

Phylogeographic Patterns of Feline Immunodeficiency Virus Genetic Diversity in the Domestic Cat

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Feline immunodeficiency virus (FIV) has a worldwide distribution among feral and domesticated cats and in many cases induces immunodeficiency disease analogous to that of human acquired immune deficiency syndrome. FIV is genetically homologous to human immunodeficiency virus (HIV) in both genome organization and gene sequence and, like HIV, exhibits enormous sequence variation throughout the range of host species. We sampled 91 feral cats from six disparate locales and studied the phylogenetic relationships of viral DNA from infected cats using both *pol* and *env* genes (520 and 684 bp, respectively). The patterns from the two genes recapitulated previously described major FIV clades and showed concordance between phylogenetic patterns of the *pol* and *env* genes. Evidence for recombination between the *pol* and *env* genes was not found among a sampling of nine isolates, although evidence for intragenic exchange within the *env* gene was observed in two isolates. A small local population of cats from a rural farm in the United Kingdom had a remarkably high FIV antibody prevalence (47%), but displayed 8-fold less overall diversity of FIV genomic variation compared with FIV from different parts of the world. We interpret this low variation as a consequence of a recent monophyletic introduction of FIV into the population. © 1998 Academic Press

INTRODUCTION

Feline immunodeficiency virus (FIV) is the cause of an immune system disease in domestic cats, which results in depletion of the CD4⁺ cells, susceptibility to opportunistic infections, and sometimes death (Dua *et al.*, 1994; English *et al.*, 1994; Pedersen, 1993; Pedersen *et al.*, 1987; Reubel *et al.*, 1994; Yamamoto *et al.*, 1989). It is analogous to human immunodeficiency virus (HIV) infection in humans and therefore is a useful animal model for acquired immune deficiency syndrome (Dua *et al.*, 1994; English *et al.*, 1994; Pedersen, 1993; Reubel *et al.*, 1994). FIV infection has been reported in domestic cats from many parts of the world (Pedersen, 1993). Feline lentivirus strains antigenically and phylogenetically related to FIV are present in 18 other felid species, and each host species appears to carry its own distinctive strain of FIV (Brown *et al.*, 1993, 1994; Carpenter and O'Brien, 1995; Carpenter *et al.*, 1996; Olmsted *et al.*,

1992). There is extensive viral variation within host species, and each species-specific strain can be divided into several subtypes or clades, similar to those that have been described for HIV-1 (Brown *et al.*, 1994; Carpenter and O'Brien, 1995; Carpenter *et al.*, 1996; Kakinuma *et al.*, 1995; Louwagie *et al.*, 1993; Sodora *et al.*, 1994; vanden Haesevelde *et al.*, 1994).

The genetic characteristics of domestic cat FIV have been revealed by complete genomic sequences of several isolates (Maki *et al.*, 1992; Olmsted *et al.*, 1989; Phillips *et al.*, 1990; Talbott *et al.*, 1989). The viral genome is made up of three major genes and several smaller regulatory genes (Olmsted *et al.*, 1989; Talbott *et al.*, 1989). The *pol* and *gag* genes encode viral enzymes and core proteins, respectively, and are relatively highly conserved (Olmsted *et al.*, 1989; Phillips *et al.*, 1990). The *env* gene encodes surface and transmembrane glycoproteins and is highly variable compared with the other two structural genes (Olmsted *et al.*, 1989; Phillips *et al.*, 1990). Within the *env* gene, nine variable regions have been defined, separated by blocks that are more conserved (Pancino *et al.*, 1993b). Sequences from regions of the *gag*, *pol*, and *env* genes from several isolates have been used to estimate the rate of evolution of FIV, to analyze the impact of natural selection on FIV evolution, and to determine the relationships among FIV isolates (Greene *et al.*, 1993; Kakinuma *et al.*, 1995; Pancino *et al.*, 1993a, 1993b; Rigby *et al.*, 1993). Phylogenetic analyses

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of *env* genes have revealed five viral subtypes (Kakinuma *et al.*, 1995; Pecoraro *et al.*, 1996; Sodora *et al.*, 1994), a number that can be expected to increase as further studies reveal additional diversity. The isolates for which complete genomes were sequenced included members of the two most common subtypes.

The rapid evolution of lentiviruses within an infected individual results in the formation of a viral quasispecies, a phenomenon well documented in HIV infection (Brown *et al.*, 1994; Cichutek *et al.*, 1992; Eigen, 1993; Pedroza Martins *et al.*, 1992). Thus a number of similar viruses (or viral sequences) may be isolated from one individual. Intraindividual sequence variation of the FIV *env* gene in domestic cats has been reported to be as high as 2.5% (Sodora *et al.*, 1994). The extent of *pol* gene variation within individual domestic cats has not been reported. In individual lions infected with lion FIV, *pol* gene variation reaches 4% (Brown *et al.*, 1994), whereas in pumas it is generally <1%, except in rare cases of individuals coinfecting with two strains of virus (Carpenter *et al.*, 1996).

