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Phylogenetic analysis of the long terminal repeat of feline immunodeficiency viruses from Japan, Argentina and Australia

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Summary. The nucleotide sequences of the long terminal repeat of five Japanese, five Argentine and three Australian isolates of feline immunodeficiency virus (FIV) were determined and compared with those of isolates previously described. The results revealed that the Japanese isolates were found to cluster with nucleotide sequence similarity of 95.6%–99.4%. The Australian isolates also clustered with nucleotide sequence similarity of 97.2%–99.4%. The Argentine isolates formed two groups; the LP9 isolate is closely related to the Japanese isolates, whereas the LP1, LP3, LP20 and LP24 isolates are distant from both the Japanese and Australian isolates. From these results, FIV can be divided into three groups, namely: (I) the Californian, Australian and British isolates; (II) the Japanese isolates and one Argentine LP9 isolate; (III) the other Argentine isolates.

Introduction

Feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus of cats, which was initially isolated in 1986 from a cat with symptoms of an immunodeficiency-like syndrome [21]. Additional isolates have been reported in the United Kingdom [7], the United States [23], Japan [12, 14] and other countries [1, 17, 25]. FIV infection has a worldwide distribution as revealed by seroepidemiological surveys [2, 6, 9, 11, 12, 26, 30]. FIV infection in cats is associated with immunodeficiency-like diseases similar to those seen with human immunodeficiency virus type 1 (HIV-1)-infection [10–12, 22, 33, 34].

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The biological and immunological similarities between FIV and HIV-1 make FIV infection of cats a useful small animal system for studies on pathogenesis, vaccination and antiviral chemotherapy of human acquired immunodeficiency syndrome.

Lentiviruses display a large degree of sequence variation. The variation is primarily ascribed to the low fidelity of their reverse transcriptase used for replication, which has limited or no proof-reading function [29]. As described for HIV-1, there is considerable genetic variation in FIV [3, 5, 13, 14, 19, 20, 23, 27, 31]. These studies provide insight into the molecular basis of viral virulence and pathogenesis, and assist in understanding the evolution and origin of FIV. Recently Sodora et al. [28] have established that FIV can be divided into three groups, A, B and C on the basis of phylogenetic analysis of FIV *env* genes. Group A consists of isolates mainly from the United States (California) and Europe. Group B is represented by isolates from Japan and the United States (not California). Group C consists of isolates from Canada (British Columbia). Rigby et al. [24] also reported that a Japanese isolate (TM2 isolate) represented a distinct group based on both *gag* and *env* sequences. Furthermore, Green et al. [4] reported that Australian and United State (California) isolates clustered and the Japanese and Maryland (not California) isolates formed another cluster on the basis of nucleotide sequence variations found within the *pol* region of FIV.

To confirm and extend these observations, we analyzed the nucleotide sequences of the long terminal repeat (LTR) of five Japanese, five Argentine and three Australian isolates of FIV. Additionally, analysis of the newly determined sequence of LTR of five isolates from Japan will elucidate the phylogenetic nature of FIV in Japan. Moreover, since no FIV isolation was reported from South America, five Argentine isolates used in this study may provide insights into the worldwide spread of FIV. We selected the LTR region for phylogenetic analysis of FIV for the following reasons: (1) the LTR is relatively variable; (2) in contrast to the *env* gene, the LTR is less affected by the immune selection; (3) because it does not code for proteins, the LTR is not affected by the triplet codon; (4) recent reports of phylogenetic analyses of FIV have focused on *gag*, *pol* and *env* regions [4, 24, 28], and there are no reports with regard to the LTR.

The nucleotide sequences of LTR from 13 distinct isolates were determined and compared with the corresponding sequences of the previously published two Japanese isolates of TM1 and TM2, two U.S. isolates of Petaluma and PPR and two British isolates of UK8 and UK14 [20, 23, 31, 32]. A phylogenetic tree based on the nucleotide sequences of the LTR was constructed in order to establish their possible ancestral relationship to each other.

