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Seroprevalence and Genomic Divergence of Circulating Strains of Feline Immunodeficiency Virus among *Felidae* and *Hyaenidae* Species†

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Feline immunodeficiency virus (FIV) infects numerous wild and domestic feline species and is closely related to human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). Species-specific strains of FIV have been described for domestic cat (*Felis catus*), puma (*Puma concolor*), lion (*Panthera leo*), leopard (*Panthera pardus*), and Pallas' cat (*Otocolobus manul*). Here, we employ a three-antigen Western blot screening (domestic cat, puma, and lion FIV antigens) and PCR analysis to survey worldwide prevalence, distribution, and genomic differentiation of FIV based on 3,055 specimens from 35 *Felidae* and 3 *Hyaenidae* species. Although FIV infects a wide variety of host species, it is confirmed to be endemic in free-ranging populations of nine *Felidae* and one *Hyaenidae* species. These include the large African carnivores (lion, leopard, cheetah, and spotted hyena), where FIV is widely distributed in multiple populations; most of the South American felids (puma, jaguar, ocelot, margay, Geoffroy's cat, and tigrina), which maintain a lower FIV-positive level throughout their range; and two Asian species, the Pallas' cat, which has a species-specific strain of FIV, and the leopard cat, which has a domestic cat FIV strain in one population. Phylogenetic analysis of FIV proviral sequence demonstrates that most species for which FIV is endemic harbor monophyletic, genetically distinct species-specific FIV strains, suggesting that FIV transfer between cat species has occurred in the past but is quite infrequent today.

Feline immunodeficiency virus (FIV) is a lentivirus that infects both wild and domestic felid species and is closely related to human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) (9, 11, 21, 26). Species-specific strains of FIV have been described for domestic cat (*Felis catus*), puma (*Puma concolor*), lion (*Panthera leo*), leopard (*Panthera pardus*), and Pallas' cat (*Otocolobus manul*) (termed FIV-Fca, FIV-Pco, FIV-Ple, FIV-Ppa, and FIV-Oma, respectively) (3, 8, 10, 11, 17, 22, 29). In domestic cats, viral infection results in an AIDS-like pathology typified by a period of latency followed by CD4 depletion, immune suppression, and a host of subsequent secondary infections (37). Because of its striking similarity to the pattern of disease progression observed with HIV infection in humans, FIV-Fca offers a promising model system for understanding many clinical aspects of retroviral immunodeficiency syndrome (37). Variant strains of FIV have been found with several of the nondomestic species of the cat family, *Felidae*, yet there has been no verifiable disease association for any of these felid species to date (3, 8–10, 17, 20, 24). Whether nondomestic cat FIV strains are less virulent, less pathogenic, or, alternatively, spreading in species with genetic adaptations that confer efficient immunological defenses is unclear. Nonetheless, the widespread FIV occurrence among *Felidae* species provides some productive epidemiological and evolutionary parallels to human and simian lentiviruses.

Serological (Western blot) evidence of FIV infection has so far been documented for 18 of 37 nondomestic feline species worldwide, and FIV strains infecting 4 of these species have been at least partially sequenced and characterized in molecular terms (3, 6, 7, 9, 10, 14, 18, 19, 20, 21, 23, 38). However, population-wide genetic data are available for only two nondomestic species, the puma (5, 10) and the lion (8, 35), and much of the previous evidence for infection in non-African species comes from captive animal sera that cross-react with

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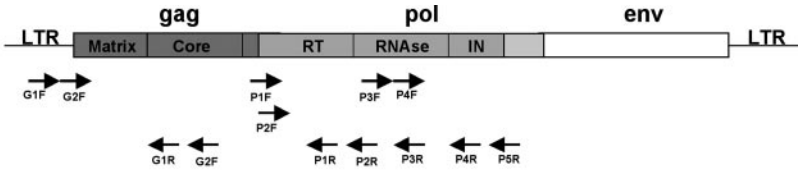

primer	sequence	5′ position
G1F	AGTKGGCGCSMGAACWSGWCYTGA	355
G2F	AATGTAWCTACAGGACGAGAA	754
P1F	TGGCCWYTAWCWAATGAAAARATWGAAGC	2407
P2F	TGAAAARATWGAAGCHTTAACAGAMATAG	2421
P3F	TAGAYTGGTHCCWGGWCATAAA	3931
P4F	GRGARGAYGCAGGATATGA	4030
G1R	GCCATRTCWGTWGRTGTTAA	1125
G2R	TCCTARYCCWTCTCTWGCYTTYTCCA	1187
P1R	GTAATTTRTCTTCHGGNGTYTCAAATCCCC	2997
P2R	GTATTYTCTGCYTTTTTCTTYTGTCTA	3427
P3R	TTRSGCSADYARRTCATATCCTGC	4061
P4R	ATTSKAAARTTTGGKCCATTATC	4760
P5R	TGGTCCATTATCWGWTTGTA	4767

1st round primers	2nd round primers	gene region	product size
GIF-GIR	none	LTR/GAG	770 bp
GIF-G2R	G2F-G1R	GAG	444 bp
P1F-P2R	P2F-P1R	POL-RT	576 bp
P3F-P3R	none	POL-RNASE	118 bp
P3F-P5R	P4F-P4R	POL-RNASE	730 bp

FIG. 1. A schematic representing the positions of the primers used for PCR confirmation on the FIV genome. The "5′ position" reflects the position with respect to FIV-Fca Petaluma strain (accession number 323933). Single round and nested primer sets that produced FIV sequence from at least one animal are described. LTR, long terminal repeat; RT, reverse transcriptase; IN, integrase.

domestic cat FIV-Fca, which is genetically and antigenically divergent from exotic cat strains of FIV (3, 8, 10).

Here, we employ a three-antigen Western blot screening (cat, puma, and lion FIV antigens) and a multigene PCR amplification of FIV genes to estimate worldwide prevalence, distribution, and genomic differentiation of FIV strains among the *Felidae* family and its closest relative, the *Hyaenidae*. The maximal sensitivity of the three-target immunological screening combined with the specificity of the PCR detection methods provides a comprehensive epidemiological and phylogenetic view of the present disposition and ancestral history of FIV.

We conclude from this survey that while FIV infects a wide variety of host species, it is currently endemic in few free-ranging populations, including (i) the large African carnivores, where FIV is widely distributed in multiple populations; (ii) most of the South American felids, which maintain a lower FIV-positive level throughout their range; and (iii) two Asian species, Pallas' cat, which has a species-specific strain of FIV, and Leopard cat, which has been shown to be susceptible to domestic cat FIV (20). Monophyly of FIV proviral sequence for most species suggests that FIV transfer between cat species is an infrequent event.

MATERIALS AND METHODS

Samples for seroprevalence and DNA analysis. Blood samples were collected from free-ranging and captive animals from around the world. Blood separation via centrifugation was performed either on site or upon shipment to the Laboratory of Genomic Diversity, NCI-Frederick. Blood products (serum, plasma, white blood cells, blood clots) were frozen at −70°C until use. Serum or plasma samples were used directly for Western blot analysis. DNA was extracted from whole blood or pelleted white blood cells.

Western blot analysis of serum and plasma samples. A three-antigen chemiluminescence Western blot screening was performed with 1,195 serum or plasma samples as follows. Viral isolates from domestic cats, pumas, and lions were grown in cell culture, and viral proteins were isolated as previously described (36). Briefly, tissue culture supernatant from virus-infected cells was centrifuged at 1,000 rpm in a Beckman GPR centrifuge for 10 min at 5°C to remove cellular material. The supernatant was then centrifuged at 27,000 rpm at 4°C for 2 h in a Beckman L-70 ultracentrifuge using an SW28 rotor. The supernatant was discarded, and the pellet was resuspended in a volume of one-twentieth of the original culture supernatant. The protein content was assayed using a Bio-Rad protein assay.

