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BIG CAT GENOMICS*

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■ **Abstract** Advances in population and quantitative genomics, aided by the computational algorithms that employ genetic theory and practice, are now being applied to biological questions that surround free-ranging species not traditionally suitable for genetic enquiry. Here we review how applications of molecular genetic tools have been used to describe the natural history, present status, and future disposition of wild cat species. Insight into phylogenetic hierarchy, demographic contractions, geographic population substructure, behavioral ecology, and infectious diseases have revealed strategies for survival and adaptation of these fascinating predators. Conservation, stabilization, and management of the big cats are important areas that derive benefit from the genome resources expanded and applied to highly successful species, imperiled by an expanding human population.

INTRODUCTION

The cat family Felidae is in big trouble. With the exception of the house cat and a few other small cat species, nearly every one of the 37 species is considered endangered or threatened by international bodies that monitor endangered species [Convention of International Trade of Endangered Species of Wild Fauna and Flora (CITES), International Union for Conservation of Nature (IUCN), U.S. Endangered Species Act, and others] (15, 58). Among the big cats, fewer than 15,000 tigers, cheetahs, and snow leopards remain in the wild. Lions are estimated at 23,000, and only about 50,000 pumas (also called cougars or mountain lions) and a comparable number of jaguars still survive. The most populous big cat species, the leopard, has an approximate census of 300,000, abundant in eastern and southern Africa, but severely endangered in North Africa, the Middle East, and most parts of Asia. The principal reasons for vulnerability of these big cats derive from their need for huge home ranges (one tiger in the Russian forest wanders about 400 square miles and a cheetah in Namibia will cover 600 square miles).

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Continuous habitat spaces sufficient to support even tiny populations of the world's most successful predators are diminishing rapidly as the far more successful primate species develops and alters the landscape of the globe. In his 2003 book *Monster of God* (78a), naturalist David Quamman summarized the conservation crisis for large predators: "The last wild free-ranging population of big flesh eaters will disappear sometime around the middle of the next century." Reversing human-caused extinction of these exquisite evolutionary creations is a lofty goal of conservationists everywhere.

Species conservation is an end, not a scientific discipline. Indeed, environmental conservation draws from many specialties of natural and social sciences, to name a few: ecology, behavior, reproduction, evolution, genetics, medicine, physiology, politics, sociology, law, history, and economics. Each discipline plays an important but different role in every situation, and the translation of each to action and species stabilization poses an ongoing challenge to conservationists and conservation biologists. In this review, we highlight the role genetics and genomics can play in dynamic situations involving the large felid species.

Although the initial goal of studies in this area stemmed from a conservation rationale and applications, additional translational benefits from studying big cat genetics rapidly became evident. First, in the postgenomic era of dense gene maps and whole-genome sequences for representative mammal species, one could explore the strategies of survival and adaptation that have benefited these as-yet poorly studied free-ranging species. For decades molecular evolutionary biologists have used evolutionary neutral genomic markers [e.g., mitochondrial DNA (mtDNA) variation, microsatellites, restriction fragment length polymorphism (RFLPs), even allozymes] as a surrogate for genome-wide diversity and variation (2, 3, 24, 86). Annotated gene maps/sequences now allow us to identify the precise genes that mediate adaptation and survival (55, 68). Second, wild animals, including the big cats, descend from the winners of historic struggles for survival. Hardwired in their genomes are naturally tested solutions to daily challenges including countless genetic and infectious diseases. Identifying how they acquired genetic resistance to diseases analogous to human pathologies is the hope of genomic prospecting, the search for naturally tested solutions to medical maladies that affect people (61, 63). Geneticists are just now beginning to mine the human genome for the footprints of ancient plagues and adaptive episodes. The big cats, studied intensely by field ecologists and conservationists, offer a parallel opportunity for genomic prospecting in a distinct, highly adapted, and heretofore successful predatory lineage (58, 90, 92).

Before the Human Genome Project, mammalian genetics was dominated by hypothesis-driven studies of mice and rat models. Humans were unsuitable for experimentation for ethical reasons, yet pedigree and population genetic association studies, driven by microsatellite [also called short tandem repeat (STR)] and single nucleotide polymorphism (SNP) methodologies, have made human genetics a rich area of biomedical discovery. Endangered cat species are also ineligible for manipulative experimentation (only studies that directly relate to species conservation are allowed/permitted for endangered species by the U.S. Endangered

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Species Act), yet pedigree, population, and molecular evolution interrogation has led to deep insight in these species. In many instances, as experts from various conservation specialties gather around a cheetah, a lion, or a tiger, there is no single primary hypothesis driving the study. Rather, committed scientists from divergent disciplines are drawn together to discover whatever they would about the marvelous specimens under study. Genetics has opened many doors of investigation, and in the following pages we review some of the most illuminating examples.

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The Beginnings of Conservation Genetics

The deleterious consequences of inbreeding were recognized by Charles Darwin, who devoted an entire chapter of *On the Origin of Species* to the dangers of interbreeding domestic animals (17). Twenty-five years ago, Kathy Ralls and her colleagues demonstrated the damage graphically by showing that among captive zoo animals, in nearly every case offspring of consanguineous matings showed a dramatic increase in infant mortality compared to offspring of unrelated parents (81). They went further by computing a quantitative estimate of the number of lethal equivalents in these species, thus providing predictive insight into the inbreeding damage in different zoo-bred species (80).

Yet one species, the African cheetah, showed little difference in the incidence of infant mortality between related and unrelated parents. All cheetah matings—unrelated and consanguineous—resulted in 30% to 40% infant mortality, the highest level seen in Ralls' survey for unrelated matings (70, 71). Cheetahs, it turned out, appeared to have naturally inbred with 90% to 99% reduction of overall genetic diversity as measured by allozymes two-dimentional protein electrophoresis (2DE) of fibroblast proteins, RFLP variants at the major histocompatibility complex (MHC), and SNP sequencing (estimated by random shotgun sequencing of cheetah genomic DNA; K. Lindblad-Toh, personal communication). Perhaps the most dramatic affirmation of the cheetah's genetic uniformity was the demonstration that reciprocal skin grafts, surgically exchanged between 12 unrelated cheetahs, were accepted by their immune systems as if they were identical twins (70). The cheetah's MHC, which specifies class I and II cell surface antigens that trigger graft rejections in all other mammals, alleles were all the same, an extraordinary discovery for a free-ranging species. Somehow the ancestors of modern cheetahs off-loaded most of their endemic genomic diversity, so breeding between siblings today has a minimal effect on an already elevated juvenile mortality.

The explanation for the cheetah's remarkable genetic depletion is now thought to derive from an extinction event that nearly extirpated cheetahs from the earth toward the end of the Pleistocene epoch around 12,000 years ago (48). Before then, at least four species of cheetahs had a range that included North America, Europe, Asia, and Africa. After the final retreat of the glaciers from the northern hemisphere, cheetahs disappeared from all but Africa and a few small areas of the Middle East and India (56). As the cheetah range contracted, the world saw the simultaneous extinction of three quarters of the large mammals living on those same continents, the most extreme species loss in the 100-million-year history of mammals on earth.