Materials and methods

Virus isolation and cell culture

FIV isolates used in this study are listed in Table 1. FIV isolates TM3, TM4, LP1, LP3, LP9, LP20 and LP24 were isolated in our laboratory. The origins of other FIV isolates

Table 1. Feline immunodeficiency viruses used in this study

Isolate	Origin	Reference
TM1	Tokyo, Japan	Miyazawa et al. (1989)
TM2	Tokyo, Japan	Miyazawa et al. (1989)
TM3	Tokyo, Japan	this study
TM4	Tokyo, Japan	this study
KYO-1	Tokyo, Japan	Hayami et al. (unpubl.)
Shizuoka-1	Shizuoka, Japan	Koyama et al. (unpubl.)
Aomori-1	Aomori, Japan	Koyama et al. (unpubl.)
LP1	La Plata, Argentina	this study
LP3	La Plata, Argentina	this study
LP9	La Plata, Argentina	this study
LP20	La Plata, Argentina	this study
LP24	La Plata, Argentina	this study
N91	Perth, Australia	Green et al. (1993)
R91	Australia	Green et al. (unpubl.)
T90	Perth, Australia	Green et al. (1993)
Petaluma	California, USA	Pedersen et al. (1987)
PPR	California, USA	Phillips et al. (1990)
UK8	Portsmouth, England	Rigby et al. (1993)
UK14	Colwyn Bay, Wales	Rigby et al. (1993)

are described in Table 1. For virus isolation, the peripheral blood mononuclear cells (PBMC) from infected cats were purified over Ficoll-Hypaque and cocultivated with a feline interleukin-2 (IL-2) dependent T-lymphoblastoid cell line (MYA-1 cells) [15]. Cultures were maintained in RPMI-1640, supplemented with 10% fetal bovine serum, antibiotics, 50 μM 2-mercaptoethanol, 2 μg/ml polybrene, and 100 units/ml recombinant human IL-2. Culture supernatant was collected after 5–7 days of cultivation and further passaged on MYA-1 cells, which were used to assess for the presence of FIV antigens by the indirect immunofluorescence assay.

DNA isolation

To obtain proviral DNAs of FIV, the FIV-infected MYA-1 cells were cocultured with uninfected MYA-1 cells at a ratio of 1 to 10. Two days after cocultivation, the extrachromosomal DNA was extracted from these cultures by the Hirt method [8]. The DNA samples were treated with proteinase K (50 μg/ml) at 37 °C for 2 h, extracted with phenol-chloroform-isoamyl alcohol (24:24:1), and precipitated with ethanol.

PCR and DNA sequencing

Two oligonucleotide primer pairs, N2–N1 and N3–N1, were used to amplify the whole LTR of one-LTR circular proviral DNA of FIV (Fig. 1). The primers, N1 and N3, were designed to identify regions that are highly conserved among FIV. The primer N3, which corresponds to the polypurine tract just upstream of the LTR and is located at nucleotide positions (np) 9091–9112 according to the numbering of the published TM2 isolate sequence

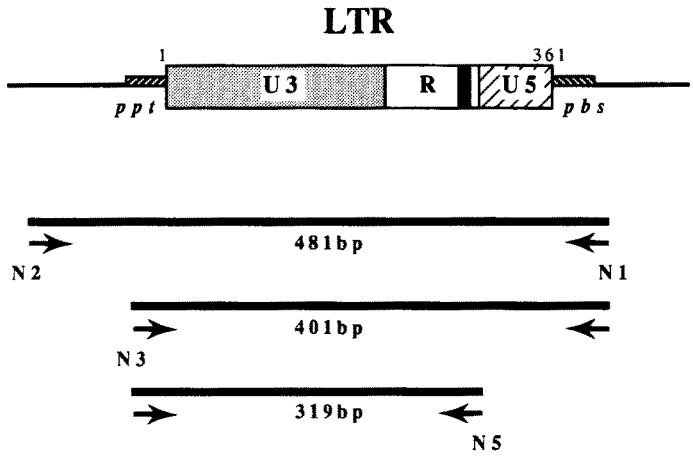


Fig. 1. Schematic representation of LTR and primers used in this study. The locations of the oligonucleotide primers used for PCR amplification are indicated below. Arrows indicate the polarity of the primers. Bars represent the position of the resultant PCR products. Each size indicated was calculated on the basis of the TM2 nucleotide sequence [13]. Shaded box within the R region represents the specific insertion (see text); *ppt* polypurine tract; *pbs* primer binding site

[13], has the sequence of 5′-CAAAAGAAAAAAGGGTGGACTG-3′. The primer N1, which corresponds to the primer binding site just downstream of the LTR and is located at np 381–361, has the sequence of 5′-GTCCCTGTTCGGGCGCCAACT-3′. The primer N2, which is located at np 9011–9032 and has the sequence of 5′-GATGGCAAATCTAGA-GAACCGC-3′, has a *Xba*I site within the sequence.

