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Genetic and Phylogenetic Divergence of Feline Immunodeficiency Virus in the Puma (*Puma concolor*)

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Feline immunodeficiency virus (FIV) is a lentivirus which causes an AIDS-like disease in domestic cats (*Felis catus*). A number of other felid species, including the puma (*Puma concolor*), carry a virus closely related to domestic cat FIV. Serological testing revealed the presence of antibodies to FIV in 22% of 434 samples from throughout the geographic range of the puma. FIV-Pco *pol* gene sequences isolated from pumas revealed extensive sequence diversity, greater than has been documented in the domestic cat. The puma sequences formed two highly divergent groups, analogous to the clades which have been defined for domestic cat and lion (*Panthera leo*) FIV. The puma clade A was made up of samples from Florida and California, whereas clade B consisted of samples from other parts of North America, Central America, and Brazil. The difference between these two groups was as great as that reported among three lion FIV clades. Within puma clades, sequence variation is large, comparable to between-clade differences seen for domestic cat clades, allowing recognition of 15 phylogenetic lineages (subclades) among puma FIV-Pco. Large sequence divergence among isolates, nearly complete species monophyly, and widespread geographic distribution suggest that FIV-Pco has evolved within the puma species for a long period. The sequence data provided evidence for vertical transmission of FIV-Pco from mothers to their kittens, for coinfection of individuals by two different viral strains, and for cross-species transmission of FIV from a domestic cat to a puma. These factors may all be important for understanding the epidemiology and natural history of FIV in the puma.

Feline immunodeficiency virus (FIV), a lentivirus related to human immunodeficiency virus (HIV) and simian immunodeficiency virus (27, 28), causes an immune system disease in domestic cats (*Felis catus*) involving depletion of the $CD4^+$ population of T lymphocytes, susceptibility to opportunistic infections, and sometimes death (8, 9, 29, 30, 33). Although mortality of FIV-infected cats is somewhat lower than that of HIV type 1 (HIV-1)-infected humans, the disease caused by FIV in domestic cats has a number of immunologic and pathogenetic similarities to AIDS in humans and may therefore be a useful animal model (8, 9, 33).

Viruses related to domestic cat FIV occur also in nondomestic felid species. Members of at least 18 of the 37 species in the family Felidae carry an FIV-related virus, as has been shown by the presence in their sera of antibodies which react with FIV antigens (2, 3, 5, 20, 28, 39). However, it has not yet been demonstrated that FIV-related viruses cause disease in any species other than the domestic cat (4, 20).

Genetic analysis of FIV isolates indicates that different felid species are infected by different strains of FIV. Comparison of the complete genome of a puma (*Puma concolor*) FIV isolate with that of a domestic cat isolate revealed the same set of genes but in a slightly different arrangement (19, 27). Analysis of *pol* gene sequences of FIV from lions (*Panthera leo*), pumas, and domestic cats indicated that each species has a specific strain of FIV (termed FIV-Ple, FIV-Pco, and FIV-Fca, respectively) and that the strains are related but quite distinct (4, 13, 28). The *pol* sequences from lion FIV-Ple isolates could be separated into three distinct clades (4). Similarly, the *pol* sequences from domestic cat FIV-Fca form two clades or subtypes (13). Analysis of the more variable *env* regions from a wider range of domestic cat samples revealed at least four viral subtypes (15, 38). The sequence studies reveal extensive viral diversity, providing an interesting parallel to studies of primate lentiviruses.

Pumas are currently found throughout most of South and Central America and in the western part of North America as far north as southern Alaska, as well as in Florida (1, 14). Historically their range extended throughout the United States and southern Canada (1, 14). Previous studies of puma FIV-Pco (19, 28) have focused on the Florida panther (*P. concolor coryi*), a relict endangered subspecies of the puma, inhabiting southern Florida (25). The Florida panthers are genetically distinct from other puma subspecies, consistent with a long period of isolation and inbreeding (25, 35). A preliminary study of Florida panther FIV-Pco (28) included two *pol* gene short sequences from Arizona and Colorado pumas, which were significantly different from the Florida panther FIV-Pco sequences. This observation, together with the extensive inter se genetic variation seen in lions and domestic cats (4, 13), suggested that an analysis of FIV which covered the entire geographic range of the puma might reveal considerably more viral variation, providing the opportunity to study patterns of lentiviral divergence in a cosmopolitan species with an extensive geographic range.

We undertook an extensive survey of FIV seroprevalence among 434 pumas from 30 different named subspecies (1, 35)

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and found the virus to be present in most parts of the species range. To genetically characterize puma FIV, we analyzed sequence data from a conserved region of the viral genome, the part of the *pol* gene encoding reverse transcriptase, from samples obtained throughout North America and parts of Central and South America. We observed greater viral diversity in the puma than has been documented in domestic cats and found that the FIV infecting Florida panthers is genetically divergent from that seen in other pumas, which allowed us to define two principal clades of puma FIV isolates. Within these two clades, however, genetic divergence of puma FIV *pol* sequences was large, in some cases as great as between lion or domestic cat clades, prompting the recognition of 15 divergent lineages or subclades of puma FIV. In addition, we describe three important epidemiological phenomena: evidence consistent with vertical transmission of FIV in the puma, coinfection of individuals by different viral subtypes, and cross-species transmission of the virus. We present a model for the evolution of FIV in the puma that is based on the relationship between the pattern of viral variation and the geographic origin of the samples, combined with comparisons of FIV from other species (4, 13, 15, 28, 38).

MATERIALS AND METHODS

Blood samples were collected from free-ranging and captive pumas from locations throughout North, Central, and South America. Additional samples were collected from leopards (*Panthera pardus*) from South Africa and domestic cats from Britain and Central and South America. Blood samples from endangered species were collected in full compliance with specific federal permits (CITES; Endangered and Threatened Species; Captive Bred) issued to the National Cancer Institute by the U.S. Fish and Wildlife Service of the Department of the Interior. Serum or plasma samples were tested for the presence of FIV-reactive antibodies by Western blotting (immunoblotting), using the domestic cat FIV Petaluma strain as a source of viral antigens, as described previously (3).

A proviral DNA fragment of approximately 520 bp was amplified from genomic DNA isolated from the leukocytes of seropositive animals, using primers described previously (4). The amplified fragment was from the relatively conserved region of the *pol* gene which encodes reverse transcriptase. The nested PCR consisted of a first round with primers 1258F and 1260R (4) and 0.5 to 1.0 μg of genomic DNA in a total volume of 50 μl. The second round combined primers 1259F and 1261R (4) with 10 μl of the first-round reaction. PCR cycling conditions were as follows: 94°C for 1 min, 37°C for 1.5 min, and 72°C for 1 min for 30 cycles, followed by 10 min at 72°C. The *pol* gene fragment from puma Pco408 was amplified by using a new primer, 1259Ext (GGAAAGGTAAAAA GAGCAGATCCTAATAATCC), as a substitute for primer 1259F. The *pol* gene fragments were cloned into pBluescript KS+ (Stratagene), and the resulting plasmids were sequenced with an ABI automated sequencer (Applied Biosystems Inc.).