In addition to rapid evolution by nucleotide substitutions and insertions/deletions, lentiviruses gain diversity through recombination (Robertson *et al.*, 1995; Sabino *et al.*, 1994; Zhu *et al.*, 1995). This may occur when individuals are dually infected with two different viral strains (Robertson *et al.*, 1995; Sabino *et al.*, 1994; Zhu *et al.*, 1995). Coinfection of domestic cats with two strains of FIV has been experimentally induced, and the hybrid viral strains produced by recombination were observed (Kyaw-Tanner *et al.*, 1994; Okada *et al.*, 1994). The prevalence of recombinant isolates in naturally infected cats is not known; however, mosaic sequences have been detected that appeared to be the result of recombination within a region of the *env* gene (Bachmann *et al.*, 1997). Additionally, strains of HIV have been reported that appear to be recombinants (Robertson *et al.*, 1995; Sabino *et al.*, 1994; Zhu *et al.*, 1995). The role of recombination in generating diversity among FIV isolates may have a significant impact on the effectiveness of vaccines, may accelerate the evolution of viruses with new characteristics of transmission and virulence, and also has the potential to confound the classification of FIV isolates.

To examine more closely the genetic variation of FIV in the domestic cat, we sequenced regions of the *pol* and *env* genes from novel FIV isolates. These data have been used to estimate the variation of FIV within individuals and within a small, well-defined population to determine the relationship of novel isolates to previously reported isolates and to assess the impact of recombination in generating viral diversity.

RESULTS

Seroprevalence of FIV in domestic cats

Two of the populations tested for FIV included seropositive individuals; these were the Barley Park Farm

TABLE 1

Prevalence of FIV Exposure in the Domestic Cat (*Felis catus*) as Determined by Western Blot Detection of Serum Antibody to FIV

Source	No. of cats that were positive	No. of cats that were tested	Positive (%)
Barley Park Farm, United Kingdom	26	55	47
Central and South American zoos	3	11	27
Sakkarbaug Zoo, India	0	3	0
Moscow and St. Petersburg zoos, Russia	0	5	0
Jacksonville and White Oak zoos, Florida	0	10	0
Thailand zoos	0	7	0
Total	29	91	32

population in the United Kingdom, in which 47% of the cats were seropositive, and the domestic cats from Central and South American zoos, of which 27% gave positive results (Table 1). None of the domestic cats from zoos in Florida, Thailand, Russia, or India were seropositive for FIV. Among the Barley Park Farm cats, there were no significant differences ($P > 0.05$) in FIV seroprevalence between male and female cats or between adults and juveniles, according to χ^2 tests.

Genetic variation of the FIV *POL* gene in a small population of domestic cats

Twenty-three of the 26 seropositive Barley Park Farm cats yielded FIV *pol* sequences. Twenty-four different FIV *pol* sequences were isolated from the 64 clones sequenced. One common sequence (GG1-10; Fig. 1) was isolated from 11 different individuals, and all the other sequences differed from this one by seven or fewer nucleotide substitutions in the 476 bp that were sequenced. Many of the changes distinguishing the derived sequences from GG1-10 were seen in only one sequence, resulting in a polytomy in the tree topology. The low level of variation among the sequences and the unstructured tree topology suggest that the isolates sequenced were recently derived from a monophyletic introduction into the population.

Variation between isolates from different individuals in the population was <2.2%. The population was estimated to have been in existence for 60 years, so FIV has been diverging in this population for 0-60 years. These figures allowed us to calculate a minimum rate of divergence of 0.04% per year. Multiple sequences from each of 11 cats revealed that intraindividual variation was <0.7%. Assuming an individual life span of 15-20 years, the estimated rate of divergence was concordant with the 0.04% per year calculated above. However, the rate presumes that the population became infected 60 years