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Forty large animal species, including mammoths, mastodons, giant ground sloths, dire wolf, massive short-faced bears, American lion, saber-toothed tigers, and cheetahs, were eliminated from North America. Similar extinction waves of large mammals occurred in South America, Australia, and Eurasia, and several large flesh-eating birds, eagles, vultures, condors, and teratorns were also eliminated (25, 32). Coalescent-based back calculations of rapidly evolving genome families (mtDNA, minisatellites, and microsatellites) in modern cheetah subspecies dated the origin of diversity at 10,000–12,000 years ago, at the precise time of the Pleistocene extinction of large mammals (19, 48). Ancestors of the cheetah had passed through a population bottleneck that dropped effective population size numbers to very few individuals over several generations and also over space, leading to a species pronounced depauperate in genetic variation (71).

The cheetah's ancestors survived their brush with extinction and by the eighteenth century increased to hundreds of thousands across Africa and parts of Asia (58). Yet cheetahs today display physiological correlations of inbreeding depression in both captive and free-ranging populations, including a high infant mortality (less than 15% fecundity in captive settings), rendering the international program to breed cheetahs less than self-sustaining (46). Compared to the other felid species, reproduction is constitutively impaired because cheetahs have tenfold reduction in sperm count, 70% abnormal sperm per ejaculate, and a high incidence of acrosomal defects (100, 101). Although the cheetah's reproductive problems were apparent from detailed physiological measurements, they apparently were not a large factor in regulating the growth of wild populations, as cheetah numbers in recent times have been more affected by habitat loss and human expansion (9, 58, 62).

The cheetah's genetic legacy taught us some important lessons for conservation management. First, if a species survives a near-extinction crisis or population bottleneck, it can carry a hangover of genetic impoverishment and associated inbreeding depression that in some, but not all, cases lowers its fitness (23, 59, 60). Second, genomes of living species retain patterns of diversity that we are learning to interpret in the context of their natural history (61, 63). Third, the outcome of historic inbreeding is different every time. In some cases, genetic reduction seems to have little effect on recovery. For example, northern elephant seals have experienced a near extinction due to overhunting and appreciable genetic reduction (8, 33). Their numbers rose to a high of 120,000 by 1960 after 40 years of protection by the U.S. and Mexican governments. In other cases, inbreeding damage can be worse. Among the big cats, only the cheetah has the entire species genetically impoverished. However, two subspecies of big cats, the Asiatic lion and Florida panther, showed even greater genetic depletion and adverse consequences than cheetahs (83, 99). The Florida panther, a small subspecies population of puma, dropped to fewer than 30 adults in the Big Cypress/Everglades ecosystem in southern Florida by the 1970s. Genetically impoverished, individuals from this weakened subspecies displayed 90% sperm abnormalities, up to 80% cryptorchidism, congenital heart defects, and an inordinately high load of deadly infectious diseases (83).

The consequences of extreme population bottlenecks and subsequent inbreeding in populations is variable, dynamic, and ranges from negligible to severe

debilitations such as reproductive impairment, increased genetic/congenital defects, and/or homogenization of usually divergent immunity genes such as the MHC. The early studies of cheetahs and other big cats made these points so strongly that some ecologists raised important questions about the relative importance of genetic influences compared to traditional ecological, demographic, or even stochastic threats of small endangered populations (9, 11, 41, 49). These arguments notwithstanding, two decades of cogent examples and discourse have provided compelling evidence for the notion that inbreeding caused by population bottlenecks exerts real and measurable influences on a population's survival (23, 24, 47, 59, 60, 62).

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Conservation Applications and a Genomics Tool Box

Genetics has offered more to conservation than simply estimating relative genetic diversity in search of historic bottlenecks. The field of conservation genetics applies both population genetics and molecular evolution to endangered species assessment and management. Examples from each of these applications have been achieved in the big cats, as indicated in Table 1. This table lists specific molecular genetic approaches meant to answer explicit biological questions not previously achievable.

Population genetic diversity has been assessed in each big cat species and subspecies using common indicators of variation, as discussed above for cheetahs, Asiatic lions, and Florida panthers. Leopards and jaguars have abundant variation, whereas tigers show signatures of genetic impoverishment comparable to cheetahs (21, 45, 96). By using phylogenetic and population genetic measures of genetic equilibrium (F_{st}, R_{st}, variance in microsatellite allele size range, and linkage disequilibrium), patterns of population isolation, gene flow, geographic partitions, and structure were revealed. Phylogeography, a term coined 17 years ago by John Avise (3), allows an interpretation of subspecies geographic isolation in a spacial and temporal context that informs the potential for an isolated subspecies to become a new species or to have accumulated habitat-specific adaptations.

Phylogeographic partitions now form the basis of subspecies recognition, a means to detect hybridization, and a framework for species-level classification (4, 67). Besides making taxonomy more explicit, molecular assessments provide specific well-defined characters that assist the legislative protection of endangered species, subspecies, and distinct populations. Molecular genetic species and subspecies characterization has been used extensively in forensic application, from identifying smuggled cat pelts to implicating marauding cougars after human attacks.

Coalescent theory is a population genetic- and phylogenetic-based approach for estimating the time elapsed since an historic bottleneck, divergence event, isolation, or immigration (35). Combined with fossil dates of phylogenetic bifurcations, these methods allow a more refined view of the time frame of historic events in groups like Felidae (Table 2). In addition, exact determination of parentage and kinship/relatedness have facilitated predictive hypothesis testing in big cat

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TABLE 1 Applications of molecular genetics to conservation issues with examples from the big cats

Applications in conservation genetics	Published examples in	References
I. Population genetic diversity estimate Surrogate for historic demographic reduction, inbreeding, and population bottlenecks	Cheetah, Asiatic lion, Tigers, Florida panther	(19, 45, 70, 71, 83, 99)
II. Phylogeography Population substructure Geographic isolation Gene flow Migration	Leopard, puma, ocelot, tiger, clouded leopard	(16, 21, 38, 45, 96, 97)
III. Taxonomy/systematics Species and subspecies definition Species and subspecies recognition Species and subspecies hybridization	Leopard, puma, ocelot, tiger, clouded leopard	(16, 37, 38, 45, 96, 97)
IV. Coalescent dating Founder effects Bottlenecks Divergence	Cheetah, leopard, puma, Asiatic lion, jaguar	(16, 19, 21, 48, 96)
V. Behavioral ecology Parentage Kinship	Puma, lion	(26, 72, 83)
VI. Medical ecology Emerging pathogens Commensal and symbiont development	CDV-lion; FeLV-Fla., panther FIV-Felidae spp; FeCoV-cheetahs	(10, 83, 84, 95)
VII. Gene mapping Adaptation and species isolation genes	Leopard, jaguars	(10, 21a)
VIII. Forensics Endangered species ID Marauding predator ID	Puma, domestic cat, onza	(18, 48a)

populations under ecological scrutiny (26). Phylogenetic tracking of microbial pathogens has offered a close glimpse of deadly outbreaks of diseases in the cats that bear genetic homology to HIV and SARS (10, 76, 95). Each of these applications extends beyond heterozygosity estimates and can reveal highly useful insight into the past, present, and future disposition of natural populations.