Sequences were aligned for subsequent phylogenetic analysis by using the algorithm of Needleman and Wunsch (22) as implemented in the PILEUP program in the Genetics Computer Group package (7). Genetic distances between pairs of DNA sequences were calculated by the DNADIST program in PHYLIP version 3.5 (11), using Kimura's two-parameter model (16). Sequences were translated to produce amino acid sequences, and genetic distances were calculated by the PHYLIP program PROTDIST, using the Dayhoff PAM matrix (6). Synonymous and nonsynonymous distances were calculated by the method of Nei and Gojobori (23) as provided by MEGA version 1.01 (17).

Phylogenetic analysis of sequences consisted of minimum evolution estimated by neighbor joining (36), maximum parsimony (40), and maximum likelihood (10). Neighbor-joining trees were estimated by the NEIGHBOR program in PHYLIP version 3.5 (11), using an empirically determined transition/transversion ratio. For maximum parsimony analysis of DNA sequences, we used PAUP version 3.1.1 (40). Maximum parsimony analysis of protein sequences was performed by constructing a topology with the PHYLIP version 3.5 program PROTPARS (11) and using PAUP version 3.1.1 (40) to calculate the branch lengths for that topology. Both neighbor-joining and maximum parsimony trees were evaluated, using 100 bootstrap iterations (10). For maximum likelihood analysis, we used the PHYLIP program DNAML to select the tree with the highest probability of occurrence given the empirical base frequencies. Percent differences between pairs of sequences were calculated with each gap equal to a single nucleotide substitution regardless of its length.

Nucleotide sequence accession numbers. Sequences reported in this paper have been submitted to GenBank under accession numbers U53718 to U53766. Additional sequences were obtained from GenBank, using either published accession numbers (4, 21, 27, 28, 31, 41) or the following: L16938, L16942, L16940, L16944, S67753, X57002, U11820, and X01762 (13, 32).

TABLE 1. Prevalence of FIV exposure in the puma

Source	No. positive	No. tested	% Positive
Free-ranging animals			
Alaska	1	1	100
Arizona	9	13	69
California	10	24	42
Colorado	6	9	67
Florida	15	62	24
Idaho	2	15	13
New Mexico	1	3	33
Oregon	1	11	9
Texas	11	30	37
Utah	1	2	50
Wyoming (Yellowstone National Park)	7	41	17
British Columbia	5	14	36
Chile	0	2	0
Total	69	227	30
Captive animals			
Canada	4	10	40
United States	4	96	4
Argentina	5	22	23
Belize	0	1	0
Bolivia	5	5	100
Brazil	2	13	15
Chile	0	12	0
Costa Rica	0	5	0
Guatemala	0	4	0
Mexico	2	12	17
Nicaragua	1	6	17
Panama	0	3	0
Paraguay	0	2	0
Peru	1	5	20
Uruguay	0	3	0
Venezuela	4	8	50
Total	28	207	14
Grand total	97	434	22

RESULTS

Seroprevalence of FIV in the puma. A total of 434 puma serum samples were tested for FIV antibodies by Western blotting, which revealed 22% antibody-positive animals (Table 1). All regions of North America included in the survey had some seropositive samples. Some regions of South and Central America had no seropositive individuals; however, in most of these regions, only a few samples were tested. An exception is Chile, whose 12 free-ranging and 2 captive animals were all seronegative. Seroprevalence was higher in free-ranging animals (30%) than in captive animals (14%).

Phylogenetic analysis of FIV *pol* gene sequences. FIV *pol* sequences were obtained from 24 of 48 tested samples of genomic DNA from seropositive pumas. We were unable to amplify *pol* fragments from the remaining samples despite attempts with additional primer pairs (see Materials and Methods), low annealing temperatures, and increased template DNA concentrations. Similarly, *pol* fragments were amplified and sequenced from 1 of 10 seropositive leopard genomic DNA samples and from 4 of 6 samples from domestic cats. Negative results may reflect divergent primer sequences, low viral load, or other factors. The origins of the animals which yielded FIV sequence data are shown in Table 2.

TABLE 2. Sources of samples which provided FIV *pol* sequences

Animal	C or F[a]	Source	Contact(s)
Pumas[b]			
Pco28	C	San Antonio Zoological Gardens and Aquarium, Texas	Kenneth Fletcher
Pco18	F	Big Cypress Swamp, Florida	Melody Roelke-Parker
Pco61	F	Everglades National Park, Florida	Melody Roelke-Parker
Pco117	F	Arizona/Nevada	Dave Gonzales
Pco141	F	Colorado	Melody Roelke-Parker and Dana Anderson
Pco144	F	Colorado	Melody Roelke-Parker and Dana Anderson
Pco145	F	Colorado	Melody Roelke-Parker and Dana Anderson
Pco161	F	Arizona	Matt Pierce and Harley Shaw
Pco163	F	Arizona	Matt Pierce and Harley Shaw
Pco245	F	Williams Lake, British Columbia	Melody Roelke-Parker
Pco247	F	Williams Lake, British Columbia	Melody Roelke-Parker
Pco253	C	Vancouver Game Farm, British Columbia	Melody Roelke-Parker
Pco323	F	Yellowstone National Park, Wyoming	Kerry Murphy and Maurice Hornocker
Pco328	F	Yellowstone National Park, Wyoming	Kerry Murphy and Maurice Hornocker
Pco332	F	Yellowstone National Park, Wyoming	Kerry Murphy and Maurice Hornocker
Pco333	F	Yellowstone National Park, Wyoming	Kerry Murphy and Maurice Hornocker
Pco336	F	Yellowstone National Park, Wyoming	Kerry Murphy and Maurice Hornocker
Pco346	F	Idaho State University	John Laundré
Pco366	F	Santa Ana Mountains, California	Paul Beier
Pco408	C	Lima Peru Zoo, Peru	Melody Roelke-Parker
Pco549	C	Juigalpa Zoo, Nicaragua	Victoriano Corazón
Pco590	C	Sonoran Ecological Center, Mexico	Samuel Ocana
Pco593	C	Parque Zoológico Centenario, Mexico	Fernando Victoria
Pco696	C	Goiania Zoo, Brazil	Helio Ferreira
Pco733	C	Houston Zoo, Texas	Joe Flanagan
Leopard			
Ppa135	F	Kruger Park, South Africa	Mitch Bush
Domestic cats			
FcaWG3	F	Barley Park Farm, England	David McDonald
FcaTK1	F	Barley Park Farm, England	David McDonald
Fca151	C	Managua Zoo, Nicaragua	Victoriano Corazón
Fca155	C	Mendoza Zoological Park, Argentina	Jorge Bustelo

[a] C, captive; F, free.
[b] Pco247 is the mother of Pco245. Pco253 was originally from Vancouver Island. Pco336 is the mother of Pco328.