Twenty milligrams of viral protein was run on a 4 to 20% Tris-glycine gel and transferred to a polyvinylidene difluoride (Bio-Rad) membrane in a Novex Mini-Cell unit as suggested by manufacturer (Invitrogen). Four-millimeter-wide strips were exposed overnight to a 1:200 dilution of serum or plasma with 5% powdered milk in Tween buffer (0.2 M Tris, 0.1 M NaCl, 0.1% Tween 20 [pH 8.0]). A series of six 10-min washes were performed with Tween buffer and then samples were labeled with goat anti-cat horseradish peroxidase-conjugated antibody (KPL laboratories) at a 1:2,000 dilution in 5% powdered milk in Tween buffer. Six 10-min washes were performed, and strips were incubated in ECL Western blotting detection reagents (Amersham Biosciences) for 2 min and then exposed to Lumi-Film chemiluminescent detection film (Boehringer Mannheim). Film was visualized and scored manually as positive, indeterminate, or negative based on the presence and intensity of antibody binding to the p24 *gag* capsid protein. An additional 1,870 samples were screened for FIV-reactive antibodies as previously described (7).

PCR amplification of proviral DNA. Genomic DNA was isolated from white blood cells of 435 seropositive (273), seroindeterminate (108), or seronegative (54) animals using Proteinase K digestion followed by a standard phenol-chloroform extraction. Nested and nonnested PCR using primers designed from the conserved reverse transcriptase region of *pol*, the RNase region of *pol*, and the p26 region of *gag* were used to amplify the FIV sequences (Fig. 1). All primers were designed from the GenBank sequences of FIV-Fca (accession number M25381 and U11820), FIV-Pco (accession number U03982), and FIV-Oma (accession number U56928). All first-round PCRs were performed using 100 ng of genomic DNA in a 50-μl reaction with 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.25 mM (each) of dATP, dCTP, dGTP, and dTTP, 2 mM of each primer, and 2 units of AmpliTaq DNA polymerase from PE Applied Biosystems. PCR cycling conditions were as follows: 3 min at 94°C; 45 cycles of 15 s at 94°, 30 s at 52°C or 50°C, and 45 s at 72°C; and a final extension of 10 min at 72°C. Second-round PCRs were done under the same conditions, with 1 μl of product from the first-round reaction used as template. These amplifications were performed with a Perkin-Elmer 9700 thermocycler and visualized with a 1% agarose gel. Samples were also run with a touchdown protocol with temperatures from 58°C to 48°C. All other cycling conditions were the same as those described above. PCR products were sequenced using standard ABI BigDye terminator reactions.

Phylogenetic analysis. Representative nucleotide species for each species from *pol-RT* were compiled and aligned for subsequent phylogenetic analysis using ClustalX (34) and verified visually. Phylogenetic reconstructions were performed using PAUP version 4.0 (32) and included minimum evolution estimated by neighbor joining (ME), maximum parsimony (MP), and maximum likelihood (ML). ModelTest (28) was used to estimate the optimal model of sequence

TABLE 1. FIV cross-reactive anibodies detected in captive, wild-born, and free-ranging feline and carnivore species and PCR verification of viral infection[a]

WB and PCR result	FIV strain	Animal	Genus and species	Result of three-antigen screening			Combined result[b]			PCR verification result[c]		
				3 positive	1 or 2 positive	total	positive	indeterminate	total	WB pos	WB ind	WB neg
WB pos/PCR pos	Ple	Lion (African)	*Panthera leo*	196	45	428	502	68	770	156/196	12/23	0/22
	Ccr	Spotted hyena	*Crocuta crocuta*	7	15	51	7	15	51	4/5	4/9	0/2
	Ppa	Leopard (African)	*Panthera pardus*	4	6	17	9	8	117	4/5	3/5	0/1
	Pco	Puma	*Panthera concolor*	18	33	124	94	57	443	4/15	2/8	0/6
	Hya	Jaguaroundi	*Herpailurus yagouaroundi*	0	0	3	6	3	36	1/5	0/3	–
	Lpa	Ocelot	*Leopardus pardalis*	0	1	4	6	6	90	2/6	0/5	0/3
	Lwi	Margay	*Leopardus wiedii*	0	1	4	4	1	65	2/3	–	0/1
	Aju	Cheetah	*Acinonyx jubatus*	2	2	62	17	10	474	6/7	1/1	0/2
	Oma	Pallas' cat	*Otocolobus manul*	7	11	26	8	11	27	7/8***	1/1	–
	Pti	Tiger	*Panthera tigris*	9	24	124	9	24	212	1/5**	0/15	0/1
	Pun	Snow leopard	*Panthera uncia*	0	0	4	3	0	72	1/2*	–	–
WB pos/PCR neg (or not done)	Hhy	Striped hyena	*Hyeana hyeana*	4	4	17	4	4	17	0/2	0/3	0/2
	Pon	Jaguar	*Panthera onca*	0	0	10	7	2	40	0/6	0/2	0/1
	Oge	Geoffroy's cat	*Oncefelis geoffroyi*	0	5	9	2	7	51	0/2	0/5	0/1
	Lti	Tigrina (tiger cat)	*Leopardus tigrinus*	0	1	7	2	1	39	0/2	–	0/1
	Fma	Sand cat	*Felis margarita*	1	5	7	1	5	13	0/1	0/3	0/2
	Fli	African wild cat	*Felis libyca*	0	0	12	1	0	16	0/1	–	–
	Ipl	Flat-headed cat	*Ictailurus planiceps*	0	0	2	1	0	11	–	–	–
	Ppa	Leopard (Asian)	*Panthera pardus*	0	0	13	1	0	66	–	–	–
WB ind/PCR neg (or not done)	Fni	Black-footed cat	*Felis nigripes*	0	3	11	0	3	11	–	0/3	0/1
	Pma	Marbled cat	*Pardofelis marmorata*	0	2	8	0	2	10	–	0/2	0/1
	Fch	Jungle cat	*Felis chaus*	0	5	11	0	5	17	–	0/4	0/2
	Lru	Bobcat	*Lynx rufus*	0	4	19	0	6	44	–	0/4	0/1
	Lyp	Iberian (Spanish) lynx	*Lynx pardinus*	0	7	48	0	7	48	–	0/7	0/1
	Fsi	European wild cat	*Felis silvestris*	0	4	8	0	4	16	–	0/3	–
	Nne	Clouded leopard	*Neofelis nebulose*	0	1	36	0	4	59	–	0/2	–
	Pte	Asian golden cat	*Profelis temminicki*	0	3	21	0	3	29	–	0/1	0/1
	Pvi	Fishing cat	*Prionailurus viverrina*	0	0	18	0	1	25	–	0/1	0/1
	Lca	Canadian lynx	*Lynx canadensis*	0	1	2	0	1	2	–	–	–
	Lco	Pampas cat	*Lynchailurus colocolo*	0	1	8	0	1	12	–	–	–
	Ple	Lion (Asian)	*Panthera leo*	0	0	3	0	1	52	–	–	–
WB neg/PCR not done	Pba	Bay cat	*Pardofelis badia*	0	0	1	0	0	1	–	–	–
	Pru	Rusty spotted cat	*Prionailurus rubiginosa*	–	–	–	0	0	1	–	–	–
	Pau	African golden cat	*Profelis aurata*	0	0	2	0	0	2	–	–	–
	Hbr	Brown hyena	*Hyaena brunnea*	0	0	2	0	0	2	–	–	–
	Ogu	Kodkod	*Oncifelis guigna*	0	0	2	0	0	2	–	–	–
	Lse	Serval	*Leptailurus serval*	–	–	–	0	0	2	–	–	–
	Lly	Lynx	*Lynx lynx*	0	0	7	0	0	7	–	–	–
	Cca	Caracal	*Caracal caracal*	0	0	22	0	0	22	–	–	–
	Pbe	Leopard cat	*Prionailurus bengalensis*	0	0	22	0	0	81	–	–	–
Total				248	184	1195	684	260	3055	188/273	23/108	0/54

[a] –, not done; *, amplified from wild-born zoo animals; **, amplified from captive-born zoo animals; ***, amplified from some wild-born and some captive-born zoo animals; WB, Western blot; pos, positive; neg, negative; ind, indeterminate.

[b] Results from the three antigen Western blot screen are combined with results from FIV-Fca only Western blots; positive is a strong positive signal against one or more antigen(s).

