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# Interspecies Transmission of Feline Immunodeficiency Virus from the Domestic Cat to the Tsushima Cat (*Felis bengalensis euptilura*) in the Wild

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**Feline immunodeficiency virus (FIV) was isolated from a wild-caught Tsushima cat (*Felis bengalensis euptilura*), an endangered Japanese nondomestic subspecies of leopard cat (*F. bengalensis*). Phylogenetic analysis of the *env* gene sequences indicated that the FIV from the Tsushima cat belonged to a cluster of subtype D FIVs from domestic cats. FIVs from both the Tsushima cat and the domestic cat showed similar levels of replication and cytopathicity in lymphoid cell lines derived from these two species. The results indicated the occurrence of interspecies transmission of FIV from the domestic cat to the Tsushima cat in the wild.**

Feline immunodeficiency virus (FIV) is a causative agent of AIDS-like disease and immunological abnormalities in domestic cats (*Felis catus*) that are similar to those caused by the human immunodeficiency virus (HIV) in humans (1, 25, 26). FIV isolates have been classified into five distinct subtypes: A, B, C, D, and E (10, 16, 21–24). Subtypes B and D are the two major subtypes that are distributed throughout the northeastern and southwestern districts of Japan (16). Other FIV-related lentiviruses detected in nondomestic wild cats, including the puma (*Puma concolour*), lion (*Panthera leo*), and Pallas's cat (*F. manul*), are known to be distantly related to FIVs of domestic cats by phylogenetic analyses (2–4, 11, 20).

A Tsushima cat (*Felis bengalensis euptilura*) that was captured in the forest of Tsushima Island for the purpose of breeding in a zoo was found to be positive for FIV antibody. The Tsushima cat is an endangered wild cat discovered in Japan in recent years, a local form of the Siberian subspecies of the leopard cat (*F. bengalensis*). It inhabits busy forest edges and paddy fields near the coast of Tsushima Island off the Japanese mainland. It is larger than the continental subspecies but smaller than another Japanese subspecies (*F. bengalensis iriomotensis*). Its population size is between 70 and 90 individuals. Therefore, this subspecies of leopard cats seems to be a relic population that separated from other leopard cats long ago (14).

In this study, the Tsushima cat virus was analyzed phylogenetically to help explain the origin and transmission of FIV or FIV-related lentiviruses. Furthermore, the growth and cytopathicity of the virus from the Tsushima cat and FIV from the domestic cat were examined in lymphoid cell lines from both species.

**Virus isolation from the FIV-positive Tsushima cat.** At the serum examinations immediately upon capture, the wild-caught Tsushima cat was positive for FIV antibody and negative for feline leukemia virus (FeLV) antigen in serological tests using a commercial test kit (SnapTM; IDEXX, Portland, Maine). After capture, the Tsushima cat was kept in a special facility in Fukuoka Municipal Zoo and Botanic Garden, completely isolated from any other animals, including domestic cats. Hematological examination of the Tsushima cat revealed a lymphocyte count of 1,480/μl, which was within the range of four previously examined healthy Tsushima cats (1,400 to 3,000/μl). A $CD8^+$ cell count, determined by using a monoclonal antibody against cat lymphocytes (Southern Biotechnology, Birmingham, Ala.), for the Tsushima cat was 435/μl, which was similar to the values in previously examined healthy Tsushima cats. A $CD4^+$ cell count could not be determined because a monoclonal antibody against feline CD4 (Southern Biotechnology) did not react with lymphocytes of the Tsushima cat.

