the Hybrid Policy leads to the following:

1) The Florida panther would receive continued protection since it clearly qualifies as a subspecies. In fact, the present population may be better off as a result of acquisition of new genes because of the multiple congenital difficulties that apparently emerged as a result of inbreeding in the ancestral *F. concolor coryi* (*2*).

2) The natural hybridization of gray wolf and coyote is limited to a narrow hybrid zone that developed recently in the Midwest. Since it does not affect the genetic integrity of either species elsewhere in their ranges, there is no justification for eliminating protection of the gray wolf species.

3) The status of red wolf is difficult because it is extinct in the wild and the captive bred survivors are likely descended from natural hybridization between coyotes and an extinct subspecies of gray wolves. The case for protection would be that the captive red wolves are the only available descendants of that historic subspecies (*13*).

4) The dusky seaside sparrow had a series of molecular characters that distinguished it from Gulf Coast subspecies but were indistinguishable from Atlantic Coast populations (*5*). Phylogenetic concordance provides justification for revising the taxonomy of the seaside sparrow complex to designate Gulf Coast and an Atlantic Coast subspecies (*2, 8*). Should either group become rare, protection under the Endangered Species Act could be contemplated.

There are many additional examples of confusion and misdirected judgments in the task of conserving endangered species (*1, 14*). It is important that legal opinions recognize the important distinction between species and subspecies. Biological species do not form hybrids that disintegrate population genetic organization, but subspecies may. The Hybrid Policy of the Endangered Species Act should discourage hybridization between species, but should not be applied to subspecies because the latter retain the potential to freely interbreed as part of ongoing natural processes.

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Genetic Introgression within the Florida Panther *Felis concolor coryi*

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*The Florida panther (*Felis concolor coryi*) is a severely threatened relict population of puma or mountain lion whose historic range has included much of the southeastern United States. The population now consists of 30 to 50 animals living in the Big Cypress Swamp–Everglades ecosystems in southern Florida. Field observations indicated the presence of two distinct morphological phenotypes that are stratified between the two adjacent areas despite the occurrence of periodic migration between them. A comprehensive molecular genetic analysis using mitochondrial DNA and nuclear markers indicates the existence of two distinct genetic stocks concordant with the morphological phenotypes. One stock confined to Big Cypress is derived from the ancestors of* F. c. coryi. *A second stock, found largely in the Everglades, is descended primarily from pumas that evolved in South or Central America, but were introduced (probably by man) in the Florida habitat very recently. The precarious genetic disposition of the few remaining authentic Florida panthers may be benefiting from the introgression of genetic materials into the wild population.*

The Florida panther (*Felis concolor coryi*) is one of 27 described subspecies of puma (also called cougar or mountain lion) that inhabit the western hemisphere (Goldman 1946). The Florida panther has always ranged throughout the southeastern United States; however, the pressure of human development has reduced the present subspecies to a relict population of <50 individuals living in the Big Cypress Swamp and Everglades National Park ecosystems in southern Florida (Belden 1986, Goldman 1946). In March 1967, the U.S. Fish and Wildlife Service listed the species as endangered. Portions of panther habitat in the two regions have received state and federal protection and four government agencies (U.S. Fish and Wildlife Service; Florida Game and Fresh Water Fish Commission; National Park Service; and Florida Department of Natural Resources) have combined resources to sustain this fragile population (Florida Panther Interagency Committee 1987). By many standards the Florida panther has emerged as a flagship species for national and international efforts to preserve endangered species and their associated ecosystems.

The Florida panther was first described by Cory (1896) and named as a distinct subspecies by Bangs (1889). The original taxonomic

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description of *F. concolor coryi* was based largely on geography and cranial morphology, specifically the occurrence of a distinctive broad, flat frontal region of the skull with rather broad and highly arched nasals (Bangs 1889, Goldman 1946). Belden (1986) reported two additional morphological characters consistently observed in the Big Cypress Swamp panthers: a ridge or whorl of hairs similar to a cowlick on the mid-dorsal thorax and an ~90° angle or kink in the posterior tail vertebrae. In an analysis of 636 *F. concolor* specimens, including 35 Florida panthers collected from 1896 to 1987, the incidence of the cowlick was 83% in Florida panthers compared with 4.8% in other subspecies (Wilkins et al. in press). The kinked tail occurred in 100% of 18 examined specimens of Florida panther from Big Cypress Swamp for which post-cranial skeletons were available (Belden 1986, Wilkins et al. in press).