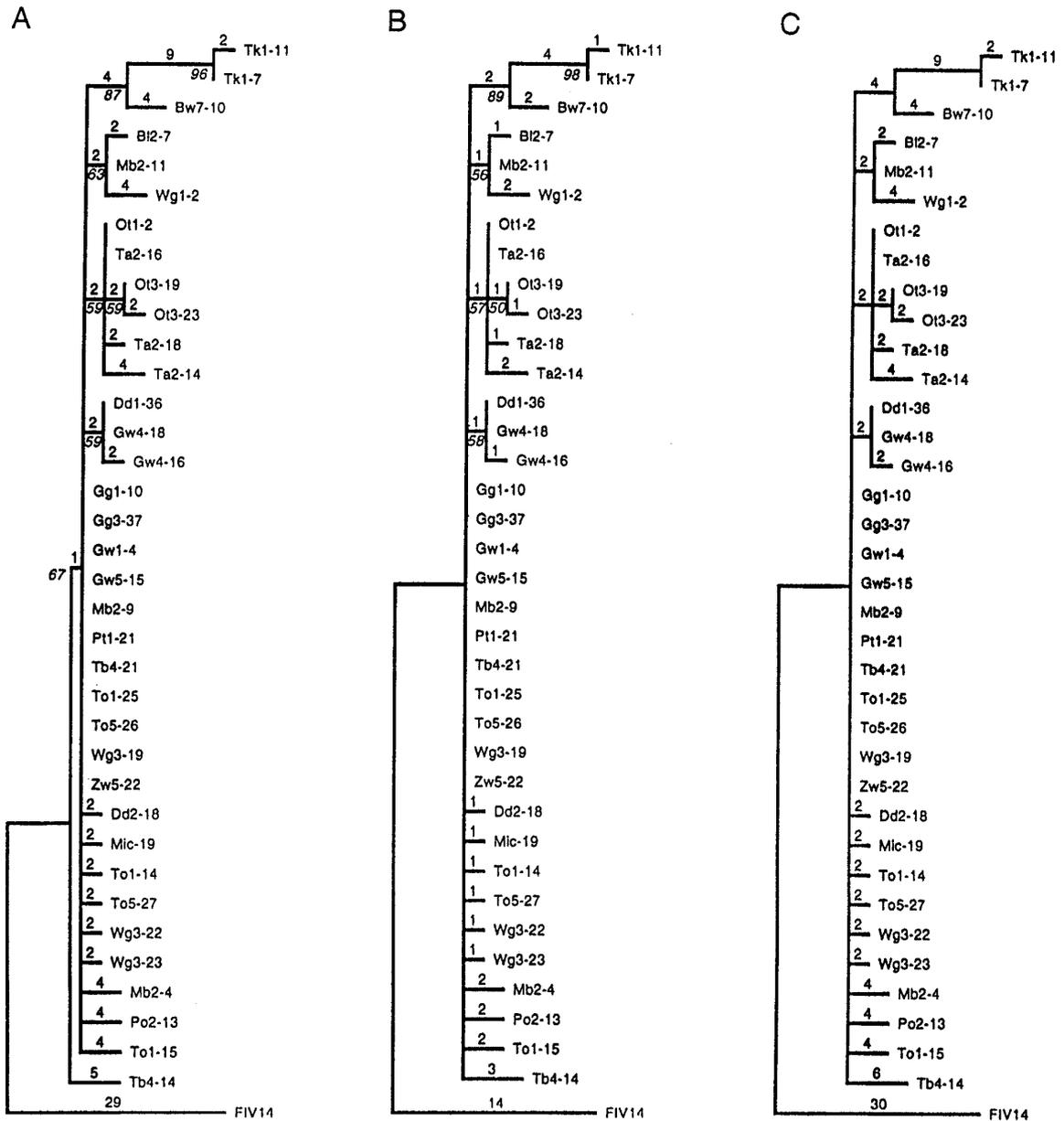


FIG. 1. Phylogenetic analysis of 36 FIV *pol* sequences from 23 cats in the Barley Park Farm domestic cat population, using the Petaluma isolate FIV14 as an outgroup. (A) Neighbor joining tree with branch lengths equal to percent sequence divergence. Bootstrap values (%) are shown at the nodes. (B) Maximum parsimony tree. Branches are labeled with the number of substitutions. Bootstrap values (%) are shown at the nodes. This is one of two equally parsimonious trees of length 50. (C) Maximum likelihood tree with branch lengths equal to the expected number of substitutions per 100 sites. Branch lengths not significantly different from zero were collapsed into polytomies. Ln likelihood = -954.3; 9271 trees were examined. Sequence nomenclature: Tk1-11 represents clone 11 from animal Tk1.

ago; any more recent introduction would increase the divergence rate estimate. Furthermore, because FIV may reduce the life span of infected cats, the actual rate of divergence may be even greater.

Relationships among domestic cat FIV gene sequences from different locations

FIV *pol* sequences were obtained from two of the three FIV seropositive domestic cats from Central and South

America. The sequences from Nicaragua (Fca151 from Managua Zoo) and Argentina (Fca155 from Mendoza Zoological Park) were analyzed with three representative sequences from the Barley Park Farm cats (Gg1-10, Wg1-2, and Tk1-11) and other previously published FIV *pol* sequences (Fig. 2). The Nicaraguan, Argentinean, and Barley Park Farm sequences were grouped with FIV *pol* sequences from California, Switzerland, and Australia. The Swiss and Californian isolates have previously