The numeric tools for phylogenetic and population inference derive from computational algorithms developed across three decades of theory and practice. In

TABLE 2 Estimated age of intrinsic genetic variation present today in big cats

SPECIES				
Common	**Latin**	**Molecular marker**	**Age (years)**	**References**
Cheetah	*Acinonyx jubutus*	mt DNA, minisatellites	10–12,000	(19, 48)
Leopards[a]	*Panthera pardus*	mtDNA, microsatellite	470–825,000	(96)
Asian leopards	*Panthera pardus*	mtDNA, microsatellite	169–400,000	(96)
Puma[b]	*Puma concolor*	mtDNA, microsatellite	200–300,000	(16, 19)
South American puma	*Puma concolor*	mtDNA, microsatellite	200–300,000	(16, 19)
North American puma	*Puma concolor*	mtDNA, microsatellite	10–12,000	(16, 19)
Florida panther	*P.c. coryi*	mtDNA, microsatellite	200	(19, 83)
Jaguars	*Panthera onca*	MtDNA	280–510,000	(21)
Tigers[c]	*Panthera tigris*	MtDNA, microsatellite	72,000–108,000	(45)
Asiatic Lions	*Panthera leo persica*	Microsatellite	1081–4279, 100	(19, 99)

[a]See Figure 3.
[b]See Figure 2.
[c]See Figure 4.

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addition, genetic markers specific for the big cats have been borrowed from the Feline Genome Project, an international collaborative effort to map and sequence the domestic cat genome (54, 66, 69). Full mtDNA sequence, MHC sequence, almost 1000 microsatellite loci, a radiation hybrid map, and a linkage map have been achieved.

Domestic cat libraries of bacterial artificial chromosomes (BACs) and plasmid artificial chromosomes (PACs), flow-sorted chromosomes, and the Y chromosome are also available, as well as a collection of more than 50,000 tissue specimens from some 10,000 individual cats at National Cancer Institute's (NCI's) Laboratory of Genomic Diversity (42, 66, 69). In August 2004, the U.S. National Human Genome Research Institute announced plans to develop a whole-genome sequence of the domestic cat by the fall of 2005. Because all cat species shared a common ancestor during the past 10 million years (see below), and because genome structure is highly conserved within Felidae (54, 68), the domestic cat genomic resources can be readily applied to nondomestic Felidae genomic enquiries. For all these reasons, the big cats are beginning to reveal the secrets of their origin, migrations, divergence, adaptations, and survival. A few specific examples follow.

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Origins of the Felidae

A recent robust molecular phylogeny based on DNA sequence of more than 16,397 nucleotides estimated that placental mammals diverge from an ancestor that lived 105 million years ago (MYA) (52, 53). The Carnivora family diverged from its closest relative, Pholidota (pangolins), 78 MYA and cat-like carnivores (cats, hyenas, mongoose, and civits) split from dog-like carnivores 55 MYA. Several waves of saber-toothed cats came and went in the fossil record since the first Nimravides of the Oligocene approximately 35 MYA. Modern felids arose in the late Miocene (around 10.2 MYA) and have evolved into the world's most widespread, adaptive, and successful carnivore families, occurring on all the continents except Australia and the poles. The abundance of species (37 modern species) combined with the recency of origin has made solving the phylogenetic hierarchy conflicting and taxonomy vexing. The conundrum has led to alternative systematics, subjective taxonomy, and contradicting nomenclature (57, 102). Our group recently produced a new molecular phylogeny based on DNA sequence analyses of 22,789 base pairs (bp) (including nineteen autosomal, five X-linked, six Y-linked, and 9 mitochondrial genes) using maximum parsimony, minimum evolution, maximum likelihood, and Bayesian positive probability influence (37). Also assessed were 35 insertion/deletion variants (indels) that support the cladogenesis. A consensus molecular phylogeny of the 37 living species of Felidae based on these methods is presented in Figure 1.

The large nucleotide data set, full taxon sampling, multiple outgroups including the banding linsang, closest relative of modern felid, and insertion/deletion affirmation provided a strongly supported topology (bootstrap resampling and Bayesian posterior probabilities), the most comprehensive resolution of the Felidae achieved to date. The divergence nodes were estimated based on paleontological dates (minimum and maximum) of 14 fossil calibration dates using a Thorne/Kishino method that permits simultaneous fossil constraints and accounts for variance in molecular divergence rates in different lineages (37).

The Felidae molecular phylogeny demonstrates eight major lineages, each receiving 100% statistical support from analyses of both nuclear and mitochondrial genes. The derived time scale and geographic range of the eight lineages allowed for a plausible phylogeographic scenario for the origins and present distribution of the group. The hypotheses, described in detail in Reference 37, begins in Asia 10.2 MYA when the great roaring cats, *Panthera* plus clouded leopard, diverged from the ancestor of other felid precursors. *Panthera* species would remain in Asia (leopard, tiger, snow and clouded leopards) and migrate to Africa (leopard and lion) or to the Americas (jaguar). Four of the other seven lineages exist today on a single continent (bay cat and leopard cat lineages in Asia, ocelot lineage in South America, caracal lineage in Africa). A minimum of 10 migrations with imputed dates based on their phylogenic splits would account for the present distribution of all modern species. The clear partition of eight Felidae lineages interpreted in a paleontologic and geographic context provide the basis for an objective generic taxonomy corresponding to each lineage (Figure 1). This study is the latest and

most comprehensive of more than 10 phylogenic advances of Felidae published by our laboratory in the past two decades. Uncertainties remain, particularly within some of the lineages, yet the consistency observed with this very large data set suggests that Figure 1 represents a close approximation to the evolution of the world's cat species.

Archeology of the Felidae Genome—Case Studies

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AMERICAN PUMAS Pumas, also called mountain lions or cougars, occupy the most extensive range of any New World terrestrial mammal, spanning 110° of latitude from the Canadian Yukon to the Patagonian pampas. Morphometric and molecular studies suggest that the pumas' origin dates to the North American Miocene when it diverged from cheetah and jaguarundi progenitors (56). Since 1948, pumas have been classified into 32 separate subspecies, geographical populations described by mammalogists keen on placing new trophies in a geographic context (103). Because subspecies have the potential to have adapted to their habitat over accumulated generations of isolation, they represent suitable units of conservation worthy of preservation (67). A few years ago, graduate students Melanie Culver & Carlos Driscoll initiated a formal study of genetic diversity among puma subspecies (16, 19). Culver assembled some 315 tissue specimens (261 contemporaneous tissues and 54 museum skins) from across the entire range of the species, including every named puma subspecies.

From analysis of three mtDNA genes (*16s rRNA*, *ATPase-8*, and *NADH5*, 891 bp) and 10 microsatellites, Culver's data reduced the 32 described subspecies into just 6 discernable subspecies based on population and phylogenetic substructure (Figure 2). Yet the distribution of genetic diversity was unusual. The puma species showed abundant diversity originating in eastern South America 200,000–300,000 years ago. But nearly all the variation came from the five subspecies south of Mexico. Pumas in Mexico, the United States, and Canada were markedly similar, showing 20- to 50-fold less diversity than in South America. Culver found 12 mtDNA haplotypes in South America, and 100 North American pumas (representing 16 traditional subspecies) shared an identical haplotype (M in Figure 2). Four pumas from Vancouver had a second genotype that differed by a single nucleotide from the common type (N in Figure 2). Microsatellite loci confirmed this finding, indicating that North American pumas had acutely homogenized genomic diversity compared to the South American subspecies. We concluded that the North American subspecies showed hallmarks of a recent population bottleneck, not unlike that observed in cheetahs and Asiatic lions.