To differentiate the Japanese and Argentine isolates from the Petaluma and Australian isolates, another oligonucleotide primer pair N3–N5 was used. The primer N5 is located at np 299–280 of the TM2 isolate and has the sequence of 5′-CAGGGTTCAATCTCAAAA-CT-3′, which corresponds to the specific insert present in R region of the Japanese and Argentine isolates (Fig. 1, shaded box).

PCR was performed according to the manufacturer's instruction using 50 pmol of each primer, 50 ng DNA, 0.2 mM each dNTP, and 1.25U *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a 50 µl total volume. The reaction was overlaid with a drop of mineral oil and cycled in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) for 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C for 30 cycles. PCR products were resolved on a 2% agarose gel and stained with ethidium bromide.

The PCR-amplified fragments were cloned into M13 or pCR cloning vector (Invitrogen, San Diego, CA). Sequencing reactions were performed by the dideoxynucleotide chain termination method using a Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The sequence was resolved on an Applied Biosystems model automated DNA sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequence data were deposited with the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession numbers (accession numbers are in parentheses): TM3 (D31929), TM4 (D31930), KYO-1 (D31931), Shizuoka-1 (D31932), Aomori-1 (D31933), LP1 (D31934), LP3 (D31935), LP9 (D31936), LP20 (D31937), LP24 (D31938), N91 (D31939), R91 (D31940) and T90 (D31941).

Phylogenetic tree construction

A phylogenetic tree was constructed using the GAP program version 7.2 of the sequence analysis software package of GCG/VAX.

Results

Sequence variation in FIV LTR

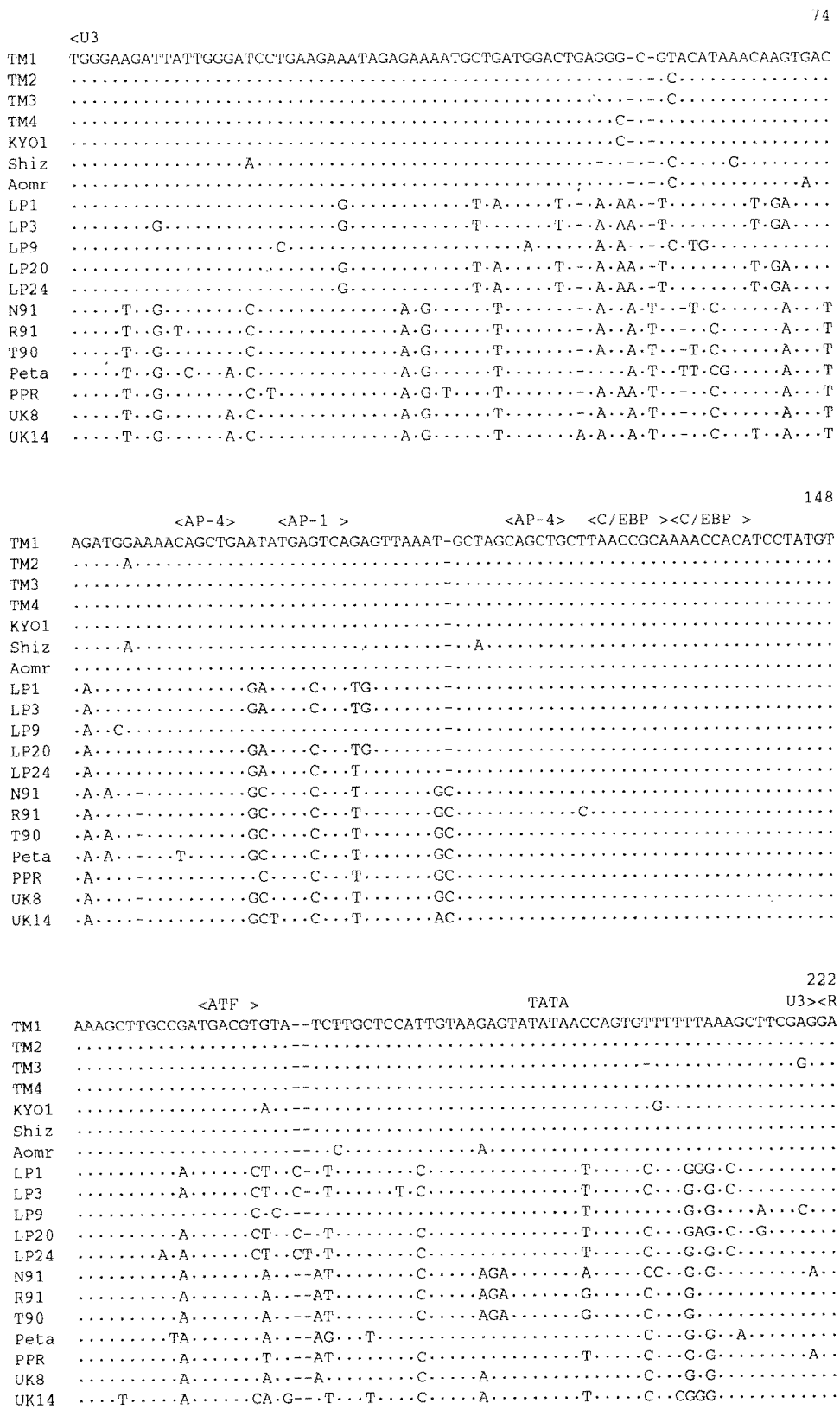
We amplified the whole LTR of one-LTR circular proviral DNA of FIV using the two oligonucleotide primer pairs, N2–N1 or N3–N1.