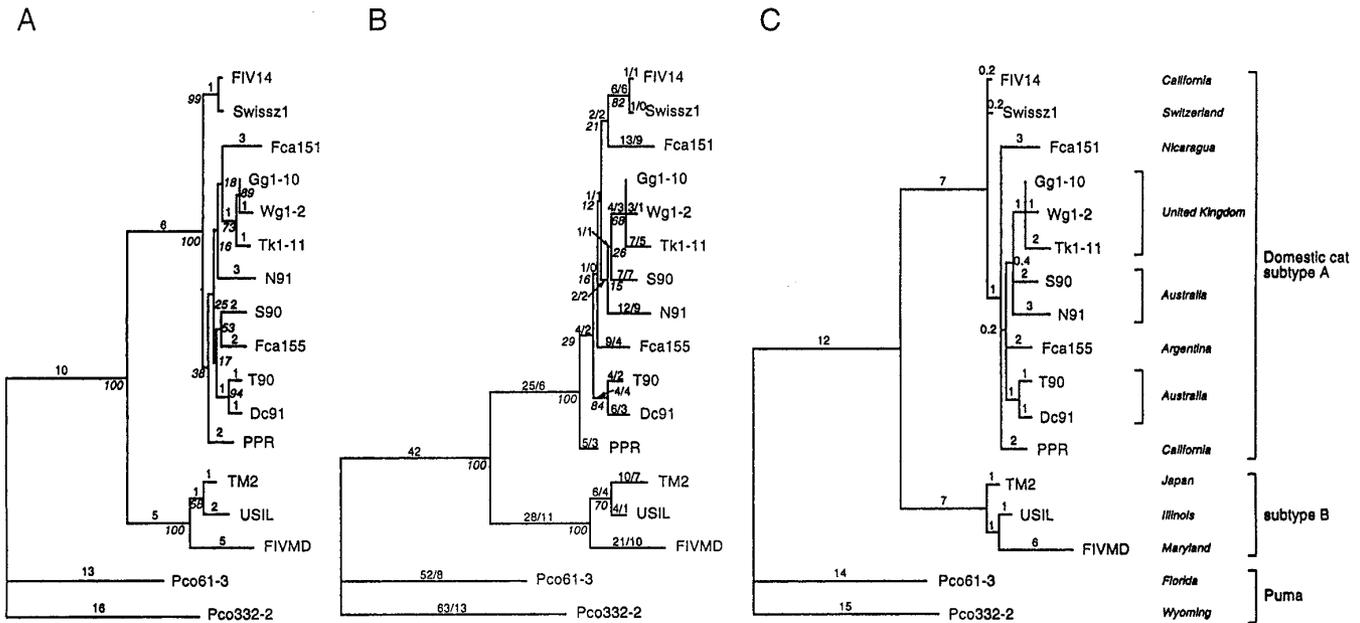


FIG. 2. Phylogenetic analysis of FIV *pol* sequences from domestic cats using puma FIV sequences (Carpenter *et al.*, 1996) as outgroups. (A) Neighbor joining tree with branch lengths representing percent sequence divergence. Bootstrap values (%) are shown in italics at the nodes. (B) Maximum parsimony tree. Branches are labeled with number of substitutions/number of homoplasies. Bootstrap values (%) are shown at the nodes. This is one of two equally parsimonious trees of length 344 whose only difference was in joining S90 to N91 or to the Barley Park Farm sequences. The consistency index was 0.750. (C) Maximum likelihood tree with branch lengths equal to the expected number of substitutions per 100 sites. Branch lengths not significantly different from zero were collapsed into polytomies. Ln likelihood = -2275.7 ; 937 trees were examined. Designated FIV subtypes (Kakinuma *et al.*, 1995; Sodora *et al.*, 1994) and geographic origins of the samples are indicated at the right.

been defined as members of subtype A based on *env* sequences (Kakinuma *et al.*, 1995; Sodora *et al.*, 1994). The three Barley Park Farm sequences formed a monophyletic group within subtype A according to all three phylogenetic methods. The relationships among other sequences within subtype A were less resolved by the topology.

The *env* gene sequences (684 bp including the highly variable regions V3–V5) were also obtained from the Argentinean and Nicaraguan isolates and from two representative samples from the Barley Park Farm cats. These were included in a phylogenetic analysis with previously published FIV *env* sequences (Fig. 3). There was some difficulty in aligning the sequences due to the high variability in the V5 (fifth variable) region, which includes a number of insertions/deletions and low sequence similarity. We therefore truncated the sequences at 622 nucleotides to remove the poorly aligned region. Alignment of the sequences revealed a 3-bp deletion in the two Barley Park Farm sequences, which was the only length variation observed in the V4 region. The V3 region had no length variation. The Nicaraguan, Argentinean, and Barley Park Farm sequences were all included within the A subtype (Fig. 3). This placed them with sequences from Europe and California and an atypical Japanese sequence (Sendai1) and separate from the U.S., Japanese and Canadian sequences forming subtypes B, C, and D. The relationships within subtype A

were not resolved as was shown by differences between the results from the three phylogenetic methods and by the low bootstrap values. Thus it was not possible to state that the novel isolates have an affinity for isolates from any particular location. The two Barley Park Farm sequences were clustered together similar to the *pol* gene analysis.