(continued from previous page): **Robert C. Belden,** *Research Biologist, Florida Game and Fresh Water Fish Commission, Gainesville, FL 32601; and* **Janice S. Martenson,** *Research Technician, Laboratory of Viral Carcinogenesis, NCI.*

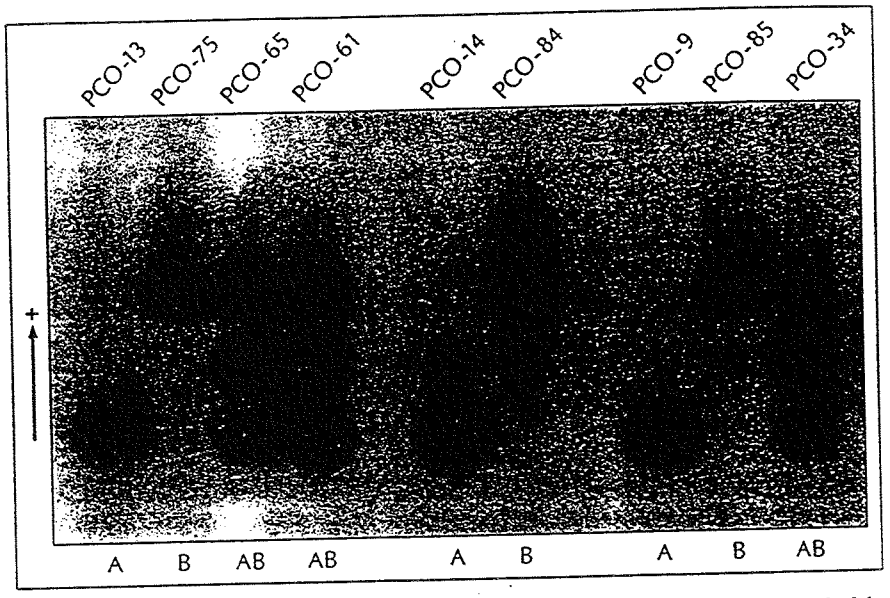

Figure 1. *Molecular genetic markers that were polymorphic in pumas.* ***Left.*** *APRT: adenosine phosphoribosyl transferase employs a ^{14}C-labeled substrate and enzyme product precipitation with lanthanum chloride (Bakay et al. 1978). Gels were loaded with red blood cell extracts from* F. concolor coryi *from the following locales: PCO-9, -13, -14, Big Cypress Swamp; PCO-61, -65, -75, Everglades National Park; PCO-34, -85, Piper captive stock.* ***Right.*** *Restriction enzyme patterns of mtDNA that were polymorphic in* F. concolor. *Twelve restriction enzymes defined 14 variable sites designated as follows: site 1-Stu I; 2-Nco I; 3-Sst I; 4-Xba I, site 1; 5-Xba I, site 2; 6-BamH I; 7-Bcl I; 8-Nde I; 9-Acc I, site 1; 10-Acc I, site 2; 11-Hpa I; 12-Dra I; 13-BstE II; 14-BstU I. Arrowheads indicate positions of 4.3 kilobase molecular weight markers. The enzyme patterns define six haplotypes distributed as shown in Figure 2. These restriction enzymes were monomorphic in the sampled pumas: Hind III, Ava I, Ava II, Apa I, Kpn I, Xho I, Pvu I, Pvu II, Bgl II, Cla I, EcoR I, EcoR V, Hinc II, Hpa II, Pst I, Sal I, Sst II.*

In 1981 the Florida Game and Fresh Water Fish Commission initiated a program of periodic capture and radio-collar tracking of individual panthers in southern Florida (Florida Panther Interagency Committee 1987, Maehr 1990). Over the past decade 33 animals have been collared and bled for clinical and genetic studies. Phenotypic observation of captured animals plus occasional mortalities revealed a notable discrepancy between the incidence of the cowlick and kinked tail in two separated populations of panthers. Nearly all the sampled animals in the larger Big Cypress Swamp population (N=23/24 individuals) retained the two morphological traits, while seven animals in the smaller Everglades National Park population (estimated at six to 10 animals) did not. These observations, plus occasional reports of releases of captive pumas of unknown origin into the Big Cypress–Everglades ecosystems (Vanas 1976), prompted a comprehensive molecular genetic analysis of the wild population to shed light on its demographic and phylogenetic history.