For virus isolation, a Tsushima cat-derived lymphoid cell line, PIPP-I, was established from a zoo-kept Tsushima cat that was seronegative for FIV, FeLV, and other common pathogens in domestic cats. The cytochrome *b* sequence from the PIPP-I cell line was amplified by PCR with primers described previously (14), and the sequence indicated that the cell line originated from a Tsushima cat. The PIPP-I cell line stained positively with monoclonal antibodies against CD8, CD9 (MM2/57; Southern Biotechnology), and interleukin-2 receptor alpha (9F23) in flow cytometric analyses (18). A feline $CD4^+$ T-lymphoid cell line, Kumi-1 (7), was also used for virus isolation. Peripheral blood mononuclear cells (PBMC) were obtained from the seropositive Tsushima cat. The cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum and were stimulated with 10 μg of concanavalin A per ml for 3 days in the presence of 100 U of human

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recombinant interleukin-2 (Pharma Biotechnologie, Hannover, Germany) per ml. The Tsushima cat lymphoid cells were cocultivated with PIPP-I cells or Kumi-1 cells 30 days after initiation of the culture. Reverse transcriptase (RT) assay as described previously (19) showed an increase in Mg-dependent RT activities in the culture supernatants of cocultures of PIPP-I and Kumi-1 cells by 20 to 30 days after initiation of cocultivation. The RT-positive culture supernatants from the lymphocyte cocultures of PIPP-I cells and Kumi-1 cells were frozen at −80°C as virus stocks designated Feu-P and Feu-K, respectively.

**Sequence analyses of viruses isolated from the Tsushima cat and FIVs from domestic cats from Tsushima Island.** For the analysis of proviral DNA of FIV or FIV-related lentivirus, high-molecular-weight DNAs were extracted from primary PBMC that were obtained from the Tsushima cat seropositive for FIV antibody and the cocultures with either PIPP-I or Kumi-1 cells. These DNA samples were used for nested PCR amplification of the FIV *env* gene spanning regions V3 to V6. The nested PCR primers used in this study were described in our previous paper (17). PCR products were directly cloned into a cloning vector (TA cloning kit; Invitrogen, San Diego, Calif.) and sequenced by using the dideoxy chain termination method. Alignments of the deduced amino acid sequences of 9 viral genomes obtained from the primary PBMC (Feu1, -2, and -3), coculture with PIPP-I cells (Feu4, -5, and -6), and coculture with Kumi-1 cells (Feu7, -8 and -9) were almost identical, showing only 1 to 23 nucleotide substitutions in the 624-bp fragment. The proviral sequences from the Tsushima cat showed relatively high amino acid sequence similarities (82.7 to 93.8%) with those of subtype D FIV strains previously reported and lower sequence similarities with those of subtype A, B, and C strains of FIV (71.6 to 80.7%) in Japan (10, 16, 17) (Fig. 1).

Furthermore, for the purpose of the investigation of the origin and transmission of FIV, blood samples were collected from 50 stray domestic cats from two villages on Tsushima Island, A and B, near the forest where the seropositive Tsushima cat was captured. Serum samples from the domestic cats showed a high frequency (11 of 51, or 21.6%) of positive results for antibodies against FIV. From the primary PBMC samples obtained from 10 domestic cats seropositive for FIV antibody, the proviral sequences of FIV were sequenced after nested PCR amplification of the *env* gene fragment. All 10 strains obtained from the domestic cats from Tsushima Island (TSU101, TSU102, TSU104, TSU107, TSU109, and TSU116 strains obtained in village A and TSU202, TSU210, TSU215, TSU226, and TSU226 strains obtained in village B) had sequences that were highly similar to those of subtype D FIVs as well as to those from the Tsushima cat obtained in this study (Fig. 1). To rule out the possibility of contamination by PCR products, we carried out three independent PCR amplifications for each of the DNA templates obtained from Tsushima cat-derived and domestic cat-derived samples and obtained almost the same results. Control PCR amplification without template DNA did not generate any amplified product.