We initially examined a group of 44 FIV *pol* sequences obtained from 16 pumas inhabiting western North America. Multiple sequences from several individuals and multiple individuals from the same geographic area were examined. The 479-bp sequences were aligned and analyzed by the neighbor-joining and maximum parsimony methods. Maximum parsimony produced 15 equally parsimonious topologies, one of which was identical to the neighbor-joining topology (Fig. 1). Relative branch lengths and bootstrap support were consistent between the results of the two phylogenetic methods. In most cases, sequences from a single individual formed a monophyletic cluster, but there were two categories of exceptions. First, there were two clusters of sequences, from animals Pco247 and Pco245 and from animals Pco336 and Pco328, in which the sequences from two individuals appeared as similar to each other as sequences from single individuals elsewhere (Fig. 1). Each of these pairs was a mother and her kitten, which suggested that transmission of the virus between mothers and kittens was occurring. Second, the topologies revealed two individuals, Pco333 and Pco145, which carried widely divergent FIV sequences. This is evidence for coinfection (or superinfection) of one individual by two different strains of puma FIV.

The different puma FIV sequences found in North America were paraphyletic with respect to geographic locales. Thus, there were two distinct phylogenetic clusters coexisting in each of Colorado, Arizona, and Wyoming. The sequence divergence between phylogenetic clusters within the same area was large (Fig. 1) for Colorado (average nucleotide divergence = 15.9%), for Arizona (average divergence = 14.5%), and for Wyoming (average divergence = 14.6%), comparable to the difference between clades of domestic cat FIV-Fca isolates (13). The prevalence of sequence clusters with such large sequence divergence within several populations suggests recent contact and FIV-Pco transmission between geographic locales.

Representative puma FIV-Pco *pol* sequences, including samples from North, Central, and South America, were analyzed with representative sequences from lions, domestic cats, and a leopard, using HIV-1 as an outgroup. Sequences of 426 bp were used, this being the length of the shortest sequences in this data set. Phylogenetic analyses using neighbor joining, maximum parsimony, and maximum likelihood yielded similar topologies (Fig. 2) which were mostly consistent among the puma sequences but differed in the relationships between the other felid species.

The results (Fig. 2) showed appreciable sequence divergence and significant cluster definition. All three phylogenetic methods detected two major clades with high bootstrap (97 and 95% by neighbor joining and 94 and 83% by maximum parsimony) and statistical significance with maximum likelihood: clade A including Florida panther FIV-Pco isolates plus a single isolate from California, FIV-Pco366-13; and clade B including all other FIV-Pco sequences (except Pco408). As in Fig. 1, the distances between many sequences are very large, comparable to or greater than distances between clades of lion and domestic cat FIV *pol* sequences (Fig. 2). If we use the amount of divergence between domestic cat clades as a thresh-

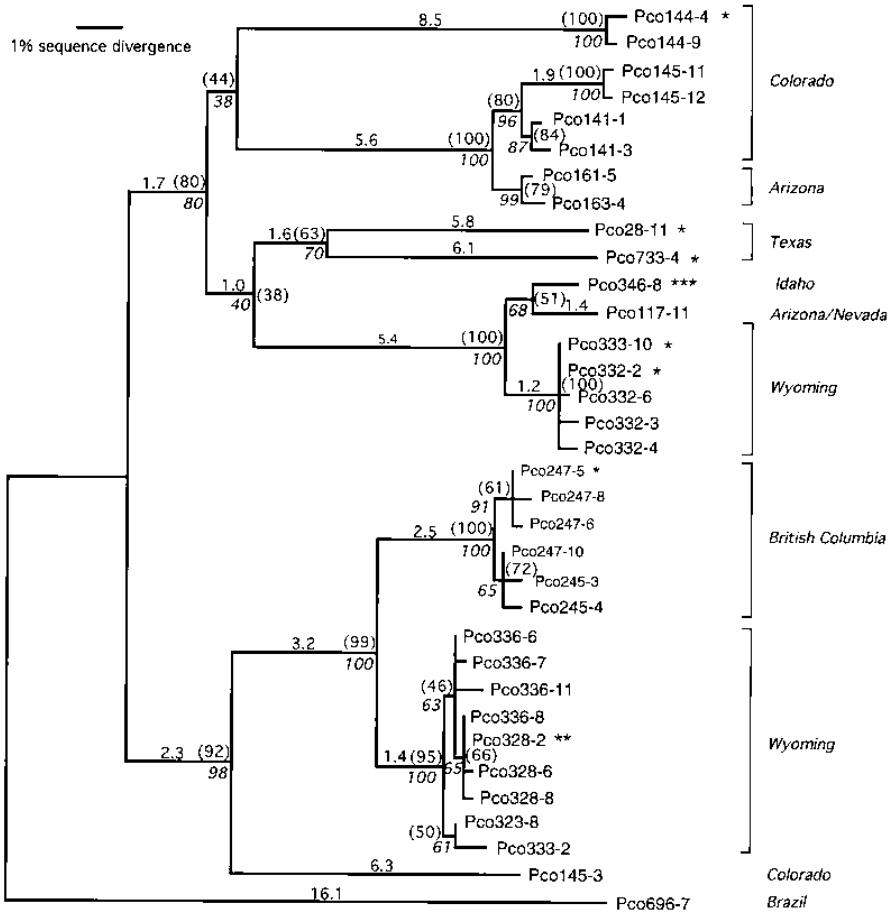
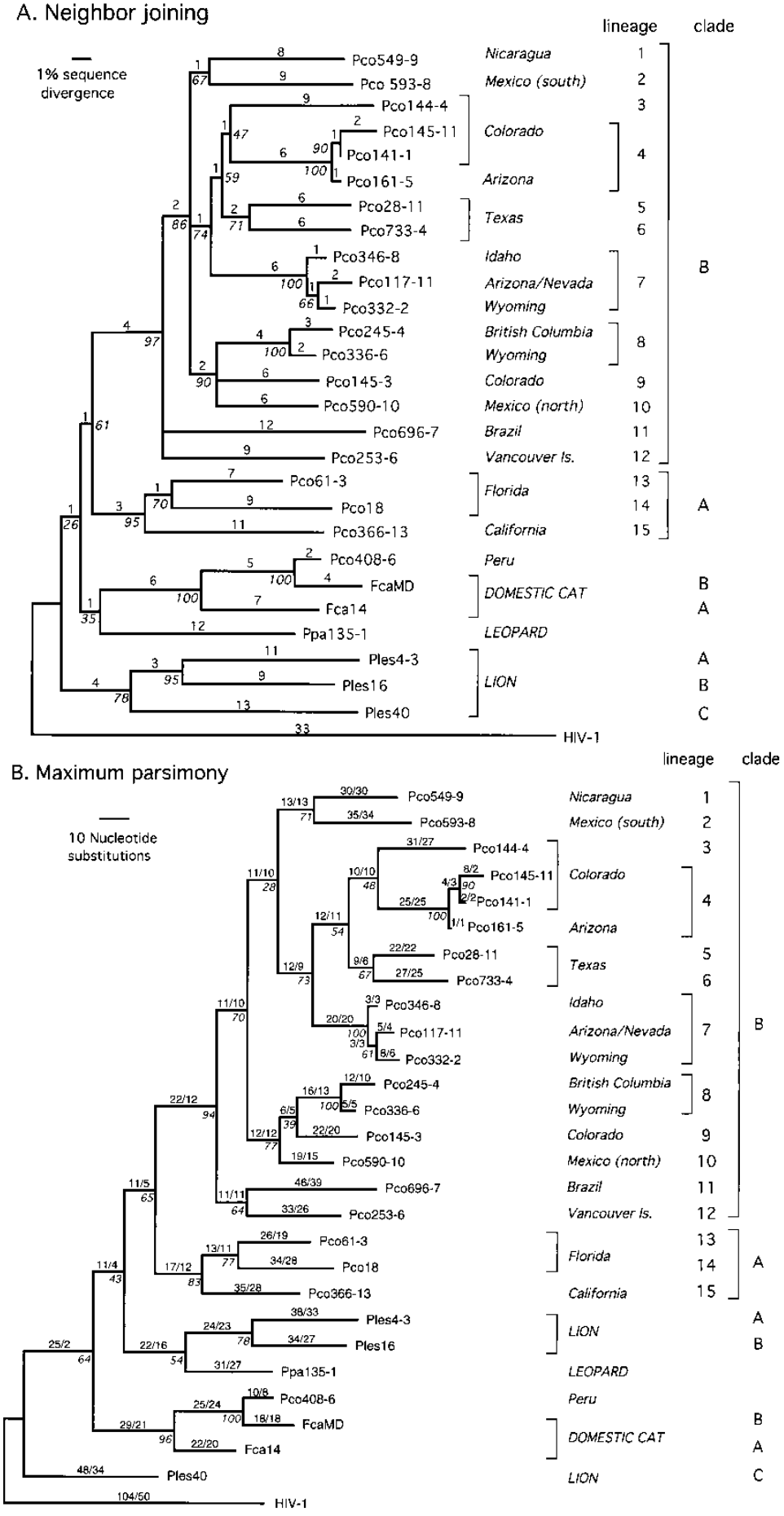