The nucleotide sequences of the LTR of FIV isolates were compared with the previously determined sequence of the TM1, TM2, Petaluma, PPR, UK8, and UK14 isolates (Fig. 2). The lengths of the LTR of FIV examined showed minor differences. As expected, most of the known enhancer/promoter binding sites were conserved (Fig. 2), namely: (1) only Petaluma had a single base substitution in the forward AP-4 site (from TGAGTCA to TGACTCA); (2) there were two types in the AP-1 site. The sequences of all Japanese isolates and one Argentine LP9 isolate were TGAGTCA, whereas those of other isolates were TGACTCA; (3) there was no difference in the backward AP-4 site; (4) there was no difference in the tandemly repeated C/EBP sites; (5) there were two types in the ATF site. The sequences of all Argentine and the British UK14 isolates were TGACGC, and those of other isolates were TGACGT; (6) There was no difference in the TATA box.

One of the most striking features was the specific insertion of 7 base pairs (bp) in the R region downstream of the poly(A) signal. This insertion was present in the R region of all of the Japanese and the Argentine isolates. Like a TAR region of HIV, it was predicted that the R region of FIV constructs secondary structure which forms a stemloop [18]. Owing to this 7 bp insertion, the predictable secondary structure of R region was significantly changed (data not shown).

As shown in Table 2, the Japanese isolates clustered as one group differing in nucleotide sequence by a maximum of 5%. The Argentine isolates had two groups. LP1, LP3, LP20 and LP24 clustered as one group differing in nucleotide sequence by a maximum of 3%. Interestingly, LP9 was more closely related to the Japanese isolates than the other Argentine isolates, since LP9 had 91.1%–93.1% nucleotide sequence similarity with the Japanese isolates but had only 85.1%–85.6% nucleotide sequence similarity with the other Argentine isolates. Like the Japanese isolates, the Australian isolates clustered as one group differing in nucleotide sequence by a maximum of 3%, and closely related to the Californian and British isolates with nucleotide sequence similarity of 93.0%–97.2%.

On the basis of the nucleotide sequence of the LTR, FIV examined in this study formed three groups I, II and III (Fig. 3). Group I consisted of the Californian, Australian and British isolates. Group II consisted of all the Japanese isolates and one Argentine LP9 isolate. Group III consisted of all the Argentine isolates except LP9 isolate.