Driscoll & Culver expanded the data set to 85 microsatellites and, based on microsatellite variance, a measure of the breadth of microsatellite allele size range that increases with time, they estimated the time of the North American puma's founder effect (19, 27). The North American puma microsatellite variance was virtually indistinguishable from the variance of the same loci in African cheetahs, indicating that cheetahs in Africa and pumas in North America both experienced a near-extinction event at the same time, 10,000–12,000 years ago and in the same

place, North America. Puma fossils much older than this period prove pumas were present in North America earlier, so it seems that whatever eliminated the cheetahs, sabertooths, mastodons, and American lions from North America also extirpated the pumas. Subsequently, pumas from South America would migrate north through a geographic bottleneck, the Isthmus of Panama. The immigrant pumas would establish large territories and disperse adolescents northward. The number of founders north of Panama remained low by behavioral reinforcement. Resident pumas north of Panama blocked immigration of additional genetic lineages from the south.

The late Pleistocene bottleneck left an imprint of high teratospermia and reproductive defects common in all North American pumas (7, 83). These conditions were exacerbated in the Florida panther, where recent persecution, depredation, and habitat loss contributed to a second bottleneck (1, 22). We now understand why the Florida panther has such extreme genetic and congenital abnormalities: It suffered two back-to-back near-extinction events, one in the nineteenth and early twentieth century and an earlier reduction 10,000–12,000 years ago.

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LEOPARDS Like the puma in the New World, the leopard spans a two-hemisphere range in the Old World, including all of sub-Saharan and North Africa, the Middle East, Asia Minor, and Southeast Asia extending north to the Amur Valley of the Russian Far East (Figure 3). Island populations occur in Java, Zanzibar, Kangean, and Sri Lanka. Except for central Africa and India, the surviving leopard populations are fragmented and endangered. The leopard species is classified as endangered in appendix I by the CITES. Pocock (79) named 27 distinct subspecies of leopard based on color pelage, morphometric measures, and geography. Two students, Sri Miththapala and Olga Uphyrkina, applied several genomic technologies (allozymes, mtDNA-RFLP, feline minisatellites, mtDNA-sequence NADH5 and control region, and feline microsatellites) to address the subspecies validity as well as to reconstruct the natural history of the world's leopards (50, 51, 96, 97).

Leopards show a great deal of molecular genetic variation across their range; for example, Uphyrkina et al. (96) describe 33 mtDNA haplotypes within 727 bp, including 50 variable SNPs in 69 leopards and 80% heterozygosity across 25 microsatellites. The mtDNA haplotype and individual composite microsatellite phylogenies revealed 9 discrete populations and this was affirmed by maximizing F_{st} and R_{st} for mtDNA and microsatellite variation, respectively. The nine subspecies (illustrated in Figure 3) extended the morphological inference and previous molecular partitions (allozyme, mtDNA RFLP, and minisatellite) to provide an explicit genetic basis for subspecies recognition (57, 58). Genomic diversity estimates of the subspecies varied from the maximum for the African subspecies *Panthera pardus pardus* to the least in a highly endangered subspecies population of Amur leopard, *P. pardus orientalis*, which showed a single mtDNA haplotype and 36% average microsatellite heterozygosity (96, 97). Coalescent back calculation places the origin of leopard diversity somewhere in Africa 470,000–825,000 years ago, with a migration out of Africa to Asia 169,000–400,000 years ago (Table 2). These

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dates are congruent with the period estimated for the human founder event of Caucasian and Asian ethnic groups 100,000–170,000 years ago (12, 39). Perhaps when modern humans migrated from Africa to populate Eurasia, leopards accompanied them.

The Amur leopard subspecies (*Panthera pardus orientalis*) survives today as a tiny relict population of 25–40 individuals 50 miles from Vladivostok in the Russian Far East province of Primorskiy Kray (97). The historic subspecies, which ranged over southeastern Russia, the Korean peninsula, and northeastern China, has a large body size, a thick coat adapted to frigid habitat, and large widely spaced thick-rimmed rosettes. The Amur leopard is considered critically endangered by IUCN, CITES, and U.S. Fish and Wildlife Service (USFWS), but receives scant financial support save for a few nongovernment organizations aimed at its conservation. Genetically, the Amur leopard shows all the hallmarks of severe inbreeding in greatly reduced mtDNA and microsatellite variability comparable to the Asiatic lion and Florida panther, although physiological correlates of inbreeding have not been medically investigated (97).

As a conservation backup, a captive population of the Amur leopard was established from nine wild-born founders in 1961 and expanded to 170 leopards. The Amur leopard studbook provided exact pedigree information on the structure of this population (14). Questions about the authenticity or genetic purity of the founders prompted a genetic analysis of the pedigree using mtDNA sequence and 25 nuclear microsatellites (97). The captive kindred showed much greater variation than the authentic Amur leopards derived from the Russian Far East population. Pedigree transmission analysis of mtDNA demonstrated that one founder leopard female retained a genotype identical to that found in the neighboring Chinese subspecies, *P.p. japonensis*. Microsatellite phylogeny showed that another male founder was also *P.p. japonensis*. The captive population had inadvertently been developed from an admixture of pure Amur leopards and the neighboring Chinese subspecies. [A similar discovery of the Asiatic lion captive population in 1987 shook the conservation community and led to a shutdown of the captive program and birth control implantation in the remaining lions (65).]

The genetic discovery of subspecies admixture in Amur leopards was disturbing to some, but has a silver lining today. The reason is that the admixed captive population would still be suitable for future reconstitution of the wild population for two reasons. First, gene flow between *P.p. japonensis* and *P.p. orientalis* occurred naturally when the widespread subspecies overlapped their ranges in the nineteenth century (Figure 3). The admixed captive population simply reflects the gene flow that occurred prior to the human-induced genetic debilitation of the Amur leopard. Second, the captive population has recovered genetic diversity and likely improved fitness by abrogating ongoing inbreeding depression of the small wild population. Because subspecies can and do mediate gene flow in natural settings, such a situation can be beneficial in a management situation. Similar logic was used to justify the 1995 reconstitution of Florida panthers by releasing eight females from the neighboring Texas subspecies, *Puma concolor stanleyii,* into Florida, a success story for conservation genetics-based intervention of a threatened species

(D. Land, personal communication; 85). (Interested readers are referred to a more thorough discussion of subspecies hybrids and U.S. Hybrid Policy in chapters 4 and 5 of Reference 63.)