[c] Number of individuals from which FIV sequence was obtained/number of individuals for which PCR was attempted.

evolution, the general time-reversible model of substitution corrected for among-site rate variation (gamma distribution), and a proportion of invariant sites. ME and ML trees were constructed using this model (see legend for Fig. 4a), with starting trees obtained by neighbor joining followed by a tree bisection-reconnection branch swapping algorithm during a heuristic search for the optimal tree. Maximum parsimony analysis employed a heuristic search of starting trees obtained by stepwise addition followed by tree bisection-reconnection branch swapping. Bootstrap analysis included 1,000 iterations for ME and MP and 100 iterations for ML. In addition, Bayesian posterior probabilities were calculated using a Markov chain Monte Carlo sampling approach with MrBayes 3.0b4 (16). Starting trees were random, four simultaneous Markov chains were run for one million generations, burn-in values were set at 45,000 generations, and trees were sampled every 20 generations.

Additional analyses were conducted on the *pol* gene sequences translated into amino acids. Phylogenetic trees were derived from the amino acid residue data using two algorithms of distance-based and maximum likelihood. The genetic distance measure of the PAM-Dayhoff model of amino acid substitution (12) was used to construct a neighbor-joining tree using the PHYLIP program, version 3.5 (13). Bootstrap analyses consisting of 1,000 repetitions were conducted for the distance-based analysis using PHYLIP. The maximum likelihood analyses of amino acids and relative bootstrap values used the PAM-Dayhoff model of substitution (12) as implemented by the ProtML subroutine of the MOLPHY program, version 2.3 (1).

Nucleotide sequence accession numbers. The novel nucleotide sequences in this data set have been deposited in the GenBank database under the following accession numbers: for FIV-Ple, accession numbers AY878208 to AY878235; for FIV-Ccr, accession numbers AY878196 to AY878200; for FIV-Aju, accession numbers AY878201 to AY878203; for FIV-Ppa, accession numbers AY878204 to AY878207; for FIV-Pco, accession numbers AY878236 to AY878237; for FIV-Oma, accession numbers AY878238 to AY878241; for FIV-Lpa, accession number AY878194; for FIV-Hya, accession number AY878195; and for FIV-Pun, accession number AY878242. Additional nucleotide sequences used in the phylogenetic analyses were obtained using BLAST (2) and BLAST 2 sequences (33) and have the following accession numbers: U53727 (Pco-163), U53722 (Pco-144), U53729 (Pco-245), U53718 (Pco-117), U53755 (Pco-733), U53751 (Pco-590), U53725 (Pco-145), U53756 (Pco-28), U53748 (Pco-336), M66437 (SIVagm), AY159322 (SIVmnd), AY159321 (SIVdrl), AF447763 (SIVcpz), AF334679 (SIVsm), AF301156 (SIVcol), AF131870 (SIVsu), AF075269 (SIVlhoest), AB100245 (HIV type 2 [HIV-2]), AF004885 (HIV-1 subtype A [HIV-1 A]), K03455 (HIV-1 B), U52953 (HIV-1 C), K03454 (HIV-1 D), AF077336 (HIV-1 F), AF084936 (HIV-1 G), AF190127 (HIV-1 H), AF082394 (HIV-1 J), AJ249235 (HIV-1 K), AJ271370 (HIV-1 N), L20587 (HIV-1 O), AF24739 (equine infectious anemia virus), M32690 (bovine immunodeficiency virus), AF479638 (ovine), AY101611 (visna), and M33677 (caprine arthritis-encephalitis virus).

RESULTS

In this study, 1,195 animals from 38 species were tested by Western blotting (Table 1; Fig. 2) for the presence of antibodies that would react to a panel of three different FIV virus isolates, FIV-Fca from the domestic cat, FIV-Ple from the lion, and FIV-Pco from the puma. The three viruses, FIV-Fca, FIV-Ple, and FIV-Pco, are genetically distant from each other and have the potential to detect antibody responses to a wide variety of viral epitopes (9). Thus, a virus closely related to FIV-Pco might be more likely to elicit an antibody response that was detectable with FIV-Pco antigen than with FIV-Fca antigen. Samples from 248 animals from 9 species showed antibody reactions to all three viral antigens, samples from an additional 184 animals from 22 species demonstrated antibody responses to only one or two of the FIV viruses, and samples from 753 animals were seronegative (Table 1).

When tested directly, species-specific viruses (i.e., FIV-Ple for lion samples and FIV-Pco for puma samples) were more sensitive than either of the nonspecific viruses (species specificity is demonstrated for lion samples tested against FIV-Ple, FIV-Pco, and FIV-Fca in Fig. 2). Of the 45 lion sera that showed FIV cross-reactive antibodies to only one or two of the three viruses, 26 reacted to FIV-Ple only, 12 reacted to FIV-Ple plus one other virus, and 6 reacted to FIV-Pco or FIV-Fca and not to FIV-Ple. Similar results, where homologous FIV antigens were most effective at detecting antibody response, were seen with pumas. Of the 33 partial positive puma samples, 11 were reactive to FIV-Pco only, 17 to FIV-Pco plus one other virus, and only 5 to FIV-Ple or FIV-Fca but not to FIV-Pco.

While the use of FIV-Pco, FIV-Ple, and FIV-Fca antigens in the Western blot screening resulted in many more samples with FIV cross-reactive antibodies than one virus alone, the significance of these partial positives is, in many cases, unclear. Samples that produced strong positive results against one FIV strain usually reacted equally well to the other two strains. Therefore, many of the partial positives were also relatively weak signals, resulting in an "indeterminate" designation. Indeterminate signals were often, though not always, negative by PCR screenings (Table 1), suggesting that many were false positives, although some may be positive for genetically divergent (from the PCR primers) variants.

A summation of results from the three-antigen Western blot screening using combined captive-born, wild-born, and/or free-ranging animals from 36 species is presented in Table 1. Twelve of the tested species, namely, the African golden cat, the black footed cat, three hyena species (brown, spotted, and striped), the caracal, the sand cat, three lynx species (Canadian, European, and Iberian), the kodkod, and the bay cat, are unrepresented in the FIV literature. Spotted and striped hyenas were the only newly tested species with convincing FIV antibody responses, although samples sizes for most of these species were too small for any negative conclusion. Indeterminate FIV antibody reactions were seen for nine species where no FIV reactivity has previously been reported; these were the black footed cat, sand cat, Canadian lynx, Iberian lynx, Pampas cat, Asian golden cat, European wild cat, jungle cat, and marbled cat.

The three-antigen FIV Western blot results were compared with previously published and new data from Western blotting using a single viral antigen, FIV-Fca (Table 1, combined results columns) (6–11, 21). In populations that were screened with the three-antigen Western blot screening and by FIV-Fca alone, slightly more FIV-reactive samples were seen with the three-antigen screening. However, strong seropositive results were highly concordant for the three- and one-antigen screenings. For example, the cheetahs in the Serengeti had 22% strong positives with the three-antigen screening ($n = 9$) and 24% strong positives with FIV-Fca ($n = 41$). Florida panthers (pumas) had 23% strong positives with three antigens ($n = 30$) and 20% strong positives with FIV-Fca alone ($n = 79$), and Tanzania lions had 83% strong positives with three antigens ($n = 145$) and 86% strong positives with FIV-Fca alone ($n = 269$).