Materials and Methods

Heparinized blood and tissue samples were collected from free-ranging Florida panthers, captive panthers of the Piper captive stock, and pumas born in the wild representing eight different subspecies o

western North America (Table 1). The Piper stock is a collection of captive Florida panthers descended from a litter born to a Big Cypress Swamp female in the early 1940s and supplemented over the next 20 years with several additional pumas including captive individual(s) from outside the range of the Florida panther (Vanas

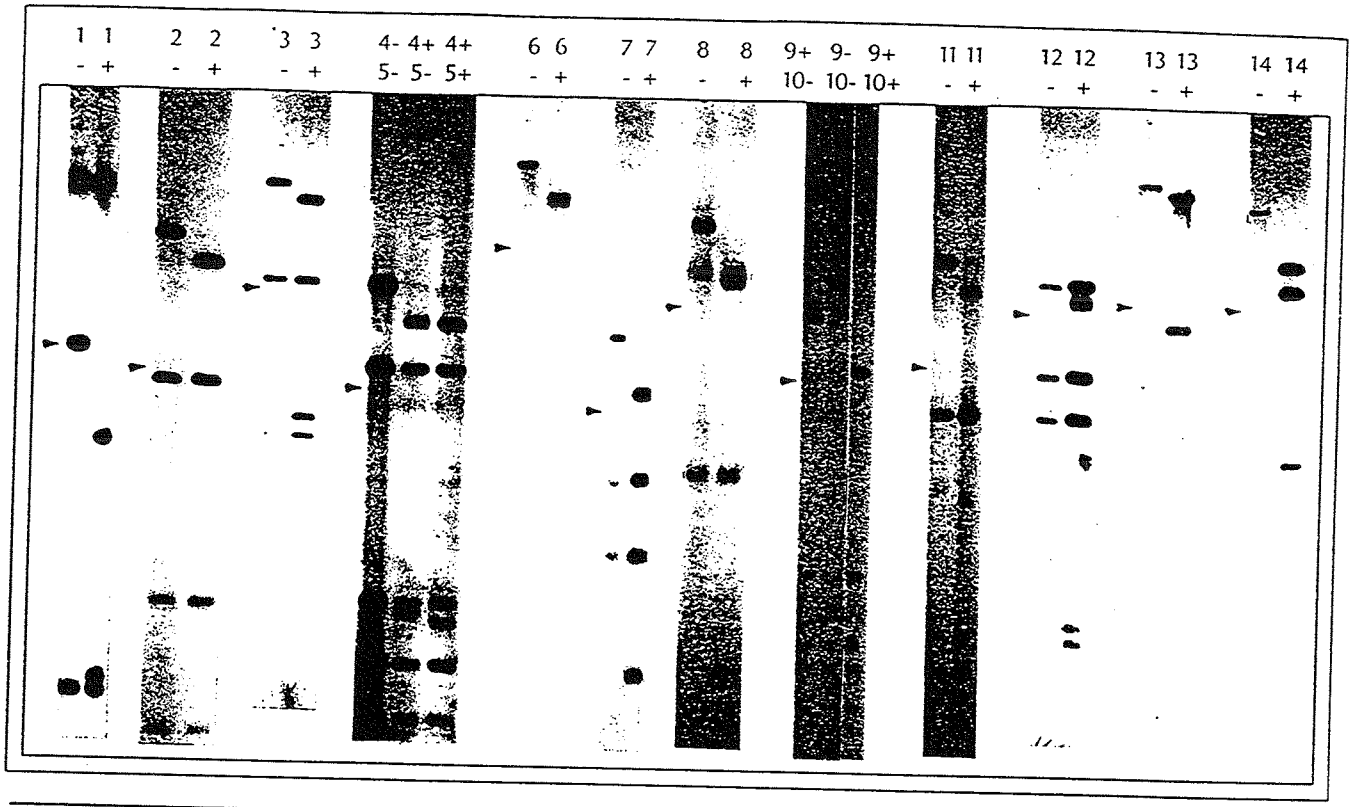

Table 1. Nuclear and mtDNA Phenotypes of Puma (*Felis concolor*) Subspecies

Location*	Subspecies	Molecular Phenotypes			
		Allozyme		mtDNA	
		APRT	Individuals	Haplotype	Individuals
FLORIDA PANTHER POPULATIONS					
Big Cypress Preserve	*coryi*	A,B	24	A,B	25
Everglades National Park	*coryi*	A,B	7	B	7
Piper captive stock	?	A,B	10	A	7
NORTH AMERICAN PUMA POPULATIONS					
Texas	*stanleyana*	A	12	C	3
Colorado	*hippolestes*	A	3	C	3
Arizona	*azteca*	A	4	C	1
Nevada, Utah	*kaibabensis*	A	2	C	1
Arizona	*browni*	A	2	C	1
California	*californica*	A	4	–	–
Southwest Oregon	*oregonensis*	A	5	C	1
East Oregon	*missoulensis*	A	1	D	1
SOUTH AMERICAN PUMA POPULATIONS					
Northern Chile	*puma*	A, B	10	E, F	5
Central Chile	*araucanus*	A	2	E	2
Southern Chile	*patagonica*	A	3	E	6