Recombination in the FIV genome

There were nine isolates for which both *pol* and *env* sequences were available, which allowed a comparison of the relationships revealed by each of the genes. The affiliation of isolates as A and B subtypes was consistent for the two genes. The relationships within subtype A were not resolved well enough to reveal any significant differences between the *pol* and *env* topologies, giving no evidence for recombination between the two genes. Examination of 15 *pol* gene sequences (those shown in Fig. 2) produced no evidence for recombination within the *pol* gene.

The V3–V5 region of the *env* gene was divided into five overlapping segments (1–240, 110–340, 240–450, 340–560, 450–684), and UPGMA (Devereux *et al.*, 1984) trees were constructed for each segment using the 42 sequences shown in Fig. 3A. Comparison of the five segment-specific trees revealed three isolates (Yokohama, Aomori1, and Fukuoka), for which the different segments

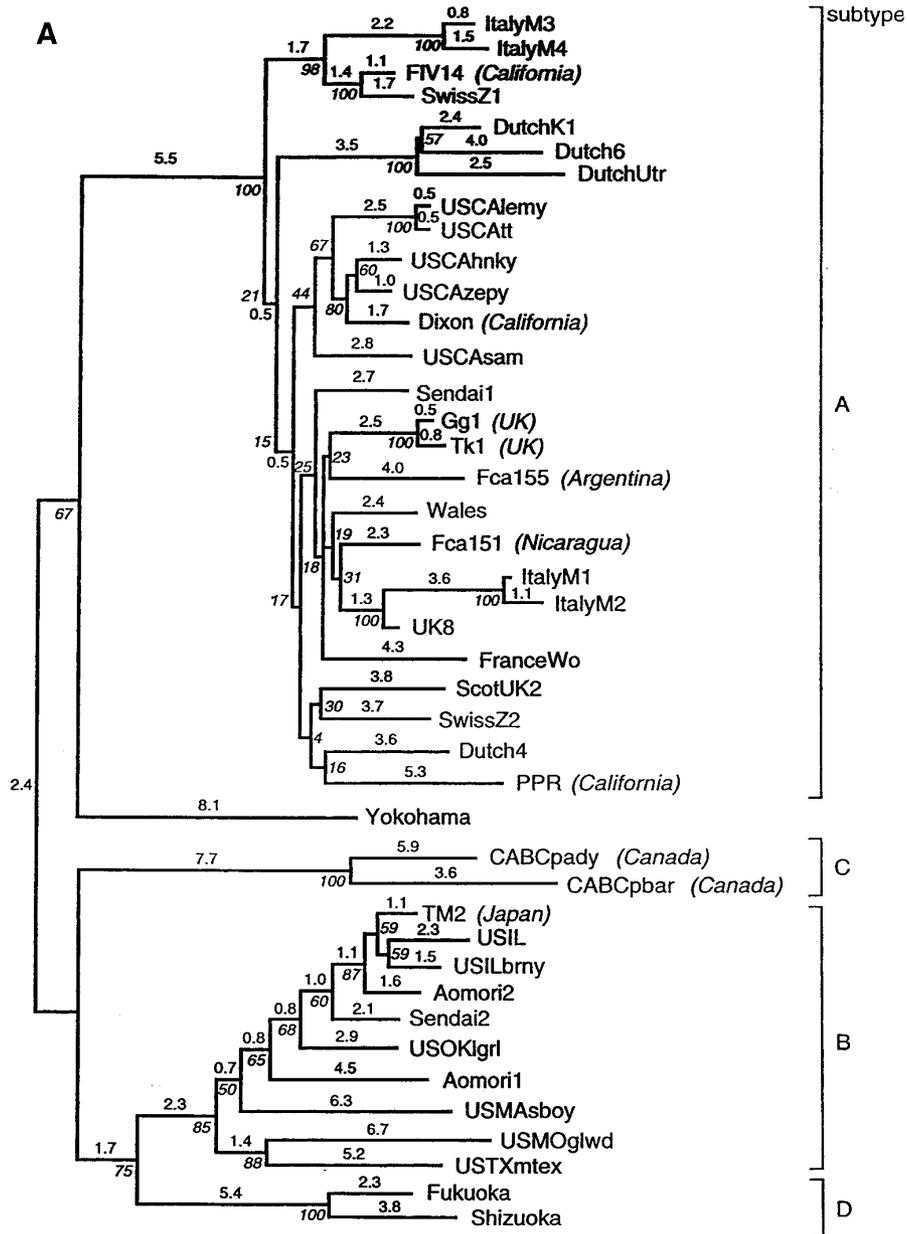


FIG. 3. Phylogenetic analysis of FIV *env* sequences (V3-V5 region) from domestic cats. (A) Neighbor joining tree with branch lengths representing percent sequence divergence. Bootstrap values (%) are shown at the nodes. (B) Maximum parsimony tree of a subset of the *env* sequences, constructed using midpoint rooting. Branches are labeled with number of substitutions/number of homoplasies. Bootstrap values (%) are shown in italics at the nodes. Tree length = 695; consistency index = 0.590. (C) Maximum likelihood tree with branch lengths equal to the expected number of substitutions per 100 sites. Branch lengths not significantly different from zero were collapsed into polytomies. Ln likelihood = -4323.1; 2399 trees were examined.