The frequency of weak (indeterminate) reactions increased when the three-antigen screening was used. For example, 22% of Serengeti cheetahs were indeterminate with the three-antigen test (weak signal against at least one antigen) while 5% were indeterminate (weak positive) with FIV-Fca alone; 40% of Florida panthers were indeterminate with three antigens, and 5% were indeterminate with FIV-Fca alone. In contrast,

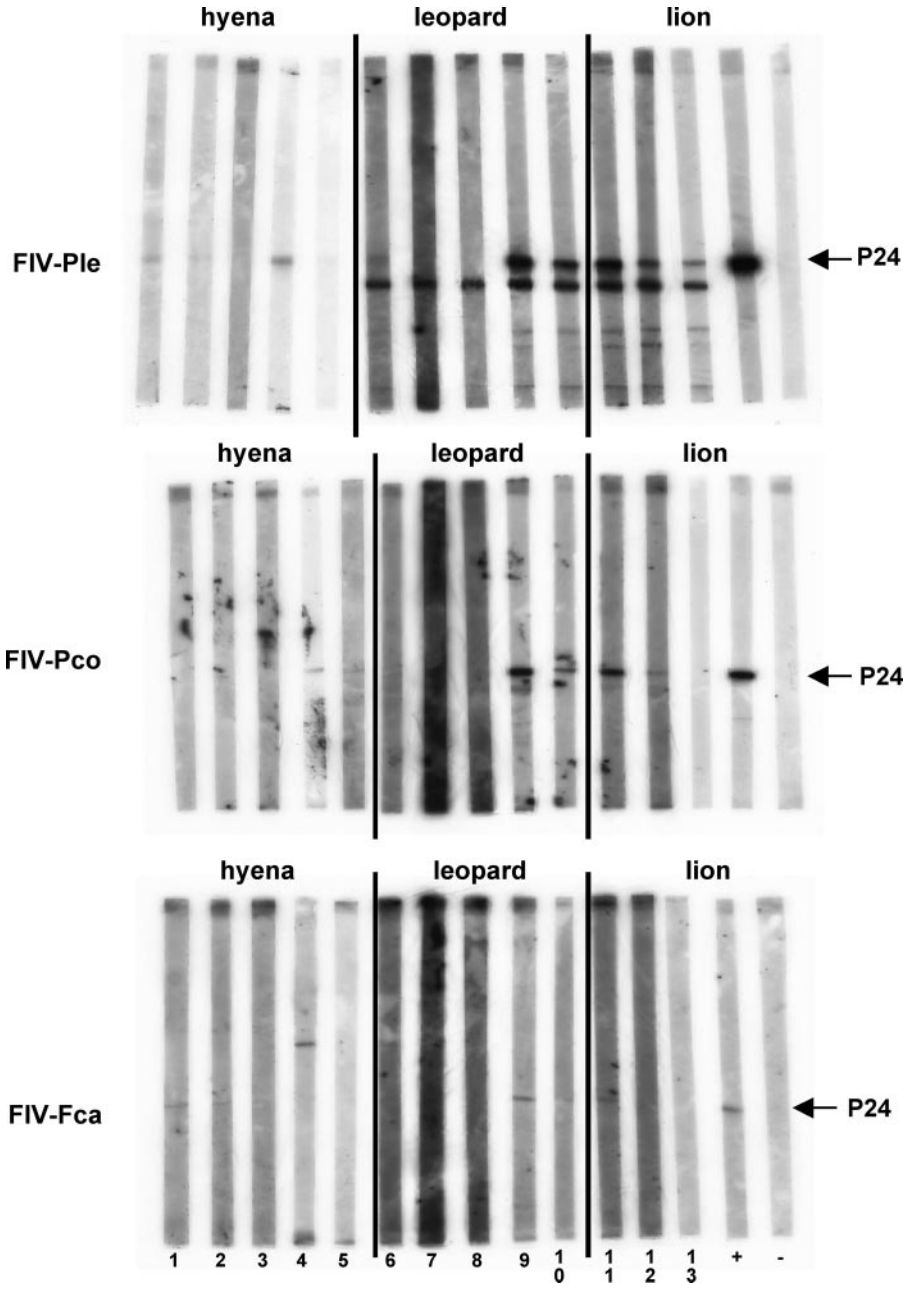

FIG. 2. Western blots showing differential antibody reactivity to the three FIV viral antigens. Lanes 14 and 15 contain serum from a known FIV-positive (Western blotting and PCR) and a known FIV-negative lion, respectively. Lanes 1 to 5 contain serum from seropositive and seroindeterminate hyenas. Lanes 6 to 10 contain serum from leopards that have strong antibody response to FIV-Ple and variable reactions to FIV-Fca and FIV-Pco. Lanes 11 to 13 contain serum from lions that have strong antibody responses to FIV-Ple and weak or negative antibody responses to the other two antigens.

7% of Serengeti lions were indeterminate with three antigens, and 8% were indeterminate with FIV-Fca alone.

Because the results from strong positives are similar with both methods, results from the 1,195 animals screened with all three antigens have been combined with results from the 1,866 animals screened with FIV-Fca alone for a total of 3,055 animals from 38 species (Table 1, combined results columns). Of these, 684 animals were Western blot positive for FIV, and 260 were indeterminate, meaning that between 22% and 31% of the animals tested have antibodies to FIV (Table 1). A majority of the positive results come from lions (502) and pumas (94). However, positive signals were seen for some individuals from 17 other species, and almost all species demonstrated some level of FIV reactivity, suggesting that FIV may be circulating in several additional feline host species. All individual animals of nine of the species screened (bay cat, rusty spotted cat, African golden cat, brown hyena, kodkod, serval, European lynx, caracal, and leopard cat) were negative (Table 1).

However, with the exception of the caracal and leopard cat, there were very few samples from these species.

PCR amplification of lymphocyte-derived DNA from 435 animals representing a subset of seropositive, seroindeterminate, and seronegative individuals (Table 1, PCR verification results) was attempted using several primer combinations within *gag*, *pol-RT*, and *pol-RNase* (Fig. 1) (35). Where available, samples from free-ranging and wild-born individuals were used. The PCR success rate (i.e., the percentage of individual animals from which FIV sequence was obtained for at least one gene region) was 69% (188/273) for seropositive samples and 21% (23/108) for seroindeterminates; all 54 seronegative samples were PCR negative at all gene regions. Most PCR-positive samples did not amplify at all gene regions, and the highest success rate was within the most conserved gene region, *pol-RT*. All products were sequenced to validate their homologies to FIV.

Table 2 presents the Western blot data (positive, indeterminate, and negatives for each species) separated into three categories of specimens, those collected from free-ranging animals, from animals held in captivity but born in the wild, and from captive-born animals. These data demonstrate a much higher incidence of FIV in free-ranging animals (40 to 50%) than in captive ones (7 to 13%). Based on positive Western blot results from free-ranging and wild-born individuals, nine species, namely, the cheetah, leopard, lion, spotted hyena, striped hyena, margay, ocelot, puma, and Pallas' cat, have FIV circulating in the wild. Indeterminate Western blot results are seen for free-ranging and wild-born individuals from an additional five species, namely, bobcat, the Geoffroy's cat, jaguar, Iberian lynx, and tiger. Finally, several species harbor FIV in captivity but not in free-ranging populations; these are the African wild cat, sand cat, flat-headed cat, tiger, and Asian leopard (Table 2). Captive-born animals have been excluded from the remainder of the seroprevalence analyses.

Species with the highest prevalence of FIV-positive animals are found in Africa (Fig. 3a). For the African lion, 68% of the animals are seropositive and an additional 6% are seroindeterminate, giving an overall prevalence of 68 to 74%. FIV is endemic in the leopard (26 to 46%) and in the striped and spotted hyena (23 to 47% and 14 to 24%, respectively). Although the cheetah has very low overall prevalence, 6 to 8%, this result reflects a geographic sampling bias, as FIV is present in high frequency (40%) in some cheetah populations (Tanzania, $n = 17$) and absent (0 to 2%) in others (Namibia, $n = 321$), and the majority of our samples come from the FIV-negative population. In contrast, FIV-reactive antibodies have not been found in the smaller African cats (serval, caracal, and African wild cat) or in the brown hyena, although few (1 to 14) samples from wild-caught animals (Table 2) were available for these species. No samples from wild-born or free-ranging animals were tested from the African golden cat, black footed cat, or sand cat.

In the Americas, FIV is also endemic in several species, although the overall prevalence is generally lower than in positive African species (Fig. 3b). The prevalence is highest in pumas, of which 25% are seropositive and an additional 15% are seroindeterminate. No seroprevalence was observed for the Pampas cat ($n = 10$), and only seroindeterminates were seen with the bobcat (14%; $n = 44$). However, all other species had low seroprevalence, ranging from 5 to 9% for the Geoffroy's cat to 19 to 28% for the jaguarundi (Fig. 3b).