FIG. 1. Neighbor-joining tree of FIV *pol* gene sequences from pumas from western North America, constructed by using the Brazilian sequence Pco696-7 as an outgroup. Pco144-4 represents clone 4 from animal Pco144. Branch lengths represent percent sequence divergence, calculated by using Kimura's two-parameter model with a transition/transversion ratio of 1. Bootstrap values out of 100 are shown in italics at the nodes. Asterisks represent additional identical sequences of clones isolated from the same animal. Maximum parsimony analysis gave 15 equally parsimonious solutions, one of which was identical to the neighbor-joining topology and had branch lengths consistent with it. The bootstrap values from the maximum parsimony analysis are given in parentheses at the nodes. Tree length = 403; consistency index = 0.640.

old, we can recognize 15 different phylogenetic lineages (subclades) within the major A and B clades of FIV-Pco (Fig. 2). Different geographic locales have multiple and distinct lineages. Thus, two distinct lineages were observed in Mexico, Colorado, Arizona, Florida, and Wyoming, with single lineages in Nicaragua, Texas, Vancouver Island, and Brazil. All puma FIV-Pco nucleotide sequences except one form a monophyletic group relative to other species' FIV sequences, suggesting that the puma FIV-Pco has evolved principally with its host species. The single exception, the Pco408 sequence, is closely related (100% bootstrap for neighbor-joining and maximum-parsimony analyses) to domestic cat FIV-Fca (Fig. 2). This virus was isolated from a captive puma in a Peruvian zoo and likely represents an interspecies infection.

Phylogenetic analysis of FIV *pol* amino acid sequences. The amino acid sequences derived from the felid FIV *pol* gene sequences were aligned (Fig. 3). The alignment revealed an insertion/deletion at position 119 which distinguished the major puma clade B sequences from the sequences from puma clade A, domestic cats (and Pco408), lions, and the leopard, as well as the HIV-1 sequence. In addition to the insertion/deletion mentioned above, there were seven additional signature (synapomorphic) amino acid residues distinguishing the two puma clades (boxed in Fig. 3). At six of the eight signature sites, puma clade A shares greater similarity with the FIV sequences from the other felids than with puma clade B (Fig. 3). There were also a few sites where the FIV sequences from Pco253 and Pco696 shared similarity with sequences from puma clade A and other felids, rather than with the clade B pumas, suggesting that the Pco253-6 and Pco696-7 sequences may represent evolutionary intermediates between the two puma clades. The sequence from Pco408 had 10 residues which were identical to those in the domestic cat sequences and different from those in all other puma FIV sequences (Fig. 3), supporting the inference that it was actually a domestic cat FIV-Fca present in a puma.

To resolve relationships between the FIV isolates of the different felid species not clear from the DNA sequences, we performed phylogenetic analysis of the *pol* amino acid sequences. Trees produced by neighbor-joining and maximum parsimony methods had similar topologies, both comprising four main groups of sequences: lion and leopard, domestic cat, puma A, and puma B (Fig. 4). There were minor differences within these groups such as the position of Ples40 in the lion-leopard group. These topologies emphasize the division of the puma FIV sequences into two major clades. Both topologies grouped the cat-lion-puma A clusters together, with relatively high bootstrap support for the outgroup status of puma clade