TIGERS Of all the big cats, or indeed of endangered species, the tiger may be both the most revered and the most feared. The remaining 5000–7000 tigers live in Asia, clinging to survival bolstered by national protection in their host countries. Traditionally, eight tiger subspecies were stipulated based on morphology habitat, and geography (90). For tigers, subspecies recognition has much relevance because, for this species, conservation is inextricably tied to knowledge of its subspecific taxonomy (89, 94). We began to apply molecular methods to tiger genetic structure two decades ago at the behest of noted tiger conservationist Ullysses S. Seal (64). Early molecular methods revealed diminished overall genetic variation and little population structure among the limited sample specimens (31, 98), leading us and others to suggest that the living tigers showed little subspecies differentiation. But that conclusion was wrong, as Shujin Luo & Jae-Houp Kim demonstrated in a recent comprehensive analysis (45). Luo & Kim assembled 134 tigers of known geographic origins, termed "voucher specimens." They sequenced 4078 bp of contiguous mtDNA, but only after they had done the same for the homologous nuclear mitochondrial (*Numt*) copies to assure that cytoplasmic sequences were interrogated properly (36, 44). Their large sequence data set was supplemented with genotypes of 30 tiger microsatellites, operative in tigers and nuclear MHC class II DRB gene variation. The mtDNA analysis (Figure 4) demonstrates phylogenetic monophyly for five modern subspecies with robust statistical support, but with one exception. The exception was the Indochinese tiger, *P.t. corbetti*, where two distinct groups were resolved, a mainland Indonesian subspecies, *P.t. corbetti,* and a peninsular Malaysian subspecies, *P.t. jacksoni* (Figure 4). The pattern was affirmed by phylogenetic monophyly and R_{st} maximumization of 30 microsatellite loci assessed in the same tigers, and supported by patterns of MHC variation (45).

Three named subspecies (Caspian, Javan, and Bali tiger) suffered extinction in the mid-twentieth century and were not included. The molecular data provided genetic support for recognition of five modern subspecies: Bengal, Sumatran, Malayan, Indochinese, and Amur tigers. Mitochondrial variation was used to create a linearized tree (93) based on the Minimum Evaluation–Neighbor Joining (ME-NJ) algorithm of Kimura 2 parameter distance as a chronometer. Tiger genetic diversity dates back to only 72,000–108,000 years ago, when a founder effect established an ancestry for all modern tigers (Table 2). The dates correspond roughly with the catastrophic eruption of the Toba volcano in Sumatra about 72,500 years ago (82). That eruption has been linked to late Pleistocene human bottlenecks as well as effects on Asian elephants. The Amur tigers show a single mitochondrial haplotype, evidence for a more recent bottleneck estimated at about 20,000 years ago, possibly consequent of the retreat of glaciers covering the region in the lower Pleistocene (45). In all, we are beginning to interpret the patterns of genomic

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diversity in the big cats to connect plausible demographic hypotheses with geological events that likely influenced fauna in these regions.

Behavioral Ecology

Long-term ecological studies of big cats offer an opportunity to study development of behavioral and reproductive strategies that work in a natural setting. Given humankind's innate fascination with the big cats, there are many high-profile longitudinal field observation projects designed to describe adaptive behaviors for foraging, defense, subsistence, and survival. Collaborations with ecologists studying each of the big cats in their native habitat have proven fruitful. Provocative evolutionary and adaptive hypotheses featured in *National Geographic* and/or The Discovery Channel now have a new accessory, the powerful technologies of modern genomics.

Each cat has its own story and space limitation precludes discussion of each; however, newer specialty journals (e.g., *Molecular Ecology*, *Conservation Biology, Conservation Genetics, Animal Conservation,* and *Trends in Ecology and Evolution*) offer scores of dazzling scenarios featuring the intersection of genomics and species behavior. One of the earliest and most dramatic successes involved a research collaboration between Craig Packer, an evolutionary ecologist leading the Serengeti lion project, and Dennis Gilbert, a student of molecular genetics (26, 72).

The Serengeti-Mara Ecosystem is a vast savannah plain of 25,000 km^2 (about the size of the state of Connecticut) in Tanzania, East Africa, defined by the migration patterns of 28 herbivore species (91). The four million-year-old ecosystem is remarkable in its pristine state, allowing daily natural challenges to the animals and species within. The keystone species, the wildebeest, numbers more than 1.3 million today, along with 240,000 zebras, 440,000 Thompson gazelles, 10,000 hyenas, and 3000 African lions. The lions have been under continuous ecological observation since the pioneering narratives of George & Kay Schaller in the late 1960s (88). Craig Packer and his research partner, Anne Pusey, took over the Serengeti lion project in the late 1970s and have monitored lion behavior and perils ever since.

Unlike nearly all other cat species, lions are social, even communal (73, 88). They live in female-dominated groups, called prides, consisting of mothers, cubs, sisters, and aunts. Prides defend a large territory and mate with resident coalitions of males who have won and now defend sexual access to the pride females. Male coalitions remain for about three years when another stronger nomadic coalition challenges the resident males and replaces them. Coalition takeovers are brutal, sometimes killing the losing males and their cubs. Afterward, the pride females synchronously enter estrus and mate with the victors, all of them, in day-long copulation encounters. Cubs are communally raised by all the females and defended by the males.

Behavioral hypotheses about the adaptive advantage of the pride organization were addressed by hypervariable minisatellite probes Gilbert had isolated from

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domestic cats and applied to parentage assessment within 11 Serengeti lion prides (26). The feline minisatellite probes were the same that showed identity in the Asiatic lions (26, 99), but for the Serengeti pride they unequivocally identified mothers, sires, and precise genealogical relationship for 200 lions, including 78 cubs. Gilbert & Packer also built a standard curve of kinship (or relatedness) versus genotypic similarity, allowing the assessment of relatedness among individuals in the population with unknown background. Accomplished in the early 1990s before microsatellites, before CERVUS and KINSHIP (computer programs that employed maximum likelihood algorithms to assess parentage of wild population), and in the face of close family relations of both the mothers (mostly sisters) and the fathers (thought to be brothers), their success represented a significant breakthrough for genomic technology. Yet the biological implications were even more revolutionary.

Packer & Gilbert interpreted the parentage and kinship measures to address prevailing hypotheses about lion behavior, particularly those based on the evolutionary notion that transmitting genes are the principal driving force of adaptation. William Hamilton's concept of "kin selection," whereby close relatives cooperate in a manner that their gene will be transmitted through matings of their closest kin, made sense for lion pride behavior—multiple males taking turns at copulation while other coalition members (were they really brothers?) patiently await their turn (29, 73).

The genetic results proved three points (72). First, all the cubs born to a pride were fathered by one of the resident coalition males with no outside male contributions, as seen in chimps, birds, and humans. Second, as expected, all female members of a pride were close relatives, so females never allowed nonrelative additions. Third, males of a coalition were never related to the females. There were no surprises here, but the assessments did affirm the occurrence of behavioral strategies hardwired in the lion's genome that precluded mating with close relatives, at least in the rich, bountiful habitat enjoyed by Serengeti lions.

The genetic results would also form a rationale for lion cooperation as related to the structure of the male coalitions (72). Years of field observation by Packer's team showed that the cohort with the most lions nearly always won a takeover encounter, so the more the better. Gilbert's kinship profiles showed that large coalitions (>4 lions) were always brothers, whereas smaller coalitions (2–3 lions) were brothers half the time, but the other half were mixtures of unrelated males. Also important, in nearly all litters, two males sired all the cubs, regardless of the coalition size. This meant that being in a small group did not greatly diminish the chance of siring cubs. For larger coalitions of brothers, outsiders were excluded, takeovers were likely, and gene transmission was assured, although often through brothers' mating, an affirmation of Hamilton's kin selection hypothesis. This early example of behavioral genomic application is now considered seminal in behavioral ecology investigations across mammals. Similar applications have been attempted for cheetahs, pumas, and tigers. Finally, noninvasive sample collections

(feces and hair samples) combined with the very efficient whole-genome amplifications methods for trace DNA specimens (6, 74) hold unusual promise for evolutionary insight into the long-hidden behavioral strategies for survival and reproduction.