There is a dearth of strong positives from any of the feline species in Asia and Europe, with the exception of the Pallas' cats, where seroprevalence is between 33% and 87% (Fig. 3c). Notably, the lion and the leopard have 0% seroprevalence in Asia, whereas FIV is endemic in their African counterparts. However, FIV seroindeterminates are found in seven species (Asian golden cat, clouded leopard, Iberian lynx, jungle cat, marbled cat, snow leopard, and tiger) at prevalences ranging from 6% for the Asian golden cat to 36% for the tiger.

Seropositive status was verified via PCR (for at least one gene region) from free-ranging cheetahs, leopards, lions, spotted hyenas, and pumas. Seropositive status of one or more wild-born jaguarundis, margays, ocelots, Pallas' cats, and snow leopards and a captive-born tiger were also verified by PCR (Table 1). *Pol-RT* sequences from these animals were aligned with FIV and other lentivirus sequences present in GenBank. Phylogenetic analysis indicates that the divergent FIV strains described here are monophyletic relative to other lentiviruses but align most closely with equine infectious anemia virus and bovine immunodeficiency virus *pol* sequences (Fig. 4a). Further, the genetic differentiation among FIV-RT sequences is comparable to the divergence among SIV-HIV isolates (Fig. 4a). The broad FIV divergence can also be seen in an alignment of amino acid residues among FIV strains, which demonstrates considerable diversity within and between strains, but very few species-specific (diagnostic) residues (Fig. 4b). The majority of changes represent homoplasies (i.e., changes that appear along more than one branch of the phylogeny) and are conservative amino acid changes that probably do not change the function of the molecule. The diagnostic residues, however, tend to be less conservative and may represent changes that are positively selected within a lineage. There is also large among site rate variation, suggesting that some regions of the molecule are under more selective pressure than others.

The lentivirus nucleotide sequences derived from six spotted hyenas (FIV-Ccr) are monophyletic (bootstrap values of 100/100/100% for ML/ME/MP, with a posterior probability of 1 for Bayesian analysis) (Fig. 4a) and specify a sister-taxon association with the multispecies FIV clade with strong ML and ME bootstrap support (bootstrap values of 89/94/65% for ML/ME/MP) (Fig. 4a). In most nucleotide trees, hyena FIV appears basal to the FIVs from the felid species (i.e., first within the FIV lineage to diverge from the common lentiviral ancestor). Within the *Felidae*, there is strong bootstrap support for the monophyly of FIV in domestic cats (FIV-Fca; bootstrap values of 100/100/100% for ML/ME/MP, with a posterior probability of 1 for Bayesian analysis) (Fig. 4a) and Pallas' cats (FIV-Oma; bootstraps values of 100/100/100% for ML/ME/MP, with a posterior probability of 1 for Bayesian analysis) (Fig. 4a), and good bootstrap support for the monophyly of FIV in leopards (FIV-Ppa; bootstrap values of 87/76/89% for ML/ME/MP, with a posterior probability of 1 for Bayesian analysis) (Fig. 4a). The cheetah FIV strain (FIV-Aju) groups with that of the leopards with good support (bootstrap values of 88/91/59% for ML/ME/MP, with a posterior probability of 0.99 for Bayesian analysis) (Fig. 4a). The greatest diversity occurs within the lion FIV (FIV-Ple) and puma FIV (FIV-Pco), and there are two distinct monophyletic groups from each of these species. The FIV

TABLE 2. Number of Western blot positive samples from free-ranging, wild-born, and captive-born animals[a]

WB and PCR result	Animal	Genus and species	Result for animal group indicated									PCR verification result[b]		
			free ranging			wild-born			captive-born					
			WB pos	WB ind	WB neg	WB pos	WB ind	WB neg	WB pos	WB ind	WB neg	WB pos	WB ind	WB neg
WB pos/PCR pos	Lion (African)	*Panthera leo*	484	50	166	2	0	12	31	9	16	156/196	12/23	0/22
	Spotted hyena	*Crocuta crocuta*	7	15	29	–	–	–	–	–	–	4/5	4/9	0/2
	Leopard (African)	*Panthera pardus*	9	7	11	0	0	8	0	1	81	4/5	3/5	0/1
	Puma	*Panthera concolor*	66	38	175	19	12	26	7	7	93	4/15	2/8	0/6
	Jaguaroundi	*Herpailurus yagouaroundi*	–	–	–	6	3	23	0	0	4	1/5	0/3	–
	Ocelot	*Leopardus pardalis*	2	0	12	4	1	49	0	5	17	2/6	0/5	0/3
	Margay	*Leopardus wiedii*	1	0	4	3	0	51	0	1	5	2/3	–	0/1
	Cheetah	*Acinonyx jubatus*	14	7	233	0	1	83	3	2	131	6/7	1/1	0/2
	Pallas' cat	*Otocolobus manul*	3	4	1	2	4	1	3	3	6	7/8***	1/1	–
	Tiger	*Panthera tigris*	0	9	6	0	10	28	9	5	145	1/5**	0/15	0/1
	Snow leopard	*Panthera uncia*	–	–	–	1	0	3	2	0	66	1/2*	–	–
WB pos/PCR neg (or not done)	Striped hyena	*Hyeana hyeana*	4	4	9	–	–	–	–	–	–	0/2	0/3	0/2
	Jaguar	*Panthera onca*	0	2	1	3	0	23	4	0	7	0/6	0/2	0/1
	Geoffroy's cat	*Oncefelis geoffroyi*	0	1	5	2	1	34	0	5	3	0/2	0/5	0/1
	Tigrina (tiger cat)	*Leopardus tigrinus*	–	–	–	2	0	31	0	1	5	0/2	–	0/1
	Sand cat	*Felis margarita*	–	–	–	–	–	–	1	5	7	0/1	0/3	0/2
	African wild cat	*Felis libyca*	–	–	–	0	0	14	1	0	1	0/1	–	–
	Flat-headed cat	*Ictailurus planiceps*	0	0	2	0	0	4	1	0	4	–	–	–
	Leopard (Asian)	*Panthera pardus*	0	0	7	0	0	33	1	0	25	–	–	–
WB ind/PCR neg (or not done)	Black-footed cat	*Felis nigripes*	–	–	–	–	–	–	0	3	8	–	0/3	0/1
	Marbled cat	*Pardofelis marmorata*	–	–	–	0	2	6	0	0	2	–	0/2	0/1
	Jungle cat	*Felis chaus*	–	–	–	0	1	3	0	4	9	–	0/4	0/2
	Bobcat	*Lynx rufus*	0	5	31	0	1	7	–	–	–	–	0/4	0/1
	Iberian (Spanish) lynx	*Lynx pardinus*	0	7	41	–	–	–	–	–	–	–	0/7	0/1
	European wild cat	*Felis silvestris*	0	0	3	0	0	3	0	4	6	–	0/3	–
	Clouded leopard	*Neofelis nebulosa*	–	–	–	0	2	32	0	2	23	–	0/2	–
	Asian golden cat	*Profelis temminicki*	–	–	–	0	1	17	0	2	9	–	0/1	0/1
	Fishing cat	*Prionailurus viverrina*	–	–	–	0	0	5	0	1	19	–	0/1	0/1
	Canadian lynx	*Lynx canadensis*	–	–	–	–	–	–	0	1	1	–	–	–
	Pampas cat	*Lynchailurus colocolo*	–	–	–	0	0	10	0	1	1	–	–	–
	Lion (Asian)	*Panthera leo*	–	–	–	0	0	16	0	1	35	–	–	–
WB neg/PCR not done	Bay cat	*Pardofelis badia*	–	–	–	–	–	–	0	0	1	–	–	–
	Rusty spotted cat	*Prionailurus rubiginosa*	–	–	–	–	–	–	0	0	1	–	–	–
	African golden cat	*Profetis aurata*	–	–	–	–	–	–	0	0	2	–	–	–
	Brown hyena	*Hyaena brunnea*	0	0	2	–	–	–	–	–	–	–	–	–
	Kodkod	*Oncifelis guigna*	–	–	–	–	–	–	0	0	2	–	–	–
	Serval	*Leptailurus serval*	0	0	1	–	–	–	0	0	1	–	–	–
	Lynx	*Lynx lynx*	–	–	–	–	–	–	0	0	7	–	–	–
	Caracal	*Caracal caracal*	–	–	–	0	0	8	0	0	14	–	–	–
	Leopard cat	*Prionailurus bengalensis*	–	–	–	0	0	45	0	0	36	–	–	–
Total			590	149	739	44	39	575	63	63	793	188/273	23/108	0/54

[a] –, not done; *, amplified from wild-born zoo animals; **, amplified from captive-born zoo animals; ***, amplified from some wild-born and some captive-born zoo animals; WB, Western blot; pos, positive; neg, negative; ind, indeterminate.