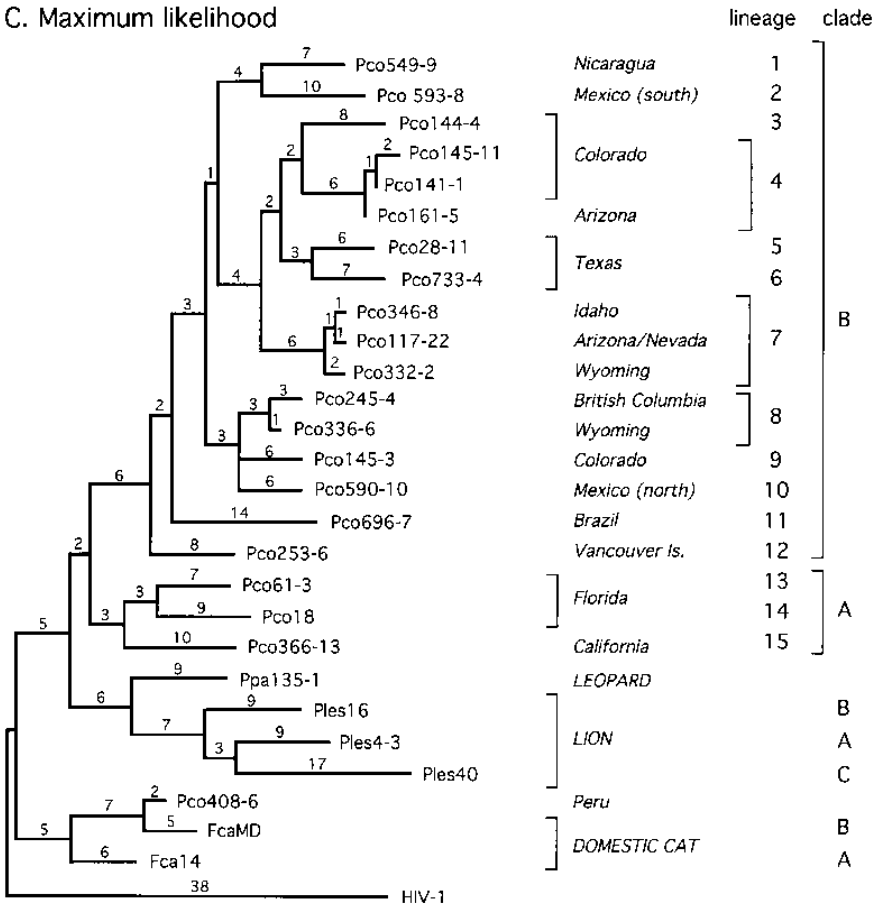


FIG. 2. Phylogenetic trees of FIV *pol* gene sequences from pumas and other felids. (A) Neighbor-joining tree. Branch lengths represent percent sequence divergence, calculated by using Kimura's two-parameter model with a transition/transversion ratio of 2. Bootstrap values (out of 100) are shown in italics at the nodes. (B) Maximum parsimony tree. Branch lengths show number of nucleotide substitutions/number of homoplasies. Bootstrap values (out of 100) are shown in italics at the nodes. Tree length = 1,083; consistency index = 0.428. This is one of four equally parsimonious trees which differ only within the puma clade B. (C) Maximum likelihood tree generated by using a transition/transversion ratio of 2. ln likelihood = −5,534.34; 2,992 trees examined. Branch lengths estimate the expected number of substitutions per site × 100. Branch lengths not significantly different from zero were collapsed into polytomies. The Ple, Fca, Pco18, and outgroup HIV-1 sequences have been published (4, 27, 28, 32).

B (86% for neighbor joining and 92% for maximum parsimony). The hierarchical relationship of domestic cat, lion, and puma clade A remained unresolved and may be best represented by a polytomy.

Quantitation of sequence variation in the FIV *pol* gene. The extent of variation in the FIV *pol* gene was estimated by calculating the percent differences between pairs of sequences from pumas, lions, and domestic cats (Fig. 5 and Tables 3 and 4). The sequence variation within individual pumas was in most cases less than 1%. However, there were two animals which exhibited much higher intraindividual variation (15 to 16%). These were the two individuals (Pco145 and Pco333) which appeared to be coinfected with two strains of puma FIV (Fig. 1). The intraindividual variation in domestic cats was also less than 1%, whereas in lions it reached 4% (4, 13).

Comparisons between *pol* gene nucleotide sequences from different individuals (Table 3) revealed high variation within puma clades A and B. Clade B included sequences that diverged up to 24%, greater variation than had been observed within the lion or domestic cat clades. In addition, the maximum divergence between sequences within puma clade B exceeded the interclade variation (13 to 17%) in domestic cats. The divergence between FIV-Pco isolates from different puma clades was also high (20 to 27%), comparable to that observed between the lion clades and considerably higher than that between the domestic cat clades. Interspecies comparisons produced similar divergence values between all three pairs of host species (20 to 33%), with the minimum values between the puma clade A and other species. Differences between the derived amino acid sequences parallel the variation observed among the nucleotide sequences (Table 4).

The percent differences between FIV *pol* sequences from different pumas were compared with those of lions and domestic cats (Fig. 5). Lion and cat FIV *pol* sequence comparisons formed bimodal distributions, made up of comparisons from within the same clade and comparisons between sequences from different clades. The distribution for the puma was more complex. The first two peaks reflected comparisons within the puma FIV-Pco lineages (subclades) and between lineages. The between lineage divergences for FIV-Pco approximated the between-clade divergence values for domestic cat (Fig. 5). A third peak, corresponding to comparisons between puma clade A and clade B, overlaps between-lineage divergence of FIV-Pco and is slightly larger than the distance between the lion

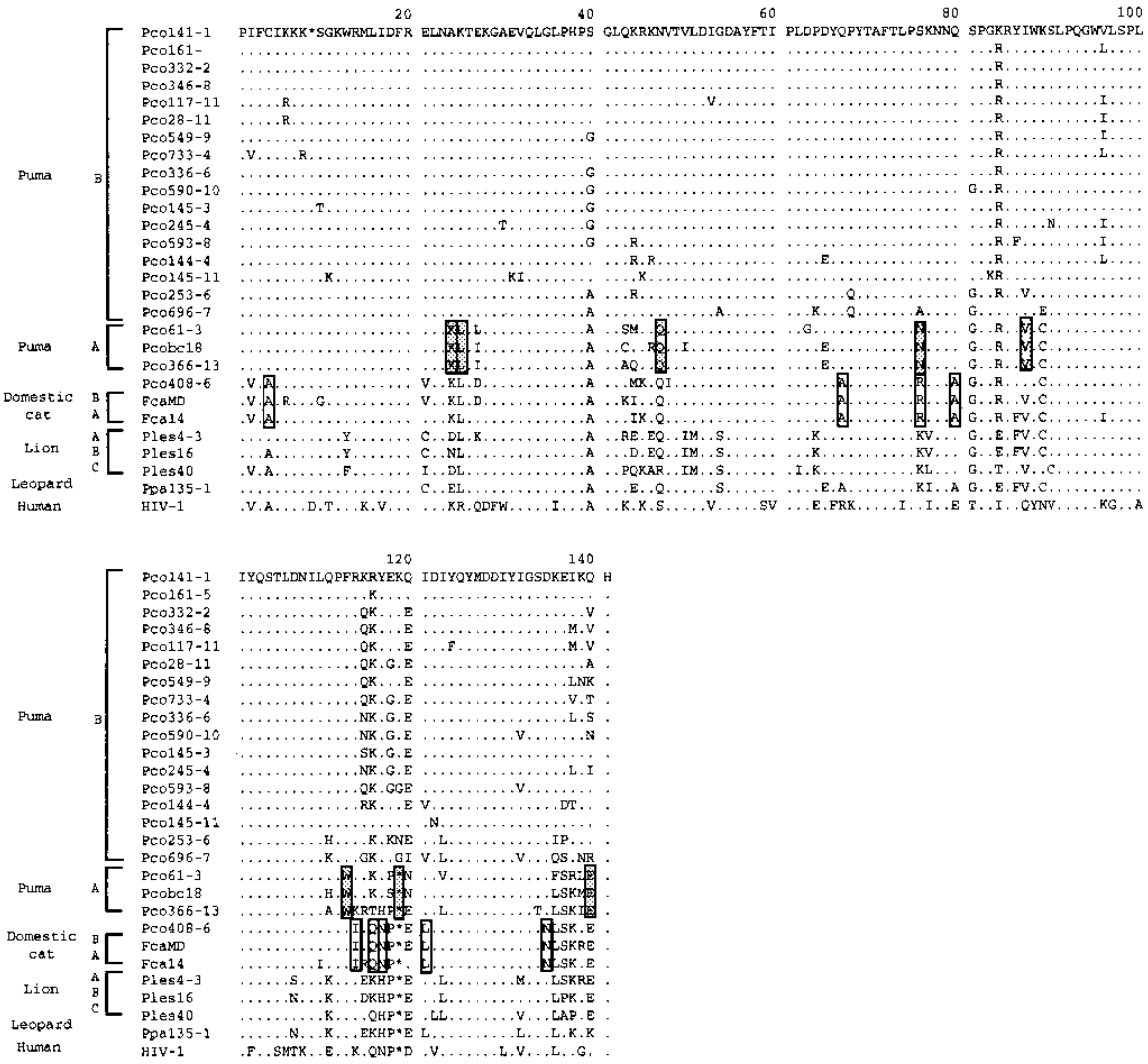


FIG. 3. Alignment of the predicted translation products of FIV *pol* gene sequences from pumas and other felids and the equivalent sequence from HIV-1. Sequences were aligned by using the PILEUP program in the Genetics Computer Group package. Residues differing from the top sequence are shown. Dots denote identity with the top sequence; asterisks denote gaps introduced to optimize the alignment. Sites which distinguish puma clade A from clade B are marked by shaded boxes; sites which group Pco408 with the domestic cat sequences are marked by open boxes.