Sources: M. Roelke, R. Belden, J. Roboski, and D. Maehr, Florida Game and Fresh Water Fish Commission; D. Jansen, Big Cypress National Preserve; O. Bass, Everglades National Park; L. Redfield and B. Wahl, private owners; M. Jones, Tallahassee Jr. Museum; R. Smith, San Antonio Zoo; R. McBride, rancher supply; A. Anderson and D. Kattner, Colorado Division of Wildlife; H. Shaw, M. Pierce, T. Boggess, and A. Fuller, Arizona Department of Game and Fish; T. Alvarado, Houston Zoo; W. Bates, Utah Division of Wildlife Resources; F. Lindsey, Wyoming Cooperative Research Unit; R. Junge, St. Louis Zoo; D. Jessup, D. Hunter, and D. Fjelline, California Department of Fish and Game; R. Gagliuso, Oregon State University; W. VanDyke and M. Henjum, Oregon State Department of Fish and Wildlife; W. Johnson and W. Franklin, Iowa State University.

1976; J. Vanas, personal communication). The Piper stock animals sampled here failed to display either the cowlick or kinked tail, an observation consistent with a between-subspecies hybridization in its recent ancestry.

Allozyme electrophoresis was conducted on erythrocytes and leukocytes and tissue culture using standard techniques (Newman et al. 1985, O'Brien 1980). Adenosine phosphoribosyl transferase (APRT) employs a ^{14}C-labeled substrate and enzyme product precipitation with lanthanum chloride (Bakay et al. 1978).

The total genomic DNA was isolated from leukocytes or tissue samples and digested with a panel of 28 restriction enzymes. Restricted DNA was separated by electrophoresis in 0.8% to 1% agarose gels and transferred to nylon filters by Southern blotting in 10 × SSC. Filters were hybridized at 65° C in 0.5 M sodium phosphate, 7% SDS, 1 mM EDTA, 1% BSA with radioactively labeled mitochondrial (mt) DNA