aligned the strains as different subtypes among the five analyses. A maximum parsimony analysis was repeated for the complete *env* gene of these isolates plus 14 published sequences but divided the sequences further into nine overlapping fragments. Two additional isolates, PPR and Sendai2, had regions that showed affinity for different subtypes in the nine topologies, identifying a total of five FIV isolates that appear to have been derived from intragenic recombination between subtypes (Table

2). The relationships within the subtypes were not sufficiently resolved to allow further indication of sequences generated by recombination within subtypes.

Alignment of the five putative recombinant sequences with index sequences characteristic of each subtype allowed estimation of the points of crossover for each recombinant sequence (Fig. 4). Four of the five recombinant sequences were from a single study (Kakinuma *et al.*, 1995), and of those, three (Aomori1, Sendai2, and

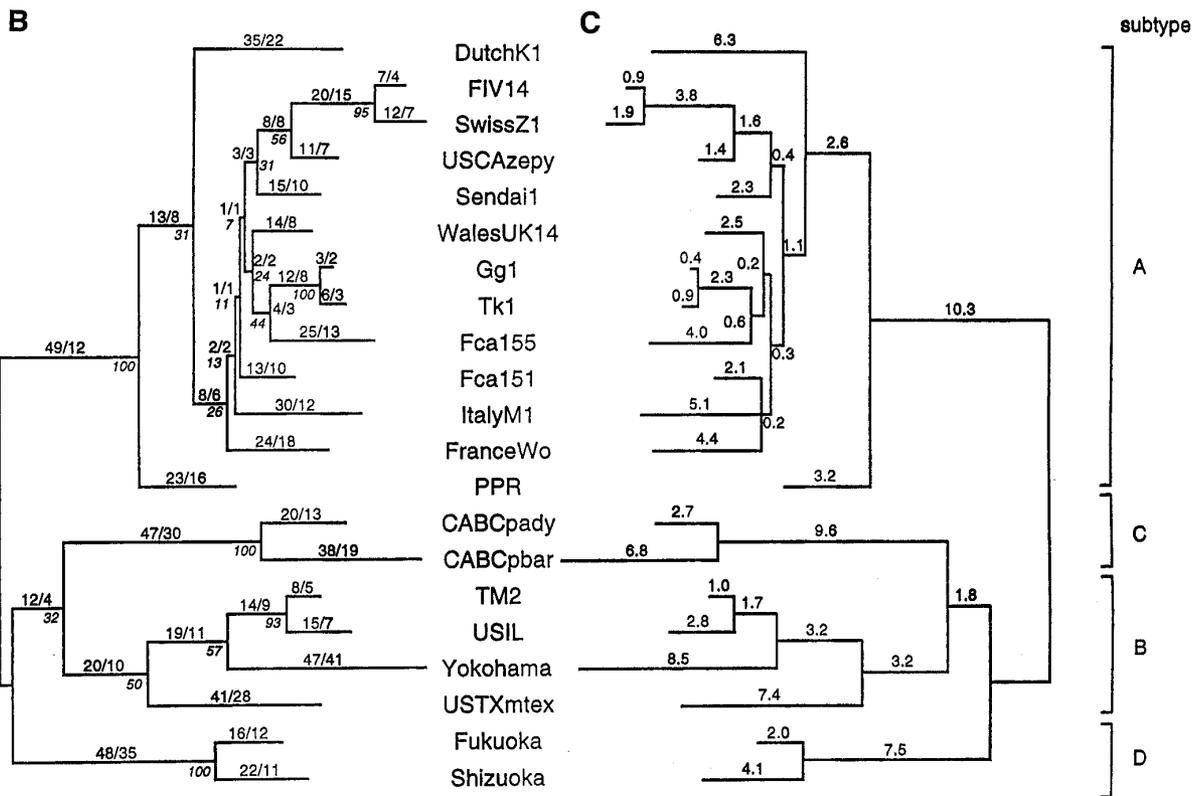


FIG. 3—Continued

Fukuoka) appeared to be the result of recombination with virus strains derived from a single laboratory that presented the study (Kakinuma *et al.*, 1995). This raised

the possibility that they could be the result of a contaminated PCR rather than genuine *in vivo* viral recombination. This suggestion is supported by the observation