FIV clades. Finally, comparisons between Pco408 (the puma with an FIV sequence characteristic of domestic cats) and other pumas gave the greatest sequence differences, consistent with the conclusion that these were equivalent to interspecies comparisons.

Synonymous and nonsynonymous nucleotide changes in FIV *pol* sequences. The proportions of synonymous and nonsynonymous sites which showed nucleotide changes (p distance) in pairwise comparisons of FIV *pol* sequences from pumas, lions, and domestic cats are plotted in Fig. 6. For each species, the amount of variation in both site categories was low within clades (or FIV-Pco lineages) and is very roughly proportionate. Once 60% of the synonymous site changes are substituted (for the most part in comparisons between clades or FIV-Pco lineages), there is a plateau of synonymous site substitution versus nonsynonymous site changes as nonsynonymous substitutions continue to increase. A comparison of the three species shows the gradation of comparative divergence (see also Table 3) whereby the domestic cat FIV-Fca has the least accumulated variation, the puma FIV-Pco has the most, and variation for the lion FIV-Ple is intermediate. For puma FIV, the between-clade (A versus B) comparisons have the highest amount of nonsynonymous site changes, with synonymous changes saturated at an earlier level of divergence. Nonsynonymous changes, which did not appear to be saturated, produced p distances in the puma and lion that were almost three times as great as in the domestic cat, suggesting a considerably longer period of FIV evolutionary divergence in pumas and lions than in domestic cats.

DISCUSSION

Phylogenetic analysis of puma FIV sequences provided evidence for three epidemiologic phenomena which may be important in the spread of FIV. First, there was indirect evidence for two instances of vertical transmission of the virus from mother to kitten, which suggested that this may be a common occurrence in this species. No inference could be made as to

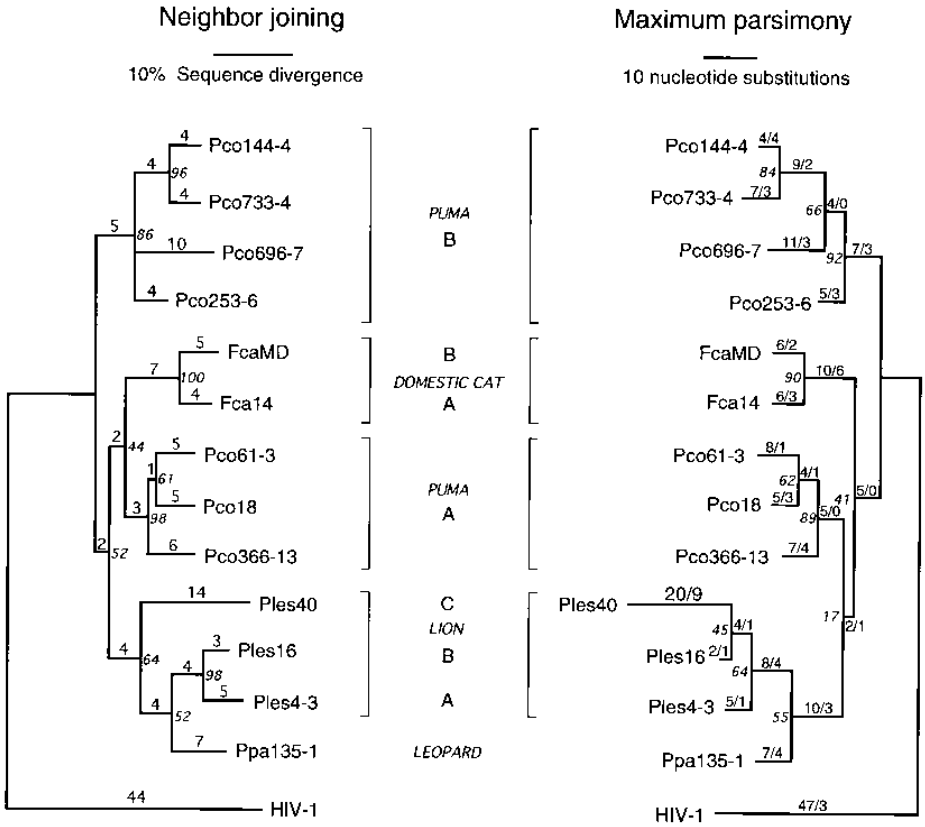


FIG. 4. Phylogenetic trees of the translation products of FIV *pol* sequences. In the neighbor-joining tree, branch lengths represent percent sequence divergence, calculated by using Dayhoff's PAM matrix. In the maximum parsimony tree, branch lengths show number of substitutions/number of homoplasies. Tree length = 208; consistency index = 0.788. This is one of two equally parsimonious trees. The topology not shown grouped puma clade A with domestic cat FIV. Bootstrap values (out of 100) are shown in italics at the nodes.

whether the infection of cubs occurs in utero, through milk, or via saliva during grooming. The close phylogenetic affinity of mother-offspring sequences (Fig. 1) may be a coincidence, but we believe that this is unlikely because other FIV-Pco isolates from the same geographic locale (e.g., Colorado [Fig. 1]) have significant (high bootstrap) distinctions unless they were close relatives. Also, there are several examples of transmission in domestic cats by biting and from mother to infant (37, 42, 43). Maternal transmission may be more likely in a solitary species like the puma, in which contact between adults is limited to mating and occasional fighting (1, 14).

Second, the analyses of viral sequences revealed coinfection (or superinfection) of two individuals each by two different strains of virus. This phenomenon has not yet been observed to occur naturally in other felid species (4) but has been experimentally induced in domestic cats (18, 26). There is evidence that coinfection occurs in humans infected by HIV-1 or HIV-2, as indicated by viral sequences which appear to have formed through recombination between two different strains (12, 34, 44).

Third, the FIV infecting one puma was characteristic of a domestic cat FIV rather than a puma FIV, suggesting a recent cross-species transmission of the virus. This result was validated by repeating the experiment with a new DNA sample and by confirming that the original DNA sample really was puma DNA by sequencing a mitochondrial gene (5a). The puma concerned was a captive animal residing in a Peruvian zoo. It is possible that a zoo animal is more likely to have contact with a domestic cat than a free-ranging animal.