[b] Number of individuals from which FIV sequence was obtained/number of individuals for which PCR was attempted.

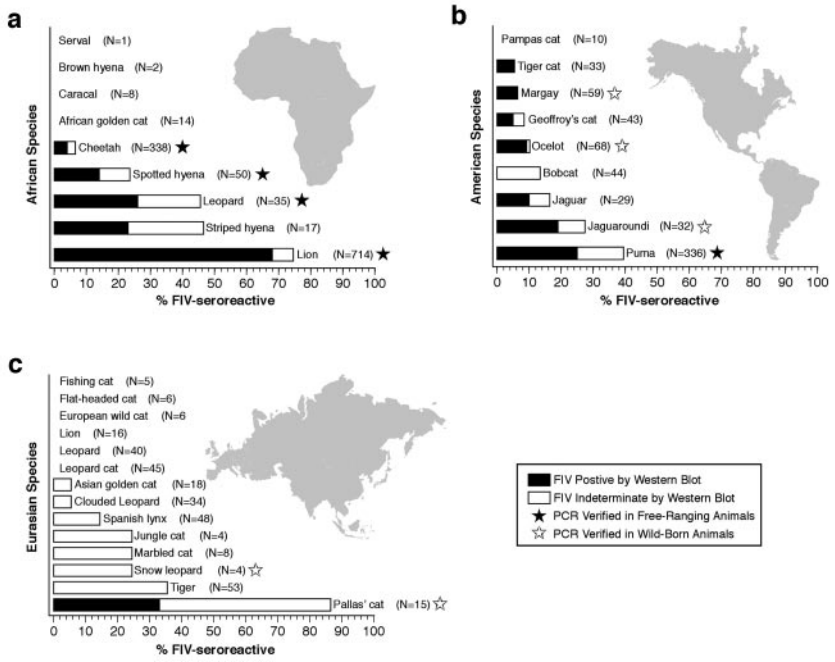

FIG. 3. Prevalence and distribution of FIV seroreactivity in free-ranging and captive but wild-born animals. The dark portion of the bar represents seropositives and the white portion represents seroindeterminates, giving conservative and liberal estimates of total seroprevalence of FIV. The number of animals tested for each species is given in parentheses. A black star indicates that PCR verification has been obtained from at least one free-ranging individual for that species. A white star indicates that PCR verification has been obtained from at least one captive but wild-born individual for that species. (a) Percent seroreactivity in African carnivores (excluding African golden cat, sand cat, and black footed cat, for which there were no wild-born samples). (b) Percent seroreactivity in American felids (excluding kodkod and Canadian lynx, for which there were no wild-born samples). (c) Percent seroreactivity in Eurasian felids (excluding lynx, bay cat, rusty spotted cat, and Chinese desert cat, for which there were no wild-born samples).

sequences obtained from the ocelot and jaguarundi are genetically distant from any other FIV type, and most likely represent species-specific strains.

Within the lions, previously described strains A, B, and C are recapitulated here (8, 35). There is strong support for FIV-Ple subtype A, found in the Serengeti, Kruger Park, Botswana, and captive lions; FIV-Ple subtype B, found in the Serengeti, the Ngorongoro Crater, and Uganda; and FIV-Ple subtype C, found only in the Serengeti. An additional three monophyletic subtypes are seen, one from Kenya, one from Botswana, and one from Kruger Park in South Africa.

These phylogenetic divisions were validated using other gene regions (*pol-RNase* or *gag*) (35) for the FIV-Oma clade, the FIV-Ppa clade, and the six distinct FIV-Ple clades (supplemental online material). The grouping of FIV-Aju with FIV-Ppa is also seen with *gag*, as is the placement of FIV-Oma basal to the FIV-Aju/FIV-Ppa clade. Additionally, the divergence of ocelot FIV from FIV sequences found in other species is confirmed with sequence from a different ocelot in a *pol-RNase* phylogeny (supplemental online material). Short sequences (90 bp) from two margays were identical to each other and confirmed the presence of FIV but were not useful for phylogenetic analysis. A *pol-RT* FIV fragment amplified from a captive but wild-born snow leopard was shown to cluster in the FIV-Ple subtype A sequences (Fig. 4a). Similarly, a 450-bp FIV *gag* sequence amplified from a captive-born tiger grouped with the "Botswana" FIV-Ple clade (supplemental online material).

Finally, phylogenetic relationships determined using predicted amino acid sequences (Fig. 4c) recapitulate those seen with nucleotide sequences, with the following exceptions: (i) amino acid analysis does not support the basal nature of FIV-Ccr (hyena), (ii) FIV-Ple (lion) is grouped in one monophyletic group using amino acid analysis, as opposed to the two separate clusters seen with the nucleotide analysis, (iii) FIV-Lpa (ocelot) and FIV-Hya (jaguarundi) group together with strong bootstrap support in the amino acid phylogeny but not in the nucleotide phylogeny, and (iv) FIV amino acid sequences from African carnivores plus the Pallas cat form a monophyletic cluster with strong bootstrap support.

DISCUSSION

An abundance of individual specimens (over 3,000 in the combined screening) from captive-born, wild-born, and free-ranging animals, the use of diverse FIV antigens, and the addition of PCR confirmation for FIV infection in many species provide the most comprehensive worldwide prevalence survey to date. Twelve of the species in this survey had no FIV data previously reported; these were the African golden cat, the black footed cat, three hyena species (brown, spotted, and striped), the caracal, the sand cat, three lynx species (Canadian, European, and Iberian), the kodkod, and the bay cat. While many of these species are quite rare and there is some difficulty in obtaining samples even from captive individuals, resulting in small sample sizes for the small African and Asian