TABLE 2
Subtypes of Segments of the *env* Gene Sequences from 17 FIV Isolates

	1: 1–517 ^a	2: 258–776	3: 517–1034	4: 776–1293	5: 1034–1551	6: 1293–1810	7: 1551–2068	8: 1810–2326	9: 2068–2585	Total: 1–2585
Aomori1	B	B	D	D	D	B	B	B	B	B
Sendai2	B	B	D	D	B	B	B	B	B	B
Aomori2	B	B	B	B	B	B	B	B	B	B
Yokohama	B	B	A	A	?	B	B	B	B	B
TM2	B	B	B	B	B	B	B	B	B	B
USIL	B	B	B	B	B	B	B	B	B	B
Fukuoka	D	D	D	D	D	D	B	B	B	D
Shizuoka	D	D	D	D	D	D	D	D	D	D
Dixon	A	A	A	A	A	A	A	A	A	A
Sendai1	A	A	A	A	A	A	A	A	A	A
UK2	A	A	A	A	A	A	A	A	A	A
UK8	A	A	A	A	A	A	A	A	A	A
FIV14	A	A	A	A	A	A	A	A	A	A
SwissZ2	A	A	A	A	A	A	A	A	A	A
Dutch113	A	A	A	A	A	A	A	A	A	A
Dutch19K	A	A	A	A	A	A	A	A	A	A
PPR	A	A	A	A	A	A	A	B	A	A

^a Numbers refer to the nucleotide positions comprising each of the nine segments of the *env* gene. Numbering is according to aligned nucleotide sequences starting at position 1 of Fukuoka.

that the Fukuoka and Sendai2 *env* genes are identical over a region of 750 nucleotides at the 3' end.

The other two examples of recombination are more persuasive. The Yokohama *env* sequence, which as a whole is grouped in subtype B, contains a region of 450 nucleotides that is characteristic of subtype A, with greatest similarity to the FIV14 sequence. Similarly, PPR (subtype A) contains a region of 240 nucleotides that causes it to be more closely associated with subtype B than with subtype A, although it is not closely related to any of the subtype B isolates so far reported (Fig. 4).

DISCUSSION

The seroprevalence of FIV in the Barley Park Farm population was, at 47%, consistent with the results of a previous study that found 53% of the same population to be seropositive (Yamaguchi *et al.*, 1996). These figures are high compared with other reports for both feral and pet domestic cats (Pedersen, 1993; Yamamoto *et al.*, 1989). This reflects the highly interactive nature of this small population centered on reliable food resources. The independence of seroprevalence from age and gender is in accordance with previous studies of this population (Yamaguchi *et al.*, 1996). The intraindividual variation in the *pol* gene (<0.7%) was similar to that reported in pumas (<1% in most individuals; Carpenter *et al.*, 1996) and less than that in lions (4%; Brown *et al.*, 1994). The levels of *pol* gene variation within and between individuals in the Barley Park Farm population gave a minimum rate of divergence of 0.04% per year.

The FIV *pol* and *env* sequences from Argentinean and Nicaraguan domestic cats are members of subtype A, which expands the range of this clade to cover parts of Central and South America as well as Europe, California, and Australia (Greene *et al.*, 1993; Kakinuma *et al.*, 1995; Sodora *et al.*, 1994). This contrasts with segments of the *env* gene from other Argentinean isolates that were members of a novel subtype (Pecoraro *et al.*, 1996) and with Argentinean long terminal repeat sequences that were members of subtype B and a novel subtype (Yamada *et al.*, 1995). The relationships within subtype A showed little structure in both the *pol* and *env* gene analyses, which suggests that FIV underwent a sudden major radiation some time after the subtypes diverged. Such a radiation is likely to have occurred within the past 140 years (as estimated from the minimum rate of divergence stated above and the 5.3% diversity observed within subtype A; Carpenter *et al.*, 1996) and may have been associated with the increasing density and mobility of the human population (and therefore cat population) during this time.

HIV isolates that are mosaics of genetically divergent viral strains have been identified (Robertson *et al.*, 1995). In FIV, we found no evidence for recombination between the *pol* and *env* genes: the A and B subtypes were

consistent between the genes. The A, B, and D subtypes were also consistent for *gag* gene sequences (Kakinuma *et al.*, 1995). However, recombination does appear to be occurring within the *env* gene of FIV, as might be expected for one of the most variable parts of the FIV genome. Similarly, recombination has been detected in the HIV-1 *env* gene, both within and between subtypes (Sabino *et al.*, 1994; Zhu *et al.*, 1995).