The puma FIV-Pco *pol* sequences (excluding Pco408, the domestic cat-type sequence) formed two major clades, one consisting of the Florida panther and California sequences (clade A) and the other (clade B) including all other puma sequences. The degree of difference between these clades is similar to that distinguishing the three lion clades (4) and considerably greater than that observed between the two domestic cat clades (13). The greatest differences between members of the two puma clades exceed the divergences between some pairs of FIV sequences from different species. The variation among 15 recognizable puma FIV lineages within the two clades is also high, exceeding that observed between clades in domestic cats. We therefore attempted to define additional puma clades, using the level of interclade variation in domestic cats as a guideline, but such divisions were not well supported by the phylogenetic trees (Fig. 2). The sequences from Vancouver Island and Brazil may be representatives of other clades, but without additional sequences, we prefer to include them in clade B and to acknowledge that the puma clades are made up of divergent lineages. The genetic variation within and between the puma clades constitutes a massive quantity of sequence diversity, and this may be an underestimate because our samples do not cover the complete geographic range of the puma, as only a few sequences were successfully amplified from Central and South America.

If FIV sequence diversity increases proportionately over time and has similar rates of evolution in the three felid species, then the fact that the FIV sequence variation (particularly at nonsynonymous sites) was greater in pumas and lions than

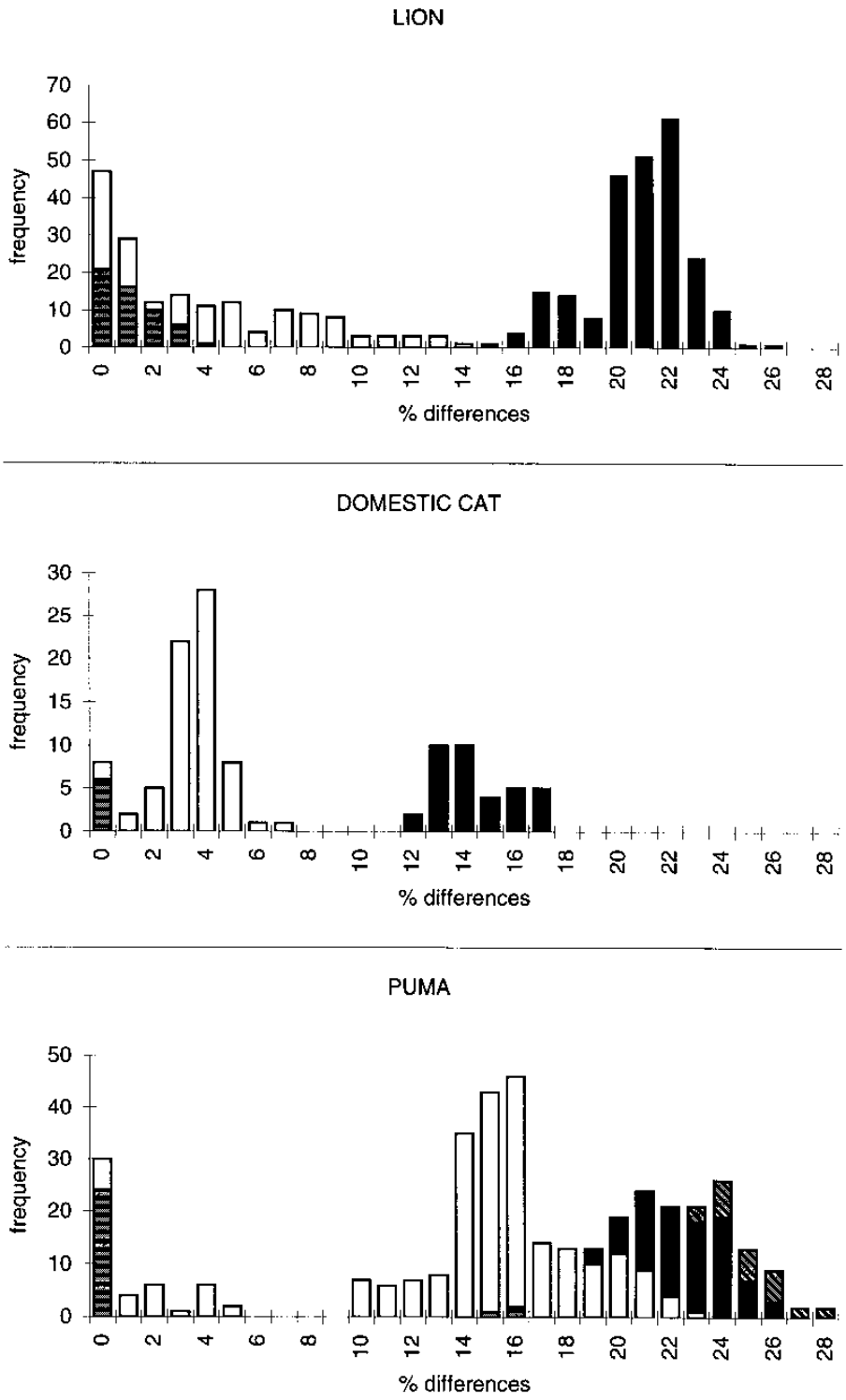


FIG. 5. Frequency distributions of the percent nucleotide sequence differences between pairs of FIV *pol* gene sequences from pumas, lions, and domestic cats. Bars: horizontal striped, within individuals; white, between individuals within clades; black, between clades; diagonal striped, between Pco408, the likely domestic cat FIV from the Peruvian puma, and other puma isolates.

in domestic cats implies that FIV has been present in the puma and lion species longer than in domestic cats. The absence of indications of disease in the puma and lion may reflect a longer period of coevolution between virus and host in these species, whereas in the domestic cat, the virus and host have not yet had time to reach a state of nonpathogenic coexistence (5, 24). However, it is by no means certain that FIV does not cause disease in lions and pumas. Although the domestic cat FIV sequences used in this analysis represent samples from different parts of the world, *pol* sequences are available for only two

TABLE 3. Nucleotide sequence variation

Clade (*n*)	Sequence variation[a]						
	Puma A, puma B	Puma B, lion A	Lion A, lion B	Lion B, lion C	Lion C, cat A	Cat A, cat B	Cat B
Puma A (4)	13.6 (1.7–17.6)	19.5–27.0	21.5–28.8	20.3–25.6	24.9–27.1	21.8–24.6	22.9–25.4
Puma B (17)	23.3	16.0 (1.9–23.6)	24.9–33.1	24.2–31.6	25.6–29.9	23.7–28.5	24.1–29.8
Lion A (11)	24.8	28.9	5.4 (1.6–10.3)	15.8–23.6	19.8–24.7	26.4–32.1	25.7–31.3
Lion B (5)	23.2	27.6	19.3	11.6 (7.2–14.1)	20.7–24.3	24.9–30.4	26.1–29.9
Lion C (7)	26.1	27.5	21.8	22.5	2.5 (0.2–7.4)	24.7–27.6	25.4–28.5
Cat A (12)	23.2	25.8	29.1	27.5	25.9	3.8 (0.4–5.3)	12.8–17.0
Cat B (3)	24.0	26.5	28.3	27.8	26.3	14.8	5.7 (2.9–7.8)

[a] Average nucleotide sequence variation (below diagonal) with range (above diagonal). Within-population variation (range) is on the diagonal.