74
<U3
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148
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TM2
TM3
TM4
KYO1
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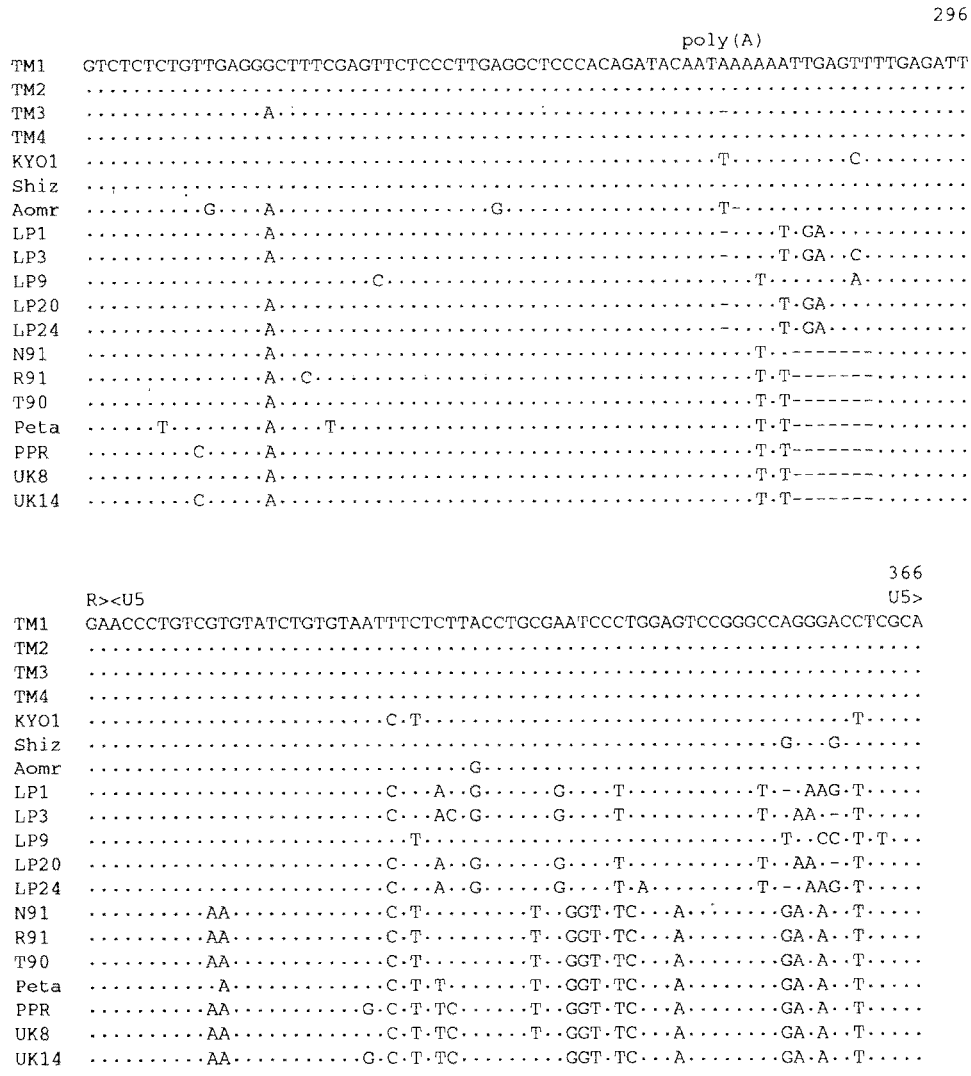

Fig. 2. Nucleotide sequence alignment of the FIV LTRs of 13 independent isolates. The alignments were compared to that of the TM1 [16], TM2 [13], Petaluma [31], PPR [23], UK8 [32] and UK14 [32]. Dots indicate identity with the TM1 isolate shown at the top. Bars represent gaps introduced to optimize the alignment. The important features are indicated above the sequences; transcriptional initiation (TATA box), poly(A) signal site and the recognition sequences of the putative enhancer/promoter proteins AP-1 (TGAGTCA), AP-4CAGCTG), C/EBP ([T/A]AACC[G/A]CA) and ATF (TGACGT). The sequences are numbered from the initiation site of the LTR. Bases 273–278 represent the POLY(A) signal site

Differentiation of the Japanese and Argentine isolates from the Petaluma and Australian isolates by PCR