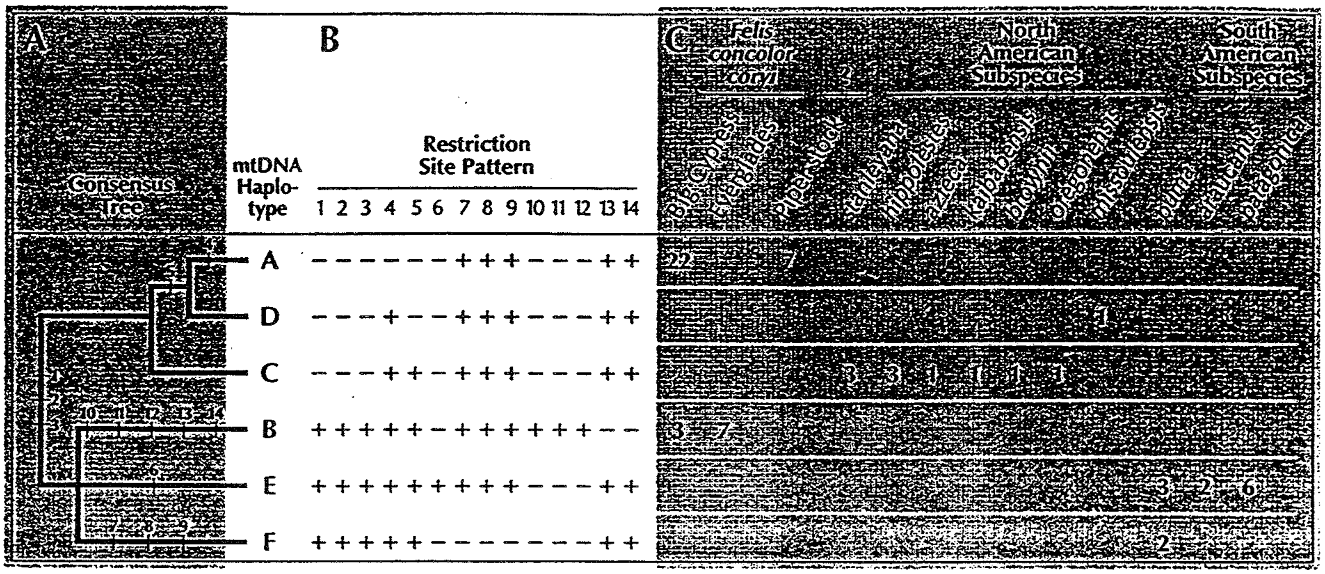

Figure 2. Topological network of six puma mtDNA haplotypes (A–F) generated by maximum parsimony. A. The Contree algorithm of the PAUP computer package (Swofford 1985) was used to produce a consensus tree with a length of 14 and a consistency index of 1.0 (no convergent site substitutions). The numbered hatchmarks on the network branches represent individual site changes shown in Figure 1, right. B. The restriction site pattern for each haplotype is indicated by the +/– list. Restriction sites are as listed in the caption of Figure 1. C. The distribution of haplotypes among different populations and subspecies in actual numbers of animals tested.

from domestic cat that was cloned into a lambda phage vector. Filters were washed at a final stringency of 0.1 × SSC, 0.5% SDS at 50° C for 30 minutes and autoradiographed for one to three days at –70°C.

To isolate domestic cat mtDNA, supercoiled mtDNA was purified from spleen tissue by a CsCl gradient. The mtDNA was digested with Xho I which recognizes a single site of the cat mtDNA, ligated with Xho I-digested λdash vector DNA, and in vitro packaged with Gigapack Gold (Stratagene). This library was screened with a purified

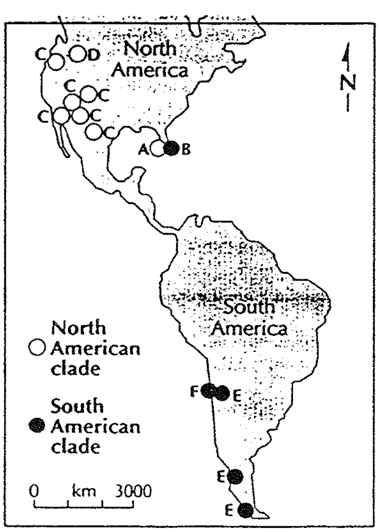

Figure 3. Geographic locations of puma/panther populations with indication of their mtDNA haplotypes. A, C, and D form the North American clade; E, F, and probably B, form the South American clade.

Results

puma mtDNA as a probe. One clone λdcmt 3-2 contained full-sized mtDNA sequences (N. Yuhki in preparation).

An electrophoretic survey of 43 allozyme loci revealed three polymorphic loci in free-ranging Florida panthers—PGD, PP, and APRT. Polymorphism for PGD and PP was common among other North American subspecies, but the APRT polymorphism was unique to the wild Florida panther populations and the captive Piper stock (Roelke 1988) (Figure 1A). All the western U.S. pumas were genetically fixed for the APRT-A allele (N=33). Furthermore, the APRT polymorphism was strongly partitioned among the wild Florida panthers. The common