**Phylogenetic analyses for the proviral DNA sequences.** Nucleotide divergences for pairs of sequences were estimated by using the neighbor-joining method in the DNADIST program from the PHYLIP software package (5). A phylogenetic tree constructed with the nucleotide sequences spanning the V3 to the V5 region of the FIV *env* gene was constructed by using the NEIGHBOR program, and the branching order reliability was evaluated by using bootstrap analysis in the SEQBOOT program (5). All of the proviral DNA sequences from the Tsushima cat (Feu1 to -9), from domestic cats in village A (TSU101, TSU102, TSU104, TSU107, TSU109, and TSU116), and those in village B (TSU202, TSU210, TSU215, and TSU226) belonged to the subtype D FIVs of domestic cats, with high bootstrap values (Fig. 2). Feu1 to -3 clones obtained from the Tsushima cat primary PBMC and Feu4 to -9 clones obtained from cocultures with PIPP-I and Kumi-1 cells clustered in the subtype D FIVs (Fig. 2). Most of the isolates obtained from domestic cats living in village A (TSU101, TSU104, TSU107, TSU109, and TSU116) formed another cluster. On the basis of the phylogenetic analysis, FIV could have been transmitted to the Tsushima cat from any subtype D-infected domestic cat. A maximum likelihood phylogenetic tree constructed by using the DNAML program (5) supported the branching orders, showing high bootstrap values ($>70$) in the unrooted neighbor-joining phylogenetic tree, with significant $P$ values ($P < 0.01$) (data not shown).

**Replication and cytopathicity of the isolates from the Tsushima cat and the domestic cat.** To investigate the interspecies transmission of FIV, we examined the replication and cytopathicity of the FIVs from the Tsushima cat and from domestic cats in lymphoid cell lines from these two species. Feu-P and Feu-K strains in the cocultures with PIPP-I and Kumi-1 cells, respectively, were used as virus stocks of FIV from the Tsushima cat. A strain isolated from a domestic cat in Fukuoka in southwestern Japan (7) for use as an FIV domestic-cat stock virus was designated reference subtype D FIV. PIPP-I cells ($10^6$ cells/ml) in a 6-well culture plate were inoculated with an equivalent number of virus particles (RT activity, 2,000 cpm) from the various FIV isolates. After adsorption at 37°C for 1 h, the cells were washed with phosphate-buffered saline and cultured in fresh RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were cultured and maintained at 37°C with medium changes every third day. RT activity in the culture supernatants and the number of viable cells counted using trypan blue staining were monitored after virus inoculation. All experiments were performed in duplicate. To rule out the possibility of contamination, we carried out the sequencing of the proviruses from the cultured cells infected with these viral strains at the end of the cultures and confirmed that there was no laboratory contamination of the virus strains used in this study. In the PIPP-I cells, both Tsushima cat-derived viruses (Feu-P and Feu-K) and domestic cat-derived virus (Fukuoka) displayed similar growth rates as shown by an increase in culture supernatant RT starting at 6 days after inoculation, reaching the highest levels ($1 \times 10^6$ to $1.5 \times 10^6$ cpm/ml) at 12 to 18 days after inoculation (Fig. 3A). The viable cell counts of PIPP-I cells progressively decreased in contrast to the increase in RT activity and finally decreased to as few as $2 \times 10^5$ cells/ml (Fig. 3B). A proportion of the PIPP-I cells producing a large amount of FIV showed shrinkage of the cells and condensation of the nuclear chromatin, which are features characteristic of apoptosis. The growth curve of the viruses and the number of viable cell counts in PIPP-I cells after infection revealed no obvious differences in these parameters between the Tsushima cat-derived and domestic cat-derived viruses. In the Kumi-1 cells from the domestic cat, viral growth rate and cytopathicity were essentially similar to those obtained with PIPP-I cells (data not shown). The results indicated that the viruses from the Tsushima cat and the domestic cat shared similar growth curves and cytopathicities in Tsushima cat and domestic cat lymphoid cells.