As described above, both the Japanese and Argentine isolates had the specific insertion of 7 base pairs in the R region. Thus, we ascertained whether PCR using the primer pair N3–N5 could differentiate the Japanese and Argentine

Table 2. Percent nucleotide similarity between LTRs of FIV

	TM1	TM2	TM3	TM4	KYO1	Shz1	Aom1	LP1	LP3	LP9	LP20	LP24	N91	R91	T90	Pet	PPR	UK8
TM1																		
TM2	99.4																	
TM3	98.6	98.6																
TM4	99.7	99.2	98.3															
KYO1	97.8	97.2	97.0	98.1														
Shz1	97.8	98.3	97.0	97.8	95.8													
Aom1	97.2	97.2	97.5	97.0	95.6	95.6												
LP1	87.6	87.0	87.5	87.6	87.3	86.5	86.4											
LP3	87.3	86.7	87.3	87.3	87.6	85.6	86.1	98.1										
LP9	93.1	93.1	93.1	93.1	92.5	92.2	91.1	85.6	85.1									
LP20	87.6	87.0	87.8	87.6	87.3	85.9	86.4	99.2	98.1	85.4								
LP24	87.3	86.8	87.3	87.3	87.1	86.2	86.2	98.6	97.2	85.4	98.1							
N91	83.1	82.9	83.1	83.1	84.0	82.3	82.0	84.0	83.7	81.5	83.4	83.7						
R91	82.9	82.6	83.4	82.9	84.0	82.0	82.3	83.7	83.4	81.5	83.1	83.5	97.2					
T90	83.4	83.1	83.9	83.4	84.5	82.6	82.8	84.3	84.0	82.0	83.7	84.0	97.7	99.4				
Pet	82.1	81.5	82.3	82.1	83.2	80.7	80.7	82.4	82.1	80.7	81.8	82.1	93.0	93.8	94.4			
PPR	82.6	82.3	83.1	82.6	83.4	81.8	81.4	84.8	85.1	81.2	84.3	84.6	95.5	94.6	95.2	92.7		
UK8	84.0	83.7	84.5	84.0	85.1	83.1	83.4	84.8	85.1	82.0	84.3	84.3	96.9	96.6	97.2	95.2	96.6	
UK14	81.0	80.7	81.5	81.0	82.1	80.2	80.4	84.6	84.3	80.4	83.7	83.8	93.2	93.0	93.5	91.6	94.4	95.5