Recombination in the FIV *env* gene was noted predominantly among the subtype B isolates. We question the validity of some of these sequences but cannot determine whether they are authentic or PCR artifacts. This highlights the necessity for extreme care when using PCR, especially when a number of similar sequences are being amplified, making the products of contamination difficult to detect. It is also possible that recombination actually does occur at high frequency in subtype B isolates.

Analyses of two of the sequences provide convincing evidence for recombination within the FIV *env* gene. One of these (Yokohama) has also been detected in a study analyzing the V3-V4 region of the *env* gene (Bachmann *et al.*, 1997). Recombination within the FIV *env* gene has been demonstrated in cats experimentally superinfected with FIV (Kyaw-Tanner *et al.*, 1994). Our results show that *env* gene recombination also occurs in naturally infected cats, which indicates that superinfection is also occurring naturally. Evidence for naturally occurring superinfection has also been found in a study of *pol* gene sequences from FIV infecting pumas (Carpenter *et al.*, 1996).

In addition to recombination between FIV subtypes, recombination may also occur between FIV strains of the same subtype, but this is more difficult to detect due to the greater similarity of the two parent sequences. However, it is recombination between subtypes that is most interesting because it is a means by which viral genetic diversity can be hugely increased, potentially altering aspects of the epidemiology of FIV, such as transmissibility, infectivity, and pathogenicity.

MATERIALS AND METHODS

Blood samples were collected from domestic cats at zoos in Central and South America, Florida, Thailand, India, and Russia and from a population of feral farm cats in the United Kingdom. This population consisted of 50–80 individuals whose behavior and disease status have been extensively studied (Yamaguchi *et al.*, 1996). Serum or plasma samples were tested for the presence of FIV-reactive antibodies by Western blotting, as described previously (Brown *et al.*, 1993).

A 520-bp proviral DNA fragment was amplified from genomic DNA isolated from the white blood cells of seropositive animals, using primers described previously (Brown *et al.*, 1994). The amplified fragment was

from a relatively conserved part of the viral genome, the region of the *pol* gene that encodes reverse transcriptase. A nested PCR was used, the first round with primers 1258F and 1260R, with 0.3–0.5 μg of genomic DNA, in a total volume of 50 μl . The second round used primers 1259F and 1261R 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 fragments were cloned into pBluescript KS⁺ (Stratagene), and the resulting plasmids were sequenced using an ABI dye primer kit and automated sequencer (Applied Biosystems).

Fragments of the highly variable *env* gene were also amplified by nested PCR, using 100 ng of white blood cell genomic DNA and published primers (Sodora *et al.*, 1994). Primers *Fenv23* and *Fenv22* were used for the first round, in a 50- μl reaction. The second round reaction used 10 μl of the first round products and modified *Fenv31* and *Fenv24* primers, which differed from those described (Sodora *et al.*, 1994) in that the restriction site was not included. The PCR conditions were 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by 10 min at 72°C. The product of ~2 kb was cloned into pBluescript KS⁺ and sequenced using an ABI dye terminator Prism kit, with the following primers: *Fenv1*, 5'-ACAAAACAATTAAGCTATTGTACAGA-3'; *Fenv2*, 5'-TTTGGATGTCATAGGAATAAATCA-3'; *Fenv3*, 5'-TGATTTATTCCTATGACATCCAAA-3'; and *Fenv4*, 5'-CGGGGTTATACCAATTTCTAA-3'. These primers produced a 684-bp sequence encompassing the V3–V5 variable regions of the gene.

Sequences were aligned using the algorithm of Needleman and Wunsch (1970) as provided by the PILEUP program in the GCG package (Devereux *et al.*, 1984). Genetic distances between pairs of DNA sequences were calculated by the DNADIST program in PHYLIP, Version 3.5 (Felsenstein, 1993), using Kimura's two-parameter model (Kimura, 1980). Minimum evolution trees were constructed by the neighbor joining method of Saitou and Nei (1987), as provided by NEIGHBOR program in PHYLIP 3.5 (Felsenstein, 1993). For maximum parsimony analysis of DNA sequences, we used PAUP, Version 3.1.1 (Swofford, 1985). Both neighbor joining and maximum parsimony trees were evaluated statistically using 100 bootstrap iterations (Felsenstein, 1985). 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.

To identify possible recombinant sequences, we divided the sequences into overlapping fragments and constructed trees for each fragment using PILEUP (Devereux *et al.*, 1984) or PAUP (Swofford, 1985). The trees were compared to reveal sequences whose positions were inconsistent. Putative sites of crossover were located by visual inspection of the aligned sequences.

Sequences reported in this paper have been submitted to GenBank under the following accession numbers: U53758, U53760, U53762–U53766, and AF051801–AF051806. Additional sequences were obtained from GenBank, using either published accession numbers (Carpenter and O'Brien, 1995; Kakinuma *et al.*, 1995; Olmsted *et al.*, 1992; Sodora *et al.*, 1994) or the following: L16938, L16942, L16940, S67753, and U11820.

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