The seropositivity of FIV antibodies in stray domestic cats from Tsushima Island was found to be very high, 21.6%, relative to those in mainland Japan and other countries (6, 8, 9, 13, 29). All of the FIV proviral genomes obtained from domestic cats of Tsushima Island belonged to subtype D, which is the most common subtype on the nearby much larger island of

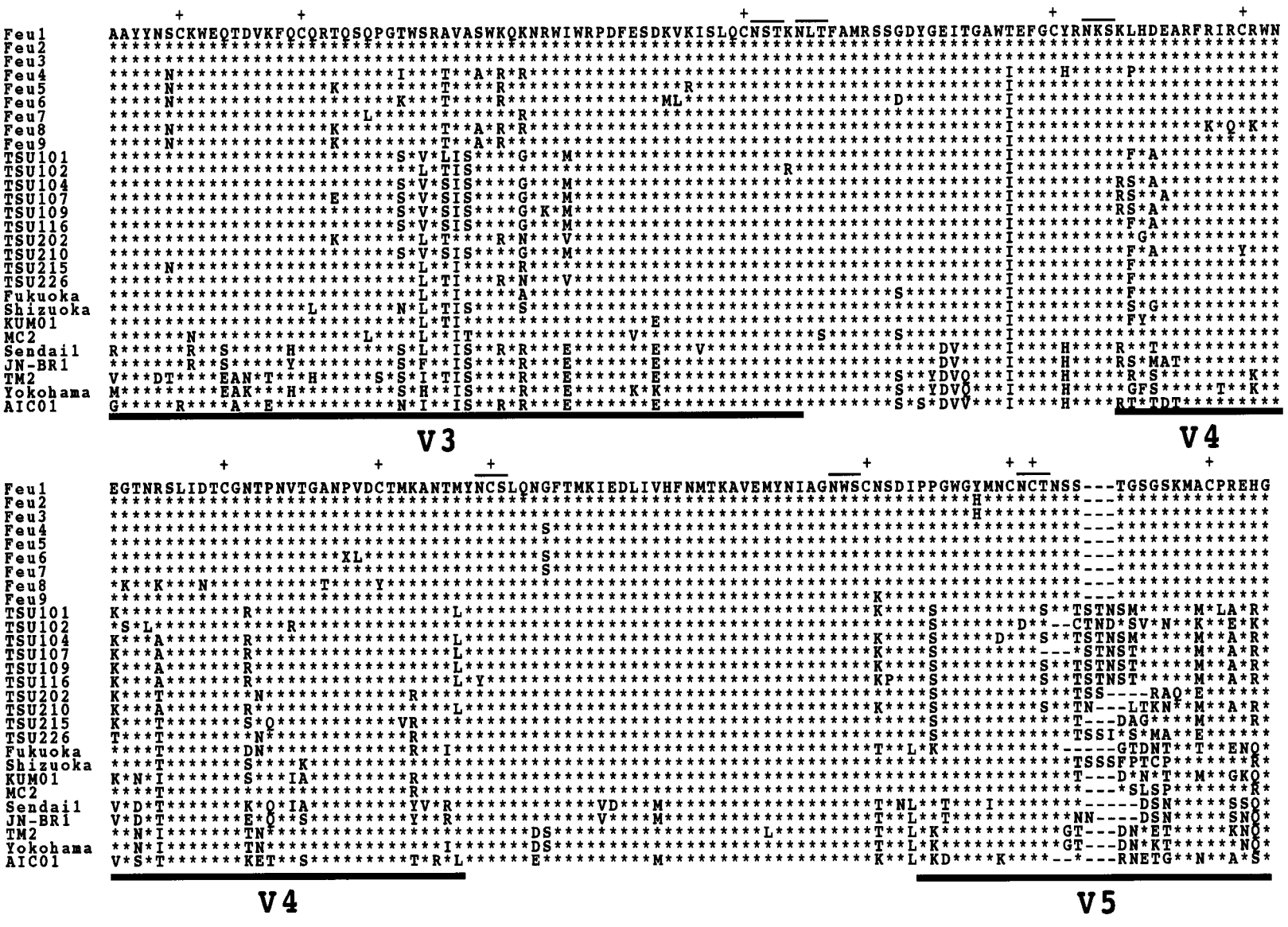


FIG. 1. Alignment of the predicted amino acid sequences of the FIV *env* gene of 9 clones from a Tsushima cat (Feu1 to -9), 10 clones from domestic cats on Tsushima Island (TSU101, TSU102, TSU104, TSU107, TSU109, TSU116, TSU202, TSU210, TSU215, and TSU226), and 9 known FIV clones, by using the CLUSTAL W program. The FIV clones previously reported include Fukuoka (GenBank/EMBL/DDBJ accession no. D37815), Shizuoka (D37811), KUM01 (AB010405), MC2 (D67062), Sendai1 (D37813), JN-BR1 (D67052), TM2 (M59418), Yokohama (D37812), and AIC01 (AB010395). Asterisks denote amino acid identity with the Feu1 strain. The horizontal lines above the sequence indicate the locations of N-linked glycosylation sites. The pluses indicate the positions of cysteine residues. The dashes represent gaps introduced for optimal alignment. The thick bars under the alignment indicate the positions of the variable regions V3 to V5 as defined by Pancino et al. (21).