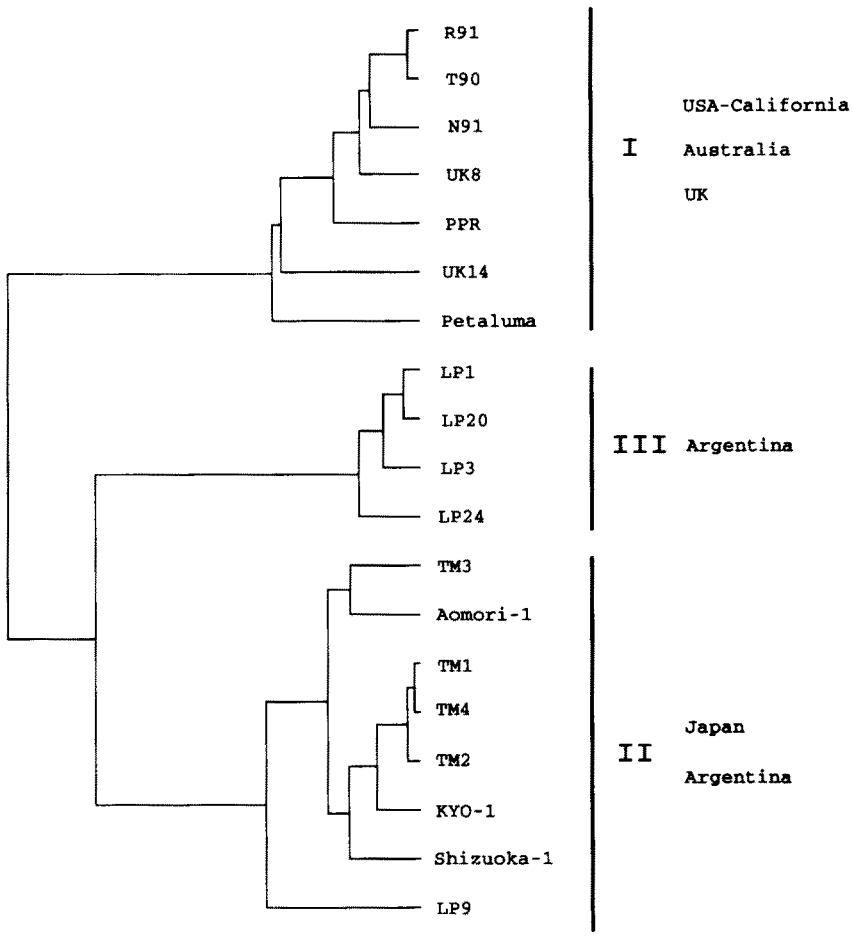

Fig. 3. Phylogenetic tree of FIV based on the nucleotide sequence of LTRs. The relationship between the FIV LTRs was examined using the GAP program version 7.2 of the sequence analysis software package of GCG/VAX

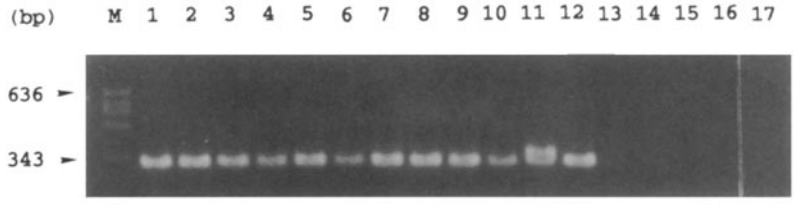

Fig. 4. PCR analysis for differentiating group I from groups II and III. PCR products amplified with primer pair N3–N5 were analyzed on a 2.0% agarose gel and visualized by ethidium bromide staining. *M* Size marker in bp; *1* FIV TM1; *2* TM2; *3* TM3; *4* TM4; *5* KYO-1; *6* Shizuoka-1; *7* Aomori-1; *8* LP1; *9* LP3; *10* LP9; *11* LP20; *12* LP24; *13* N91; *14* R91; *15* T90; *16* Petaluma; *17* MYA-1 (negative control). It is evident that the primer pair used did not amplify viruses in group I (*13–16*)

isolates from the Petaluma and Australian isolates. As shown in Fig. 4, both the Japanese and Argentine isolates were clearly amplified, but the Petaluma and Australian isolates were not. These results indicated that PCR can be used to differentiate groups II and III from group I.

Discussion

In this study we determined the nucleotide sequences of LTR for five Japanese, five Argentine and three Australian isolates of FIV and compared those with other known FIV sequences (Fig. 2). These results revealed that the Japanese isolates clustered and formed a group (Fig. 3). The Japanese isolates revealed striking genetic differences from the Australian, Californian and British isolates with variability of 14.9%–19.8%. The Australian isolates clustered with nucleotide sequence similarity of 97.2%–99.4%. Despite the remarkable geographic distance, the Australian, North American (California) and British isolates showed a considerable genetic similarity, thus suggesting a common ancestor. The Argentine isolates did not cluster into one group. The LP9 isolate is closely related with the Japanese isolates rather than the other four Argentine isolates (Fig. 3).

A phylogenetic tree analysis based on the nucleotide sequences of LTR demonstrated that FIV could be divided into three groups (Fig. 3). The distribution of FIV groups showed some geographic clustering. Group I included Californian, Australian and British isolates. Group II included Japanese and one Argentine isolates, and group III Argentine isolates. Groups I and II correspond to groups A and B of phylogenetic analysis of FIV *env* genes reported by Sodora et al. [28], respectively. Group III has never been reported. Although we did not examine the LTR of group C viruses reported Sodora et al. [28], group III viruses appear to be distinct from group C viruses, since the nucleotide sequence of the *env* gene of Argentine isolates (group III) are substantially different from that of group C viruses (Pecoraro et al. unpublished data). As a whole, FIV could be divided into four groups at present.

The phylogenetic location of one of the Argentine isolates suggests that cats carrying the LP9 isolate may have accompanied people who traveled or migrated from Asia to South America. Much more sequence data of FIV from around the world will be required to augment our understanding about subgrouping and the evolutionary history of this feline lentivirus.

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