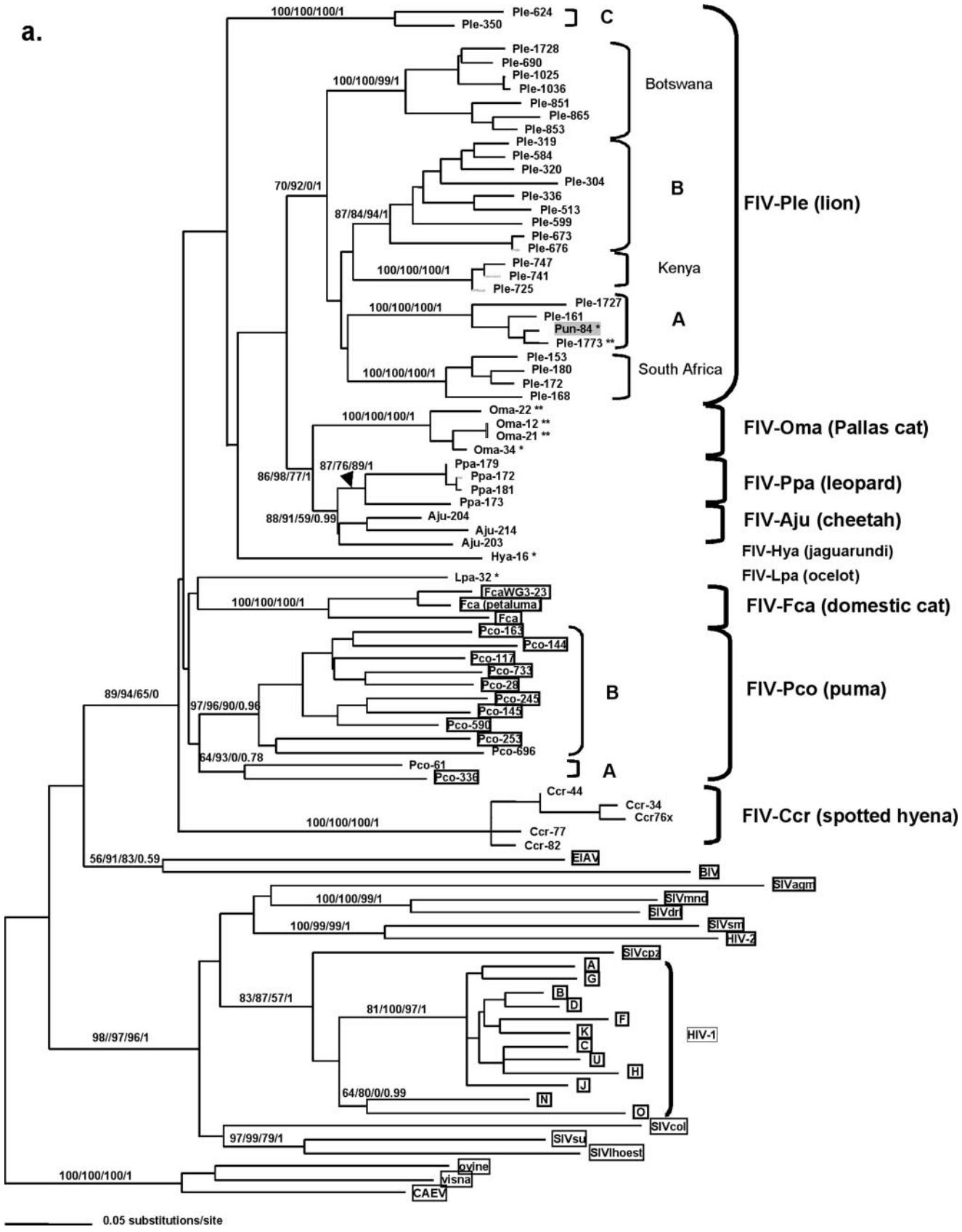

FIG. 4.

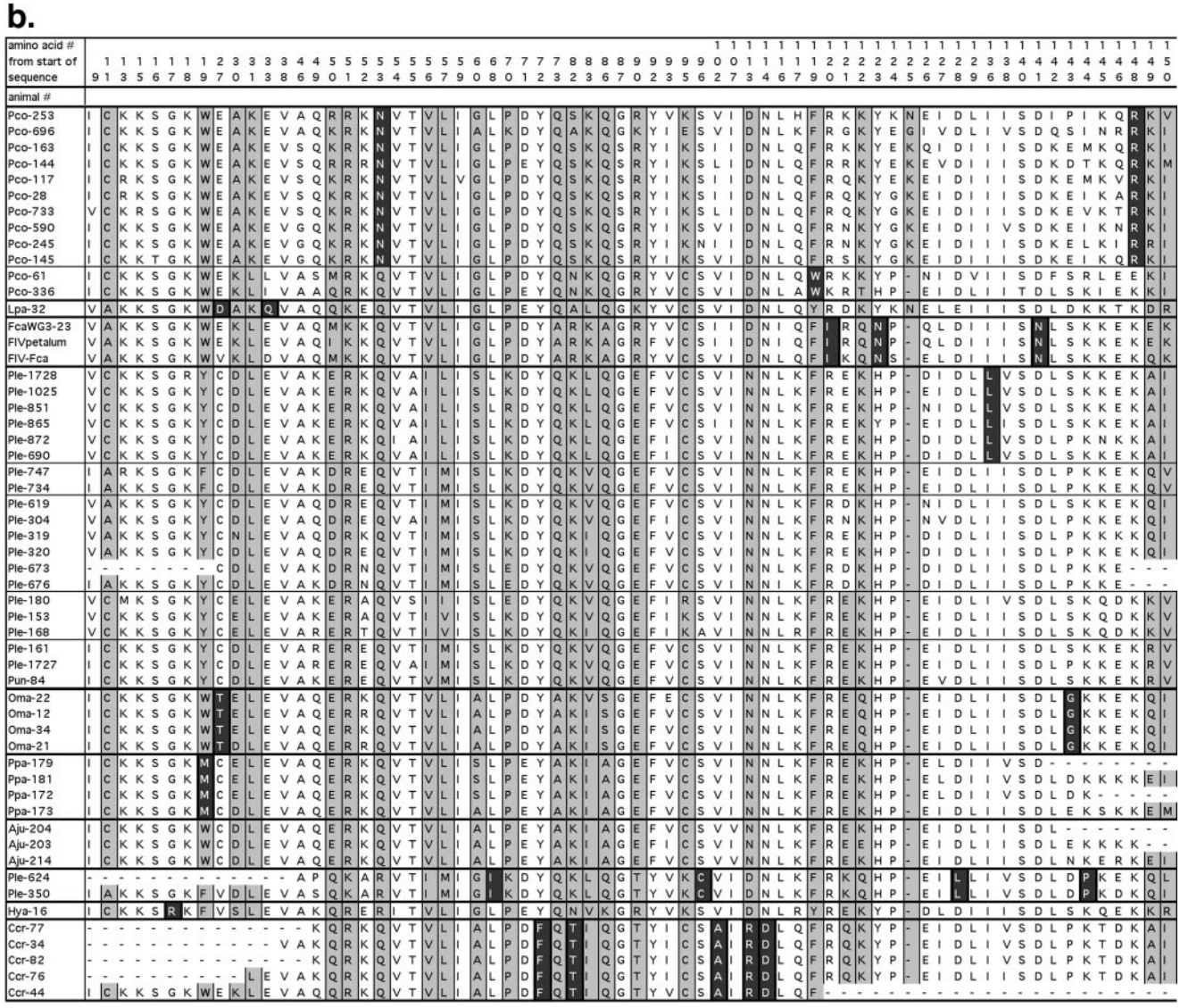

FIG. 4. (a) Phylogenetic tree for FIV *pol-RT* nucleotide sequences (476 bp included in analysis). Shown here is the single ML tree. MP, ME (neighbor joining based on the Tajima Nei algorithm for distance measure), and Bayesian trees gave similar topologies. Bootstrap values and posterior probabilities are included at all nodes with bootstrap support (ML/ME/MP/Bayesian). Taxa are designated with the species code followed by the source animal identification number. Sequences downloaded from GenBank for comparison are boxed; all other sequences are novel. A single asterisk indicates a sequence obtained from a captive but wild-born animal; a double asterisk indicates a sequence obtained from a captive-born animal. All other novel sequences are from free-ranging animals. Analysis used empirical base frequencies, an estimated shape parameter of 0.6618, and an estimated substitution matrix as follows: A/C = 2.0642, A/G = 7.2581, A/T = 1.4798, C/G = 4.0169, C/T = 14.0129, and an estimated proportion of invariant sites of 0.1525. (b) Alignments of the predicted amino acid translation products of FIV sequences from individual animals. Single-letter amino acid codes are used. Only variable amino acids are included. Dashes indicate missing data or a gap introduced to optimize the alignment. Sites that distinguish multiple FIV strains from each other are shown as black letters on a grey background, and species-specific differences are shown as white letters on a black background. (c) Phylogenetic tree for FIV *pol-RT* amino acid sequences (159 amino acid residues were included in the analysis). Shown here is the single maximum likelihood tree with maximum likelihood bootstraps from 1,000 replicates. Abbreviations are as for panel a.

cats in particular, a considerable number of free-ranging striped hyenas (19), spotted hyenas (51), and Iberian lynx (48) were obtained for this study.