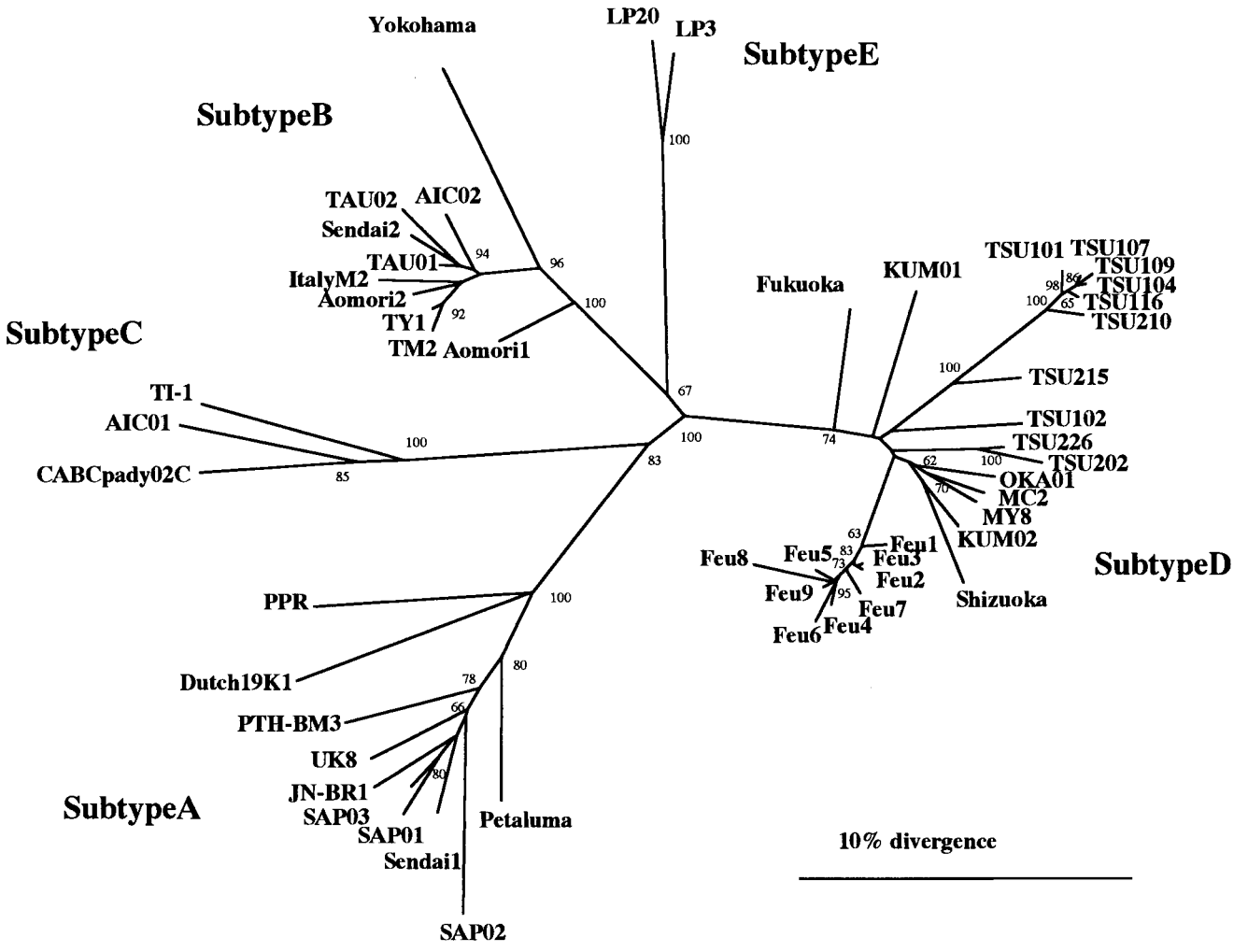


FIG. 2. An unrooted neighbor-joining phylogenetic tree of the FIV *env* gene covering variable regions V3 to V5. Nucleotide divergences were estimated by the DNADIST program from the PHYLIP software package (5). The phylogenic tree was constructed by using the NEIGHBOR program, and the branching order reliability was evaluated by bootstrap analysis in the SEQBOOT program (5). Virus clones obtained in this study are Feu1 to -9 from the Tsushima cat and TSU101, TSU102, TSU104, TSU107, TSU109, TSU116, TSU202, TSU210, TSU215, and TSU226 from domestic cats on Tsushima Island. The FIV clones previously reported are Petaluma FIV14 (GenBank/EMBL/DDBJ accession no. M25381), TM2 (M59418), Yokohama (D37812), Shizuoka (D37811), JN-BR1 (D67052), MC2 (D67062), MY8 (D67063), TY1 (D67064), Sendai1 (D37813), Sendai2 (D37814), Aomori1 (D37816), Aomori2 (D37817), Fukuoka (D37815), PPR (M36968), UK8 (X69496), Dutch19k1 (M73964), ItalyM2 (X69501), LP3 (D84496), LP20 (D84498), TI-1, CABCpady02C (U02392), SAP01 (AB010402), SAP02 (AB010403), SAP03 (AB010404), PTH-BM3 (AB010401), TAU01 (AB10405), TAU02 (AB10406), AIC01 (AB10396), AIC02 (AB10397), OKA01 (AB010400), KUM01 (AB010398), and KUM02 (AB010399). The numbers at each branch point indicate the bootstrap values (5) preserved through greater than 60 in 100 bootstrap repetitions.

Kyushu. The subtype D viruses endemic on Tsushima Island were assumed, therefore, to have been introduced from domestic cats from Kyushu. Though it is not clear when the subtype D FIV was introduced to Tsushima Island, there have been close relations via traffic by sea and air for a long time between the people living on Kyushu and those living on Tsushima Island. Therefore, domestic cats infected with subtype D FIV endemic in Kyushu could be easily introduced to Tsushima Island. The fact that there are only two species belonging to the *Felidae* family would support the theory that FIV could have been transmitted to the Tsushima cat from any subtype D-infected domestic cat. We found that 16 wild-caught Tsushima cats other than the animals in this study were seronegative for FIV antibody, indicating that FIV infection is not common in the Tsushima cat. Moreover, many species belonging to the *Felidae* family were reported to be infected with lentiviruses that were different from each other. The areas where each species lives are isolated from other species. It is conceivable that the FIV-infected Tsushima cat found in this study might have accidentally come into contact with a seropositive domestic cat in the forest or adjacent villages; recent deforestation and development on Tsushima Island have eroded the forests that Tsushima cats inhabit, making this scenario probable. A large part of the conservation of the rare Tsushima cat may rest with conservation of its environment, which serves as an important barrier to contact between this highly endangered species and infectious domestic cats.