TABLE 4. Amino acid variation

Clade (*n*)	Sequence variation[a]						
	Puma A	Puma B	Lion A	Lion B	Lion C	Cat A	Cat B
Puma A (4)	10.1 (2.5–13.3)	15.7–23.9	18.0–26.2	18.7–25.0	21.6–25.2	17.1–21.5	16.5–20.3
Puma B (17)	19.0	7.7 (0.6–17.0)	20.3–34.1	19.3–33.6	22.1–29.3	19.7–27.0	20.8–26.4
Lion A (11)	20.8	25.7	6.3 (1.6–16.4)	5.7–23.8	15.1–25.4	21.6–30.3	20.9–28.7
Lion B (5)	21.7	25.3	11.8	9.5 (2.9–13.7)	15.8–22.6	20.9–27.4	20.1–26.6
Lion C (7)	23.7	26.3	17.8	18.6	3.2 (0.7–7.2)	23.0–26.6	20.9–25.9
Cat A (12)	19.2	22.7	25.7	24.5	24.6	3.0 (0.6–5.7)	5.1–10.8
Cat B (3)	18.2	22.8	23.6	24.1	23.1	8.0	3.8 (1.3–5.7)

[a] Average amino acid variation (below diagonal) with range (above diagonal). Within-population variation (range) is on the diagonal.

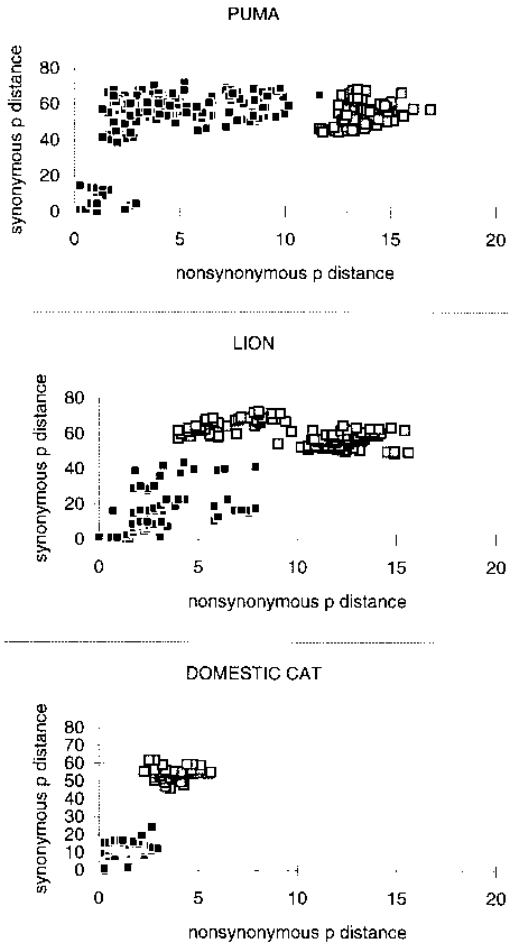


FIG. 6. Synonymous and nonsynonymous p distances between pairs of FIV *pol* gene sequences. Each point represents one pairwise comparison. The synonymous (or nonsynonymous) p distance is the proportion of the possible synonymous (or nonsynonymous) changes which have occurred. Pco408 was not included in these comparisons. Black squares, comparisons within clades; white squares, comparisons between clades.

of the four domestic cat FIV subtypes which have been characterized primarily by using *env* sequences. However, domestic cat *env* subtypes C and D do not appear to be any more divergent than subtypes A and B (15, 38), and so the domestic cat FIV diversity may be adequately represented by the sequences used in this analysis.

The relationships between FIV sequences from different felid species were not well resolved by the sequence data. The most obvious interspecies relationship was the grouping of the leopard sequence with the lion sequences, which corresponded to their common geographic origins. The leopard was from South Africa, as were some of the lions, the other lions being East African (4). The sequence differences suggested that the leopard FIV is not closely aligned to any single lion clade (Fig. 4) but rather is divergent from lion FIV-Ple of all three clades. From this level of divergence, it is likely that leopards also retain a specific strain that has evolved in that species for at least as long as the time separating the three lion FIV clades.

The pattern of variation of FIV sequences in the puma has a complex relationship with geographic origin. The large group of FIV sequences from western North America and Central America exhibited considerable diversity, but the relationships between viral isolates within this group do not appear to coincide with geographic origin. For example, sequence lineages from Arizona (lineages 4 and 7 [Fig. 2]), Colorado (lineages 4 and 9), and Wyoming (lineages 7 and 8) are not monophyletic. This may reflect the migration which occurs between puma populations when young adults seek to establish new home ranges (1, 14). The puma FIV-Pco sequences from Wyoming showed a surprising amount of diversity considering that the samples came from within the relatively small area of Yellowstone National Park. This diversity may be due to the fact that pumas were eliminated from Yellowstone during the 1920s and for the following 50 years (14). The park was eventually recolonized naturally by animals arriving from other areas (14). Individuals from different areas may have brought different types of FIV with them into Yellowstone.

The Florida panthers, an endangered relict population, have been geographically isolated from other North American pumas for approximately 80 years as a result of depredation and

habitat destruction (1, 14, 35). The present Florida panther populations are themselves made up of two distinct populations which evolved in different parts of North America and were recently mixed by a release program (25, 35). This may explain the magnitude of sequence difference (15% nucleotide divergence) between the two sequences shown in the phylogenetic analysis: Pco18, from an authentic Florida panther from Big Cypress Swamp, and Pco61-3, from a Florida panther × central America subspecies intercross from the Everglades (25). It was unexpected that the Californian puma should carry an FIV strain much like that of the Florida panthers rather than one like that of the animals from nearby areas. The California sequence (Pco366-13) had numerous differences from each of the Florida sequences (Fig. 3), and so perhaps the similarities which define puma clade A are simply retained ancestral features which have been lost in many of the B clade sequences. The Californian puma was a free-ranging animal, and there is no information to suggest it was not characteristic of its population.

The phylogenetic topologies, amino acid sequence alignment, and percent differences between clades and species collectively indicate that the puma clade A may represent an intermediate between clade B and the FIV isolates from other species (Fig. 4). Additionally, the Vancouver Island and Brazilian sequences may represent intermediates between the two puma clades (Fig. 3). These aspects lead to the following speculation for evolution of FIV-Pco in the puma. Puma clade A may represent an ancestral type of puma FIV which was once widespread but is now represented only by sequences from Florida and California. A second wave of viral proliferation in pumas is represented by Brazil and Vancouver Island sequences, while a widely dispersing type is represented by the western North America and Central American sequences. This model accounts for the fact that FIV-Pco strains from Florida and California, and to a lesser extent Brazil and Vancouver Island, which share common ancestral features, are found at the periphery of the puma's range, being separated by a central core of more recently evolved FIV-Pco strains.

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