Biomedical Ecology

The domestic cat has become a major model for several devastating infectious agents. A generation of researchers has studied feline leukemia virus, a cancer-causing retrovirus, to discover oncogenes, the mediators of cancer and carcinogenetic processes. The discovery of feline immunodeficiency virus (FIV) in a California pet cat in 1987 led to a comprehensive characterization of a natural model of pathogenic lentivirus-based immunodeficiency that parallels human AIDS (77). Several nondomestic cat species (including lions, leopards, pumas, and cheetahs) are endemic with monophyletic variants of FIV, but evidence for lethal pathology has not been found in these species, as if they have evolved genetic resistance to a fatal environmental pathogen (10, 95).

The feline panleucopenia virus abruptly jumped from cats into dogs to cause an epidemic canine parvovirus that decimated tens of thousands of puppies in 1978 (75). In another chilling viral emergence, canine distemper virus, a morbillivirus related to human measles, emerged from domestic dogs to infect spotted hyenas and to kill one third of the Serengeti lion population in a nine-month interval of 1994 (84). Each of these pathogen outbreaks, with the benefits of PCR and molecular phylogenetic tools, allowed unparalleled access to the progression of a deadly epidemic. But perhaps one of the most chilling virus episodes in big cats would reveal some important lessons about the severe acute respiratory syndrome (SARS) outbreak that began in November 2002, in southern China.

SARS first appeared as a flu-like disease caused by a new human coronavirus in Guangdong Province in southern China (20, 78). In the space of nine months, the virus traveled to 29 countries, infected more than 8000 people, and caused nearly 800 deaths (13). The virus spread with alarming speed among health care workers, through casual contacts, and across the globe, causing mass human suffering and huge economic costs. Unconfirmed published reports indicate a virus phylogenetically close to the SARS virus was discovered in samples collected in Chinese food markets from Himalayan palm civits, suggesting a host reservoir for the deadly virus (28). The epidemic subsided by May 2003, presumably consequent of draconian quarantine measures. There is still little clear understanding of the precise mode of transmission, no truly effective diagnostic test, no vaccine, and no efficacious treatment for SARS.

The SARS outbreak caught many by surprise because human coronaviruses are well known as the cause of a third of common colds but are rarely deadly. Virologists who study domestic animals are familiar with coronaviruses, which occur in livestock, dogs, cats, and poultry, though seldom cause fatal diseases

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(34). But there were exceptions. For example, in pigs, a single SNP variant of porcine coronavirus led to virulent pathogenic enteric coronavirus (5, 87). The second exception involved a devastating feline coronavirus outbreak in cheetahs documented in a drive-through wild animal park in Winston, Oregon (30, 70).

Wildlife Safari had grown by the early 1980s to be the most prolific cheetah-breeding facility in the world, holding some 60 cheetahs. In May 1982, two young cheetahs arrived from the Sacramento Zoo and rapidly developed fever, severe diarrhea, jaundice, and neurological spasms. Both died and were diagnosed at autopsy with the wet form of feline infectious peritonitis (FIP), a disease caused by a feline coronavirus in domestic cats (30). Within six months, every cheetah in the park developed antibodies to FIP virus (FIPV), diarrhea, jaundice, weight loss, gingivitis, and renal and hepatic pathology. Within two years, 60% of the cheetahs died of FIP (30, 70). To our knowledge, this was the worst outbreak of FIPV in any cat species. In reported domestic cat outbreaks, mortality seldom exceeds 5%; for cheetahs, it was 60% fatal.

The Winston FIP outbreak preceded PCR and advanced phylogenetic methods, but when SARS appeared in 2003, we revisited archival specimens from that event to characterize the nature of the cheetah coronavirus (76). Sequence analysis of three viral genes was obtained from five cheetahs, and they all fell near or within the group of domestic cat FIPV sequences (Figure 5). We detected no differences in a genetic sampling between the deadly cheetah virus and the more innocuous domestic cat virus. The reason for the extremely high morbidity and mortality in cheetahs, we suggested, was the genetic homogeneity of the cheetah's genome, or more precisely, within the usually diverse genes of the immune system. Population genetic diversity provides a broad moving target for evolving pathogens, so when a microbe evolves a strategy to abrogate immune defenses of an individual, the genetically diverse population may still be protected. For cheetahs, once virulence was achieved in the first victim, the conditions for transmission, pathogenesis, and morbidity in the immunologically indistinguishable other cheetahs were set.

The parallels and lessons for SARS were multiple. First, the deadly coronaviruses were introduced to cheetahs from domestic cat reservoirs and to people from palm civit cats. Second, in cheetahs and humans, the coronavirus is highly contagious, spreading rapidly in close quarters in weeks, if not days. Third, although mortality in humans with SARS symptoms and in house cats with FIPV was low (10% in humans, 5% in cats), cheetahs with the FIP virus exhibited the opposite extreme, with 90% morbidity and more than 60% mortality. Fourth, vertebrate coronavirus design is efficient in pathogenesis, catholic in species tropism, and frequently deadly. Fifth, genetics matters for the virus and for the host. A few mutational steps in the virus can alter pathogenicity appreciably (5, 87) and, as for cheetahs, genetic uniformity makes epidemics much worse. It seems that immune defense variation in people and domestic cats protected them from a deadly disease, to which most exposed and genetically impoverished cheetahs succumbed.

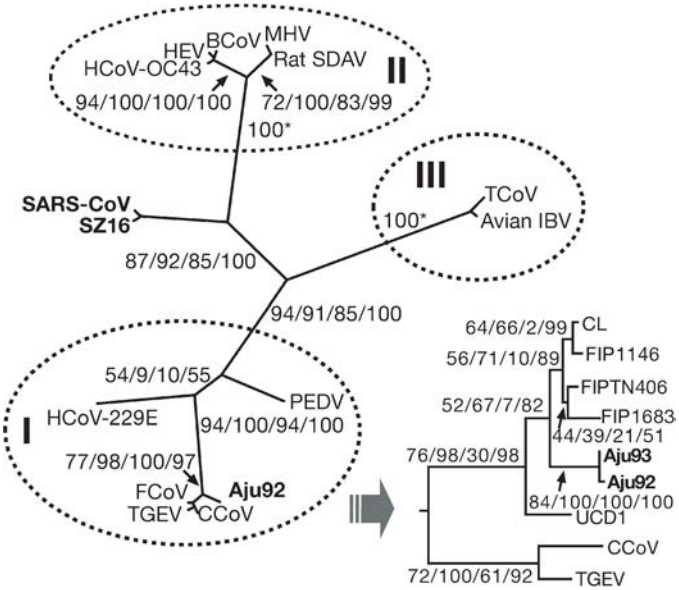

Figure 5 Phylogenetic tree of human and domestic animal coronavirus sequences (*pol lb* gene), including the Aju-CoV from cheetahs involved in the 1982 feline infectious peritonitis (FIP) outbreak in Oregon (76). Numbers plotted along the branches indicate bootstrap values and Bayesian posterior probabilities shown as percentages depicted in the following order, ML/MP/ME/Bayesian. The three major coronavirus antigenic groups (34) are indicated by hatched circles and roman numerals. Abbreviations are as follows: human conroavirus 229E (HcoV-229E), canine coronavirus (CcoV), feline coronavirus (FcoV), porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), human coronavirus OC43 (HcoV-OC43), bovine coronavirus (BcoV), porcine hemagglutinating encephalomyelitis virus (HEV), rat sialodacryoadenitis (SDAV), mouse hepatitis virus (MHV), turkey coronavirus (TcoV), and avian infectious bronchitis virus (avian IBV). Inset: CL FIP1146, FIPTN406, FIP11683, UCD1–4, Dahlberg, and Welcome are feline CoV strains. Aju-92–93 represents sequences from cheetahs that died in the 1982 outbreak in Winston, Oregon (30, 70).