Domestic cats can be infected with puma and lion lentiviruses; however, the inocula used to demonstrate these infections were viruses isolated by cocultivation with domestic cat PBMC and the cat lymphoid cell line 3201 (27, 28). In general, adaptation of virus for other host species has been shown to be an important factor for interspecies transmission. However, in the present study, FIVs from both the Tsushima cat and domestic cats showed similar replicative capacities in both PIPP-I and Kumi-1 cell types, indicating that interspecies transmission

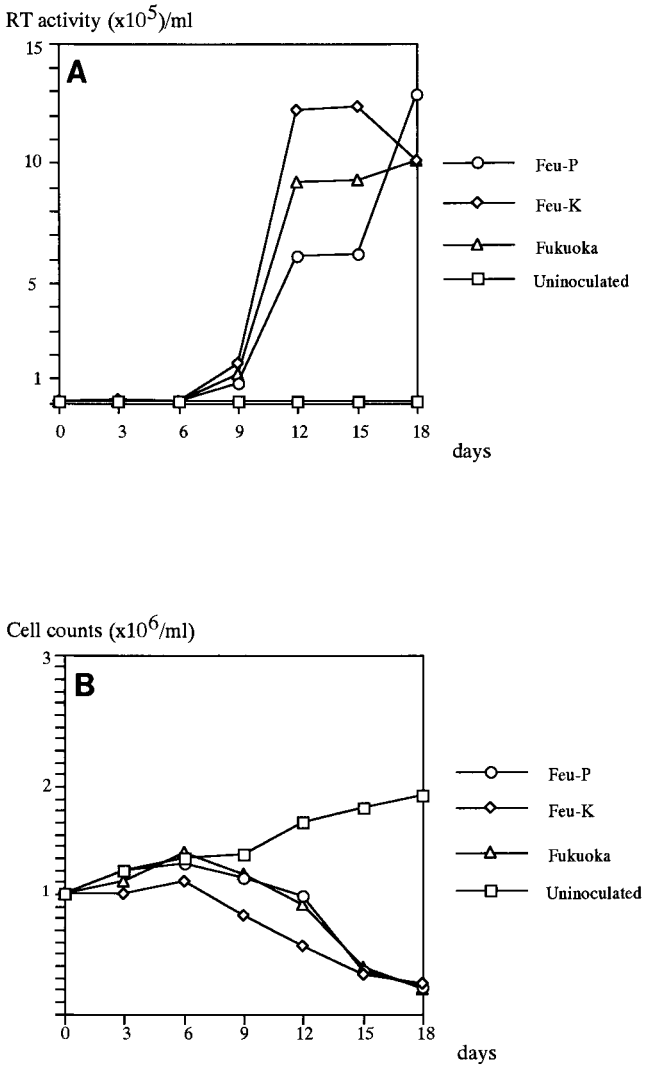


FIG. 3. Replication of the FIV isolates and the viable cell counts in PIPP-I cells after infection with the FIV isolates. (A) Production of FIV as examined by RT assay of the culture supernatants from PIPP-I cells. (B) Counts of viable PIPP-I cells. Feu-P, Tsushima cat-derived FIV isolated by cocultivation with PIPP-I cells; Feu-K, Tsushima cat-derived FIV isolated by cocultivation with Kumi-1 cells; Fukuoka, domestic cat-derived FIV isolated by cocultivation with Kumi-1 cells.

of FIV between domestic cats and Tsushima cats was highly likely. FIV-related lentiviruses in the puma and lion apparently do not induce any symptomatic disease in their natural host or in experimentally infected domestic cats (27). The case is different with simian immunodeficiency virus (SIV) infection; SIV from sooty mangabeys induced an AIDS-like disease in Asian macaques, though the virus did not cause any disease in its natural host (12, 15). Our data indicated that FIV from the domestic cat was cytopathic in cells cultured from the lymphoid cells of Tsushima cats, yet we presently do not know whether FIV can induce disease in the Tsushima cat; continued observation of possible disease progression in this FIV-infected Tsushima cat should eventually reveal the link, or lack of a link, between this virus and disease in this host.

The present study is an example of interspecies transmission of a pathogen from the domestic cat to an endangered nondomestic cat species in the wild. For the conservation of a diversity of wild animal species, it will be important to control the threat to endangered species from diseases carried by related domestic species.

**Nucleotide sequence accession numbers.** Nucleotide sequences of the FIV genome obtained in this study have been deposited in the DDBJ database under accession no. AB02110 through AB02118.

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