A total of 1,195 animals from 36 species were screened with three antigens (FIV-Fca, FIV-Ple, and FIV-Pco). The three-antigen screening was clearly more sensitive at detecting species-specific virus (i.e., FIV-Ple in lions and FIV-Pco in pumas), but it also resulted in up to 20% more indeterminate results in most populations. The general increase of indeterminate signals (i.e., low-level antibody responses to one or two

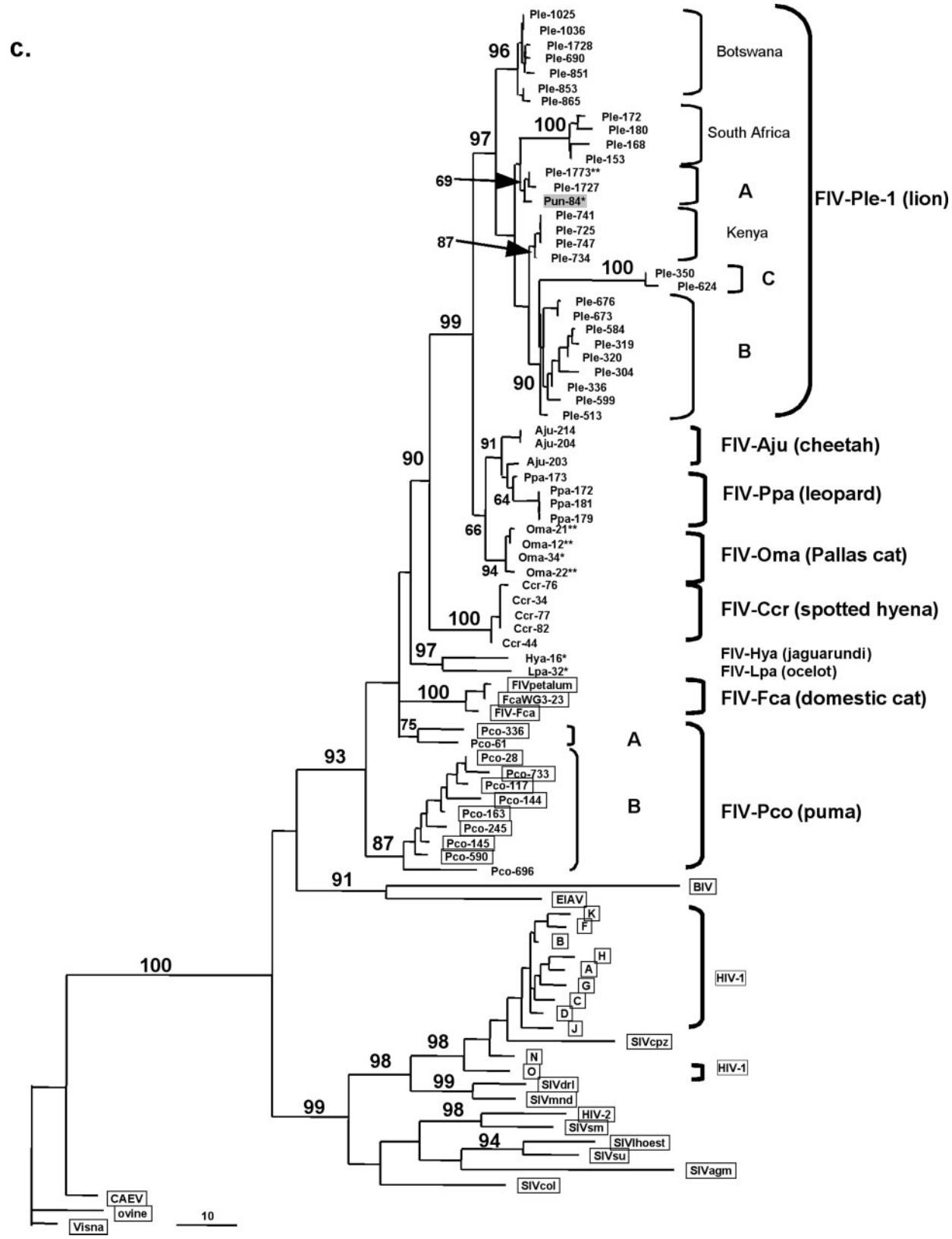

FIG. 4—*Continued*.

antigens, but negative responses to the others) in the three-antigen Western blot screening may represent the higher sensitivity of this screening or may merely reflect more opportunity for nonspecific cross-reactivity. Most (79%) of the samples classified as indeterminate were PCR negative, suggesting that many weak signals were false positives. Alternatively, some individuals may harbor a strain of FIV that is both antigenically (resulting in weak Western blot signals) and genetically (resulting in failure of primer binding) divergent from the viruses used in these screenings. The complete lack of indeterminate signals from some species despite large sample sizes (for example, sera from 0/202 Namibian cheetahs, 0/66 Asian leopards, and 0/81 leopard cats were seroreactive) implies that the higher levels of indeterminates in other species (for example, sera from 24/212 tigers and 6/44 bobcats were indeterminately seroreactive) are significant and suggestive of actual FIV exposure.

FIV infection was confirmed via PCR in 69% ($n = 273$) of the Western blot-positive animals and 21% ($n = 108$) of Western blot indeterminates. PCR verification of FIV infection in free-ranging animals has been obtained for cheetahs, leopards, and spotted hyenas as well as lions and pumas, whose FIV status and phylogeny is well documented (5, 8, 10, 14, 24, 35). Species-specific FIV strains also were amplified from wild-born jaguarundis, ocelots, and Pallas' cats, suggesting that these strains circulate in free-ranging populations. Western blot positives in free-ranging animals also indicate that FIV is endemic in these three species as well as in the spotted hyena and margay.

Western blot positives from wild-born animals suggest that additional species-specific viruses may exist in free-ranging populations of Geoffroy's cats, jaguars, margays, and tigrinas, pending Western blot and PCR verification from free-ranging individuals. Western blot-indeterminate results from free-ranging and/or wild-born individuals (without PCR verification) were found in an additional eight species, namely, the bobcat, Asian golden cat, clouded leopard, jungle cat, Iberian lynx, marbled cat, and tiger.

Phylogenetic analysis indicates that the lentiviruses infecting all feline species and the spotted hyena are related, suggesting either an ancient introduction of this virus to the *Felidae* and *Hyaenidae* (i.e., prior to the *Felidae*/*Hyaenidae* split) or cross-species transmissions, some of which must have occurred before the end of the last glaciations, around 10,000 years ago, when lions and cheetahs disappeared from the new world. As with SIV (27, 31), the FIV phylogeny does not exactly mirror that of its feline host species, and some feline species (puma and lion) harbor more than one distinct FIV type. In fact, there is no indication in the FIV phylogeny of any concurrent evolution between virus and host, as is seen in some branches of the SIV/HIV phylogeny (4, 15), nor is there a strict geographic distribution of viral strains, as would be expected with more recent introductions. However, there is some geographic partitioning reflected in the amino acid phylogeny, including an old world/new world split (Fig. 4c). In addition, the strains infecting cheetah (FIV-Aju) and leopard (FIV-Ppa) are closely related, perhaps reflecting the similar ecological niche occupied by these two species. There is no obvious explanation for the grouping of Pallas' cat FIV (FIV-Oma) with that of cheetah and leopard. FIV-Oma has now been confirmed for free-ranging Pallas' cats in Mongolia (M. Brown, et al., unpublished results), indicating that this strain does not reflect interspecies transmission in captivity.

While only results from animals living in the wild (free-ranging) can indicate the prevalence and potential threat of FIV to wild populations, the spread of FIV in captive animals is interesting. These animals are in unnatural proximity to a variety of related species, including domestic cats, and might acquire a form of FIV that they would not normally encounter or contract in a natural setting. For example, a captive puma in an Argentine zoo has been shown to harbor domestic cat FIV, although the FIV strains found in wild pumas (FIV-Pco) are quite distant from FIV-Fca (10). Here, we also report a captive snow leopard and a captive-born tiger, both infected with a FIV-Ple-1 clade A sequence similar to that seen in both free-ranging and captive lions. No FIV has been found in either of these species in the wild (wild-born or free-ranging tigers, $n = 34$; wild-born snow leopards, $n = 3$) (Table 2); however, they are clearly susceptible to cross-species transmission under certain circumstances. Cross-species transmission has also been observed in the wild, where a population of leopard cats became infected with FIV-Fca presumably obtained following exposure to infected domestic cats (20).

Given that only nine species have been shown to harbor species-specific virus in the wild, the potential for new and perhaps devastating outbreaks in naïve species is of great concern. This danger is heightened by the encroachment of humans and their domestic animals into the habitat of most wild felines. In addition, the ever-decreasing habitat of most feline species may serve to increase inter- and intraspecies aggression, further facilitating the spread and cross-species transmission of infectious disease. Finally, decreased genetic diversity in most wild populations reduces the ability of these species to adapt to new viral outbreaks (25, 30), decreasing the likelihood that naïve species will be able to undergo viral/host coevolution rendering their strain of FIV relatively benign, as has presumably occurred with the lion and the puma in the past (9).

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