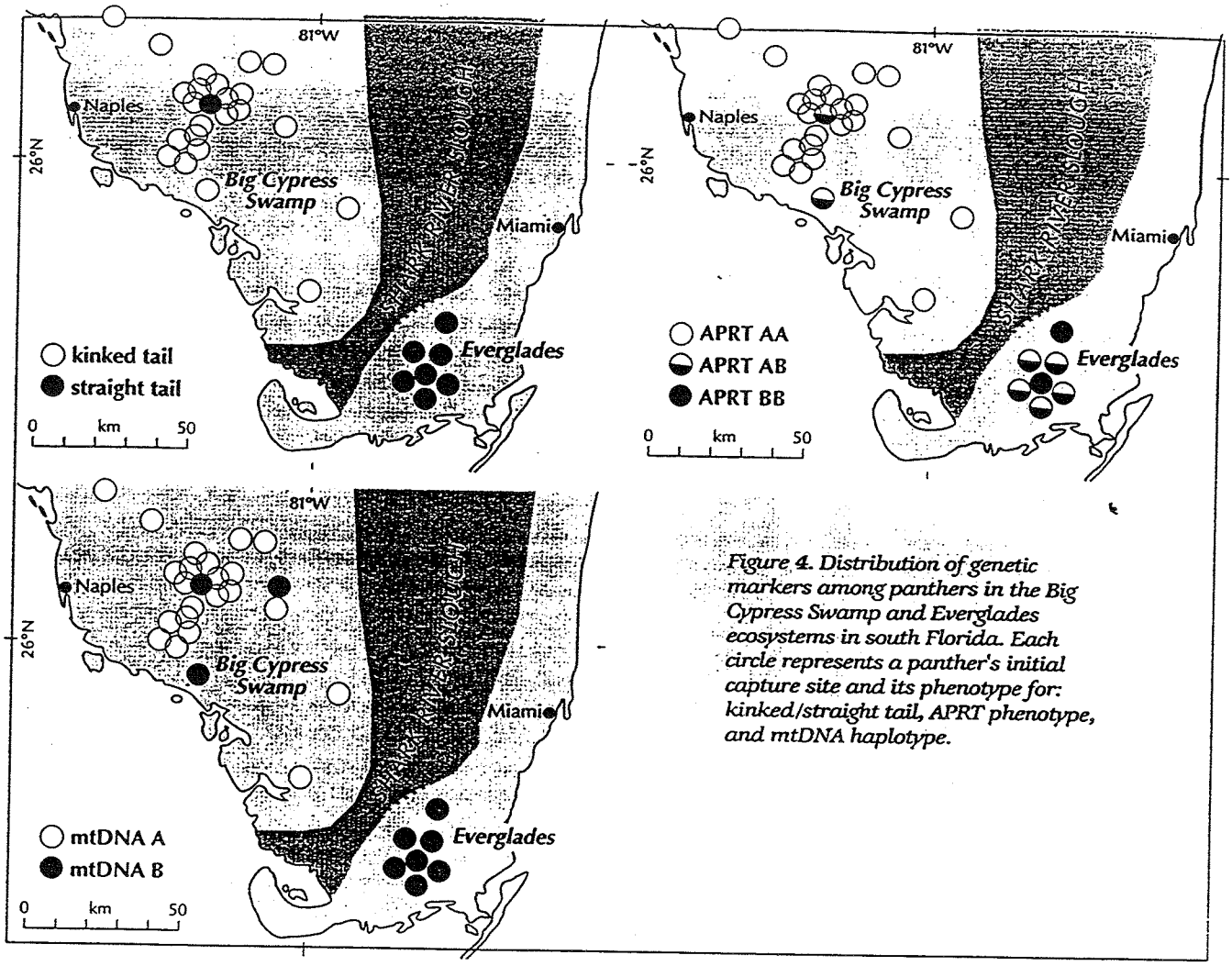

Figure 4. Distribution of genetic markers among panthers in the Big Cypress Swamp and Everglades ecosystems in south Florida. Each circle represents a panther's initial capture site and its phenotype for: kinked/straight tail, APRT phenotype, and mtDNA haplotype.

western allele, APRT-A, was nearly fixed in the Big Cypress Swamp panthers (22AA:2AB:0BB), while every animal in the Everglades National Park population had at least one APRT-B allele (0AA:5AB:2BB). The captive Piper stock contained both APRT alleles (0AA:8AB:2BB). In addition, the APRT-B allele was also found in a sample of one South American subspecies, *F. c. puma*, with an incidence of 5AA:1AB:4BB (Table 1). Two other South American subspecies samples, *F. c. araucanus* and *F. c. patagonica*, were monomorphic for APRT-AA.

The appearance of the novel (to North America) APRT-B allele and the absence of the *F. c. coryi* morphological characters in the Everglades National Park and Piper animals and the reverse in the Big Cypress Swamp panthers raised the possibility that the wild population had been recently supplemented with pumas from a different subspecies. To address this question, the authors undertook a more thorough genetic analysis of the Florida panther population using restriction site polymorphisms of mtDNA. This method has proved particularly valuable lately in resolving demographic and phylogenetic partitioning of recently separated populations and subspecies (Avise 1989, Brown et al. 1979, Harrison 1989, Moritz et al. 1987, Wilson et al. 1985). The power of this technique is due to the relatively rapid rate of evolution of mtDNA sequences in vertebrates. Furthermore, because the mtDNA molecule is maternally inherited, genetic

diversification is immediately apparent without the complications of allele segregation and recombination that affect nuclear sequences.