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CONCLUSIONS

The examples we cite in this review represent the beginning of genomic archeology and genomic prospecting in the big cats. In many ways, we are only now beginning to explore and interpret the footprints of defining events in a species' natural history. The exercise for geneticists is reminiscent of the first paleontologists who discovered an unfamiliar molar in a fossil bed. With time, they gained confidence

to reconstruct the animal that left the dentition by comparative inference among living relatives of the long-extinct species. As precision and interpretation improve, we will undoubtedly achieve a more focused glimpse of the potential of genetic interpretation.

The big cat examples featured here offer a sampling of the power that genomic technology brings to wildlife. In Table 1, we feature early applications that have influenced species and subspecies recognition, legislative protection, behavioral insight, and biomedical assessment. The human, mouse, and domestic cat genome projects serve as models, providing tools and empirical inference to reveal the background of wild species. The challenge for the future is to apply the genetic tools and computational algorithms to the most basic puzzles in biology, the lynchpins for survival and adaptation among mammals.

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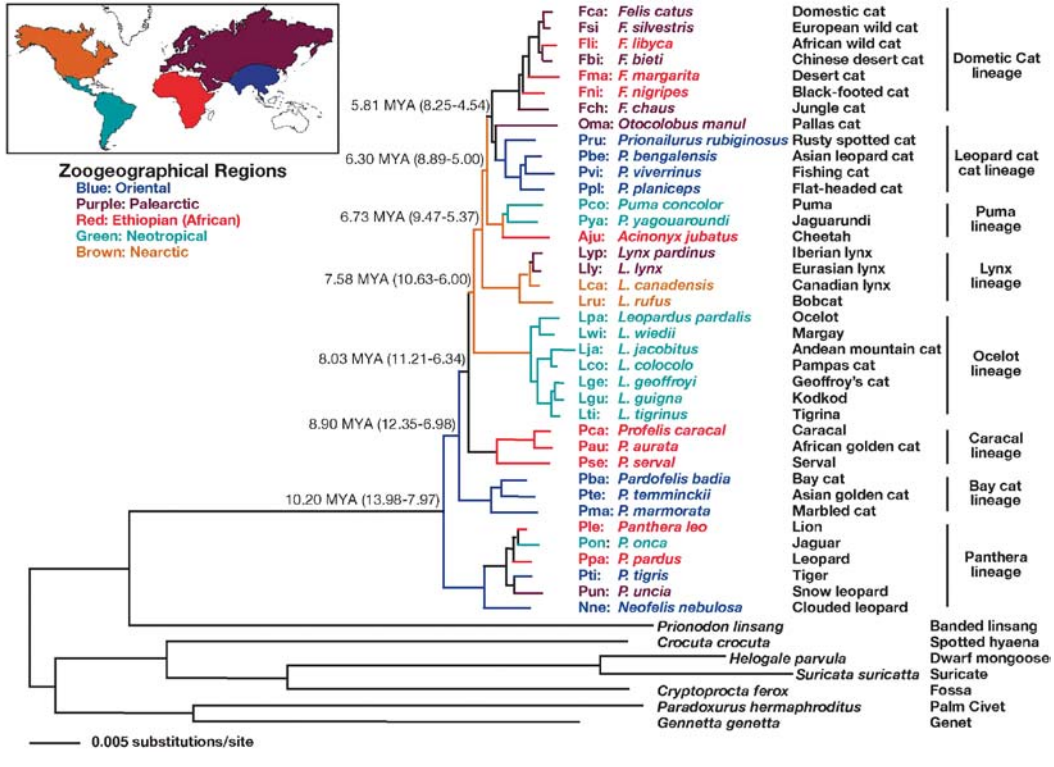

Figure 1 Phylogenetic relationships among 37 Felidae species and 7 outgroup taxa based on a maximum likelihood tree derived from 22,789 base pairs (bp) (37). Terminal species are labeled with three-letter codes, scientific name, and common name. Species are grouped into eight major lineages. The scientific names and branches are color coded to depict recent and historic associations with biogeography regions (Oriental, Palearctic, Ethiopian, Neotropical, Nearctic) through the course of Felidae radiation, as inferred from current distributions, fossil records, and phylogenetic analysis (37).

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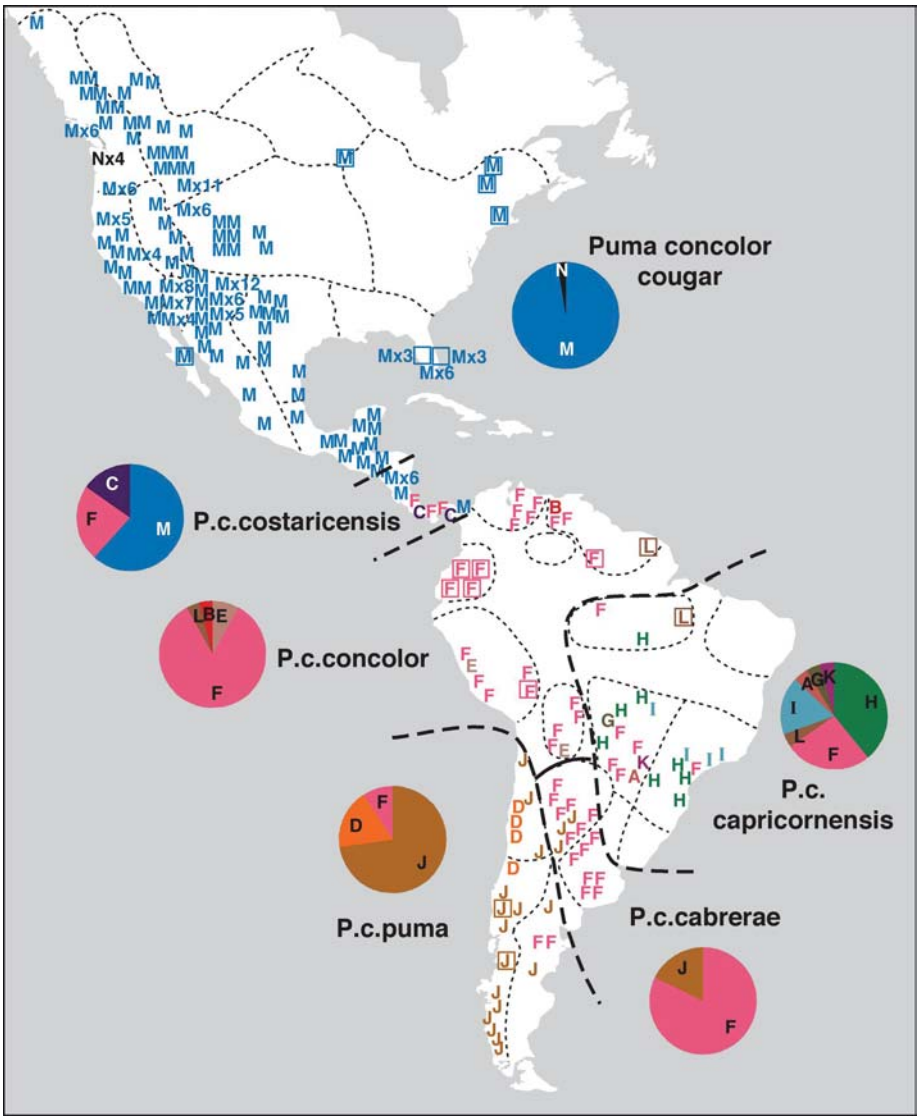