Total cellular DNA was extracted from leukocytes or tissue specimens taken from the Florida panther populations, the Piper animals, and representative individuals from seven North American and three South American subspecies (Table 1). Sample DNA was digested with 28 restriction endonucleases, subjected to electrophoresis in an agarose gel, transferred to nylon membranes and hybridized with a full-length molecular clone of mtDNA from the domestic cat *Felis catus*. A total of 109 restriction sites was scored in the analysis, representing 610 base pairs of recognition sequence or 3.7% of the 16 500 base pairs in cat mtDNA. Of the 109 restriction sites, 14 were variable in this sample, and representative patterns are illustrated in Figure 1B.

Among the 63 surveyed individuals, six distinct haplotypes were found. The restriction-site pattern and distribution of haplotypes is presented diagrammatically in Figure 2. The three Florida populations had only two haplotypes, A and B, that differed from each other at 10 different sites. The genetic difference (π) between these haplotypes was estimated using maximum likelihood (Nei & Tajima 1981, 1983) as 0.007, a rather large difference for a small isolated population. In addition, for the free-ranging panthers the two haplotypes were partitioned precisely in accordance with the APRT genotype, and by and large, along the geographic boundaries heretofore described (Figure 3). Within the Big Cypress Swamp population, 22 of 25 animals examined were mtDNA haplotype A and APRT-AA and all exhibited both the kinked tail and cowlick. The remaining three Big Cypress Swamp animals were mtDNA type B. Blood samples from two of these were available and both were APRT-AB; one of these lacked the kinked tail but had a cowlick and the other had a kinked tail and a cowlick. The remaining mtDNA type B was derived from a panther tongue confiscated in a legal action concerning illegal hunting and could not be scored for APRT or kinked tail. The pelt however displayed a prominent cowlick. Conversely, the seven panthers living in the Everglades were all mtDNA type B, APRT-AB or BB, none exhibited a kinked tail, and only one had a cowlick. All the Piper animals were mtDNA type A, APRT-AB or BB, and negative for the two morphological traits.

In order to interpret genetic partitioning in the Florida panthers, the authors also examined 12 pumas from seven different North American subspecies plus 13 pumas from three South American subspecies collected in Chile (Table 1). Two new haplotypes, C and D, were discovered in North America, each of which differed from the Big Cypress Swamp mtDNA type A by one or two restriction sites. In South American pumas two distinct haplotypes, E and F, were discovered that differed from each other and from North American mtDNA haplotypes at multiple sites (Figure 2A).

A topological network of restriction-site changes based upon the principle of maximum parsimony (that is, the tree that exhibits the shortest length of discrete character changes) using the PAUP phylogenetic algorithm (Swofford 1985), showed a striking agreement between mtDNA haplotypes and geographic locale. There was a clear North American clade (mtDNA type A, C, and D) and a South American clade (type E and F). The single exception was the Everglades National Park-specific type B. This haplotype had several unique or evolutionarily derived restriction sites (sites 10 to 14 in Figure 2) that are not informative as to the origin of type B. However, the B haplo-

type shared, with the E and F haplotype, each of the three restriction sites that discriminated the North American and South American clades. Recognizing that the authors' sample of puma subspecies outside Florida is incomplete, the shared derived or synapomorphic characters of the mtDNA type B with the E–F South American clade affirm the South American affinity of the B mtDNA haplotype.

Discussion

The results of the genetic analysis suggest that the free-ranging Florida panther population is comprised of two genetically distinct stocks that evolved separately, perhaps in different hemispheres, and were combined recently in Florida. If mtDNA in the puma has accumulated mutations at the rate estimated for other mammals (Brown et al. 1979, Wilson et al. 1985), ~2 to 4% sequence divergence per million years, then the Everglades National Park and the Big Cypress Swamp haplotypes may have last been in contact 175 000 to 350 000 years ago. Computation of the allozyme genetic distance (Nei 1972, 1978) between the two Florida subpopulations (D=0.14) supports such an estimate, placing the divergence date at 250 000 years ago.

The close phylogenetic proximity of the Big Cypress population with representatives of other North American subspecies indicates that the Big Cypress population is descended from the historic *F. concolor coryi*. The origin of the Everglades pumas with the B mtDNA haplotype and the APRT-B alleles is not obvious, but a likely candidate would be the captive Piper stock. The Piper stock and the Everglades pumas both have a high frequency of APRT-B, an allele not found elsewhere in North America, but seen in a limited sample from South America (Table 1). Furthermore, releases have been documented of seven Piper animals (including three females) into the Everglades National Park between 1957 and 1967 (unpublished archives, Everglades National Park, National Park Service, Washington, D.C.). The fate of the released animals is not known. The presence of the mtDNA haplotype A in the Piper animals does not support, but does not preclude, such a hypothesis because the pedigree of Piper animals here tested can be traced to a single great-grandmother of the youngest individuals sampled (Roelke 1988). If the Piper animals were de facto hybrids of distant subspecies, they would have possessed two or more mitochondrial types. The putative wild released animals would have retained the introduced mtDNA B type.

An alternative explanation for the accumulated results is that at one time historic *F. concolor coryi* possessed all the fixed genetic variants described here and that a series of population bottlenecks and genetic founder effect led to differential fixation of the two populations. The authors believe this scenario unlikely for several reasons. First, the four diagnostic genetic traits (kinked tail, cowlick, APRT-B, and mtDNA haplotypes) are remarkably concordant among the individuals in the studied populations (Figure 3). Second, the mtDNA genetic distance between the A and B haplotypes is very large, corresponding to a separation of >250 000 years and no intermediate haplotypes were observed in Florida, or, for that matter, in North America. Third, the mtDNA haplotype B is not similar to any other North American haplotype—in fact, the genetic distance between A and B is 5 to 10 times the distance between any of the North American haplotypes. Fourth, the maximum parsimony phylogenetic analysis (Figure 2)

clearly places the origin of haplotype B with the South American haplotype lineages. And, fifth, the nuclear marker, APRT-B, of the introgressed Everglades population was not observed in a survey of 33 individuals from eight different North American subspecies, but is prevalent in at least one South American subspecies (Table 1).

The conclusions of this report could be interpreted in the following perspective for conservation efforts on behalf of this endangered species. First, although the genetic differences between the two Florida subpopulations are diagnostic of their historic separation, the two stocks have shared a common ancestor recently enough to confidently exclude the development of reproductive isolation mechanisms (~ 200 000 years ago, comparable to the time of separation of human racial groups). As such, there would be no genetic rationale for avoidance of interbreeding between the lines in nature or in captivity. Second, the hybrid formation may actually increase the chances for population persistence. The Big Cypress Florida panther is clearly suffering from a history of inbreeding, as seen in reduced allozyme variation relative to other puma subspecies (Roelke 1988). Apparent consequences of this inbreeding include an extremely high level of developmental abnormalities in spermatozoa (>90%) and an elevated incidence of cryptorchidism (52%) among Big Cypress animals (Miller et al. 1990, Roelke 1989). These genetic defects could severely limit the reproductive potential of the surviving animals and could conceivably be improved by the introduction of new genetic material. Third, subspecies hybridization is a common phenomenon in nature and has likely occurred during subspecies evolution of the puma as well (Ferris et al. 1983, Goldman 1946, Lamb & Avise 1986, Powell 1983, Spolsky & Uzzell 1986, Wayne et al. 1990). This is not the first case in which mankind has inadvertently hybridized different stocks or populations of species (Avise et al. 1987; Avise & Nelson 1989; Janczewski et al. in press; Marker & O'Brien 1989; O'Brien, Joslin et al. 1987; O'Brien, Martenson et al. 1987; O'Brien, Roelke et al. 1985; O'Brien, Wildt et al. 1987), often with an increase in fitness. In some cases it actually appears that hybrid contact has played a critical role in species evolution (Bush 1975, Sharmin et al. 1990). Finally, the data here support the co-occurrence of two genetic "stocks" in Florida, but do not provide definitive evidence for or against individual hybridization between the stocks. Moreover, if evidence for genetic hybridization were developed, it would be restricted to racial or subspecies hybridization within the species *Felis concolor*.

The Florida panther is primarily a symbol of a fragile ecosystem. As the top species in a trophic chain, its survival serves as a keystone for preservation of the Everglades and Big Cypress ecosystems. Irrespective of the recent phylogenetic history of the panthers, the goal of preserving this tenuous population is a worthwhile effort that puts to the test our resolve to reverse a global extinction process for which mankind is clearly responsible.

Acknowledgments

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