Figure 2 Range map and geographic partitions of six validated puma subspecies as defined by analysis of mitochondrial DNA (mtDNA) and microsatellites (16). Letters indicate captive location and mtDNA haplotype of individuals sampled throughout the range. Pie charts reflect mtDNA haplotype frequency for each subspecies; note the relative genetic uniformity of North American puma populations (see text).

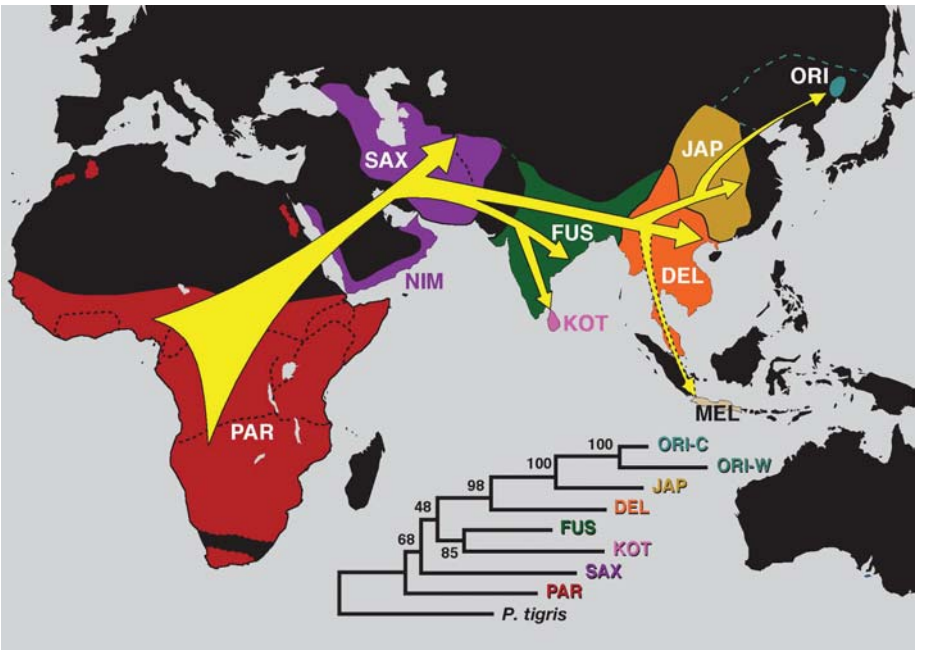

Figure 3 Range of modern leopard subspecies revealed by mitochrondrial DNA (mtDNA) and microsatellite phylogenetic analyses. The subspecies indicated by separate colors are *Panthera pardus pardus* (PAR), *P.p. nimr* (NIM), *P.p. saxicolor* (SAX), *P.p. fusca* (FUS), *P.p. kotiya* (KOT), *P.p. melas* (MEL), *P.p. delacouri* (DEL), *P.p. japonensis* (JAP), and *P.p. orientalis* (ORI). Arrows indicate imputed historic migration out of Africa and across east Asia. The width of the arrows corresponds to relative genomic diversity of mtDNA and microsatellite loci. Topology of subspecies shows an mtDNA phylogenetic hierarchy consistent with geography and postulated migration events (96, 97).

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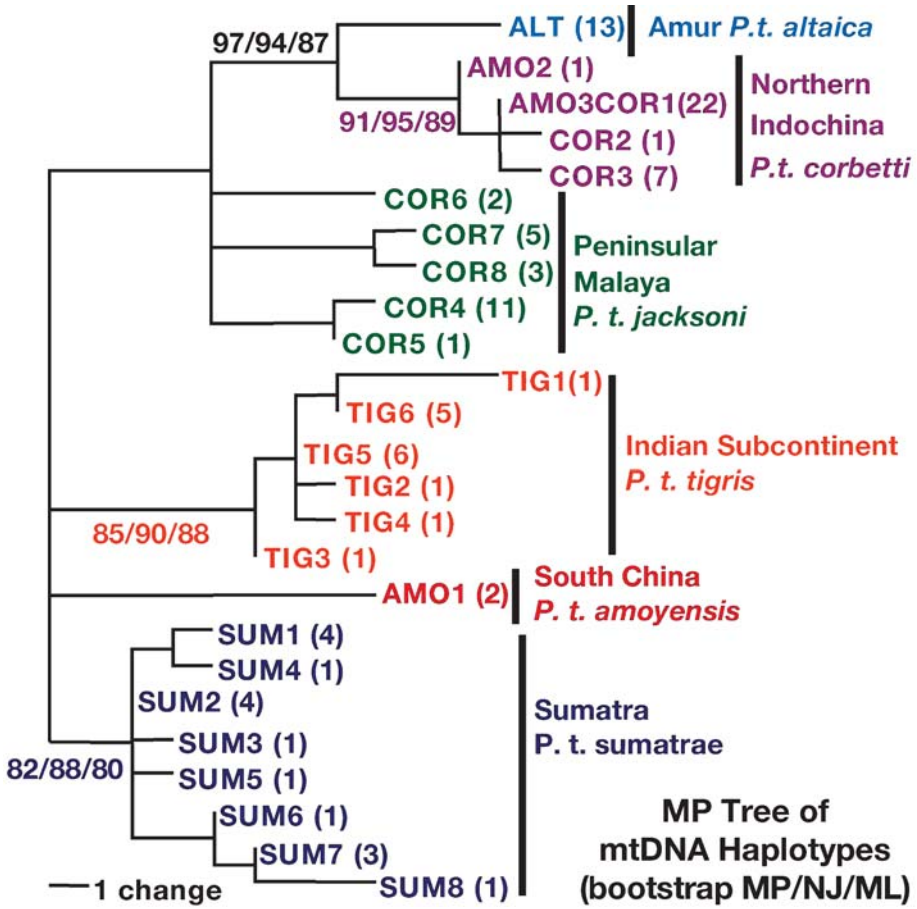

Figure 4 Phylogenetic relationships based on maximum parsimony (MP) among the tiger mtDNA haplotypes from 4078 bp mitochondrial DNA (mtDNA) sequence (45). Branches of the same color represent haplotypes of the same subspecies. Numbers below branches represent bootstrap support from 100 replicates using the MP method, followed by bootstrap values using the ME/ML analyses (only those over 70% are indicated). Numbers in parentheses represent number of individuals sharing the same haplotype. Remarkably, there was no overlap in mtDNA haplotype occurrence among subspecies (45).

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