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Genetic and biological characterization of feline foamy virus isolated from a leopard cat (*Prionailurus bengalensis*) in Vietnam

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ABSTRACT. Foamy viruses have been isolated from various mammals and show long-term co-speciation with their hosts. However, the frequent inter-species transmission of feline foamy viruses (FFVs) from domestic cats to wild cats across genera has been reported. Because infectious molecular clones of FFVs derived from wild cats have not been available, whether there are specific characteristics enabling FFVs to adapt to the new host species is still unknown. Here, we obtained the complete genome sequences of two FFV isolates (strains NV138 and SV201) from leopard cats (*Prionailurus bengalensis*) in Vietnam and constructed an infectious molecular clone, named pLC960, from strain NV138. The growth kinetics of the virus derived from pLC960 were comparable to those of other FFVs derived from domestic cats. Phylogenetic analysis revealed that these two FFVs from leopard cats are clustered in the same clade as FFVs from domestic cats in Vietnam. Comparisons of the amino acid sequences of Env and Bet proteins showed more than 97% identity among samples and no specific amino acid substitutions between FFVs from domestic cats and ones from leopard cats. These results indicate the absence of genetic constraint of FFVs for interspecies transmission from domestic cats to leopard cats.

KEY WORDS: domestic cat, feline foamy virus, infectious molecular clone, inter-species transmission, leopard cat

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Foamy viruses (FVs) are classified as the subfamily *Spumaretrovirinae* in the family *Retroviridae*. Different FV species have been isolated from primates, cattle, horses, and cats [8, 13, 31, 34]. These lineage-specific FVs have co-evolved with their hosts and circulate within each species [11, 27, 30]. FVs are considered to be non-pathogenic, although they induce severe cytopathic effects (CPEs) with syncytia-formation in cell culture [18]. The genome of FVs has long terminal repeats (LTRs) at both 5′ and 3′ ends and contains three structural and enzymatic genes, *gag*, *pol*, and *env*, which are common in retroviruses. Frequent recombination events play an important role in increasing the genetic diversity of Env of FVs [1, 15]. In simian FVs (SFVs), recombination in *env* regions generates different subtypes [5, 28]. Also, FVs have two additional genes, *tas* and *bet*. Tas is a trans-activator regulating viral gene expression by binding to the U3 region of the LTR or the upstream of the internal promoter of the FV genome [37]. Bet is an inhibitor of APOBEC3s, which are host restriction factor, against retroviruses [10].

Feline FVs (FFVs) have infected domestic cats (*Felis catus*) globally, including Asian countries [23], Australia [34], Germany [3], Turkey [14], and the USA [26]. FFVs are classified into two subtypes, the F17/951-type and FUV-type, depending on the amino acid sequences of Env [33, 36]. FFVs also transmit from domestic cats to wild cats including pumas (*Puma concolor*) [12, 15], leopard cats (*Prionailurus bengalensis*) [23, 24], and European wild cats (*Felis silvestris silvestris*) [4]. In Japan, FFV infections were reported in Iriomote leopard cats (*Prionailurus bengalensis iriomotensis*) and Tsushima leopard cats (*Prionailurus bengalensis euptilurus*) [23, 24], which are subspecies of leopard cats [21]. While phylogenetic analyses of FFVs from wild cats have been reported in pumas, Iriomote leopard cats, and leopard cats in Vietnam [12, 15, 24], the molecular characteristics underlying the inter-species transmission is not fully understood. Since domestic cats have opportunities to contact wild cats, leading to interspecies transmission of viruses, we expect the risk of interspecies viral transmission to increase with land development and urbanization [17, 32, 35]. Therefore, studies focusing on the viral characteristics underlying inter-species transmission are important for veterinary research.

In this study, we constructed an infectious molecular clone of an FFV isolated from a leopard cat in Vietnam, sequenced its complete genome, and examined the growth property of the clone-derived virus. We also sequenced FFVs isolated from another leopard cat and domestic cats from Vietnam, and analyzed their phylogenetic relationships and molecular characteristics.

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MATERIALS AND METHODS

Cell lines and viruses

Crandell feline kidney (CRFK) cells (ATCC CCL-94) were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum, and penicillin (10,000 units/ml) and streptomycin (10,000 µg/ml) (Nacalai Tesque, Kyoto, Japan) at 37°C in a humidified atmosphere of 5% CO_2 in air. A green fluorescent protein-based FFV-infection indicator cell line, termed FFG [25], was grown under the same conditions.

Two FFV-related viruses from leopard cats (*Prionailurus bengalensis*) caught in Hanoi (northern Vietnam) and Ho Chi Minh City (southern Vietnam), were designated as strains NV138 and SV201, respectively. Three isolates from domestic cats (*Felis catus*) in Hanoi designated as strains VN114, VN119, and VN150 were reported previously [22, 23]. A Japanese isolate, strain Sammy-1, was reported previously [7, 9]. For isolation of FFVs, concanavalin A-stimulated peripheral blood mononuclear cells (PBMCs) from individuals were cultured with CRFK cells. When the cytopathic effects (CPEs) showing syncytia-formation appeared in CRFK, the infected cells were collected and stored at −80°C. All isolates were used within 5 passages. An American isolate, strain Coleman, was kindly supplied by Dr. J. M. Gaskin (University of Florida, USA).

PCR-amplification of FFV proviral genome and sequencing

FFV isolates: strains NV138, SV201, VN114, VN119, VN150, and Sammy-1, were used to inoculate CRFK cells. FFV-infected cells were cultured for 4–6 days post-infection, when the apparent CPEs were observed. Then, genomic DNAs were isolated from the cells using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA).

To clone the 5′ and 3′ halves of FFVs into the pSP73 vector (Promega, Madison, WI, USA), we designed primers for PCR based on the study of Kraberger *et al.* [15]. The PCR primers used to amplify the 5′ and 3′ halves of the viral genome were 5′-TCGAGCTCGGTACCCTCTCACAGAGGAGAATACTCTCTGC-3′ (forward) and 5′-CTCTAGAGGATCCCCGCCTAGATGGTCCACTATAATTACA-3′ (reverse), and 5′-TCGAGCTCGGTACCCTGGAGGGAAATTCCTCCTTCCCGAG-3′ (forward) and 5′-CTCTAGAGGATCCCCGCGGCCTATACCTGGGATAGGTTAG-3′ (reverse), respectively. The PCR conditions were as follows. The reaction mixture (total of 25 µl) consisted of 1 µl of DNA template (1 ng), 12.5 µl of KOD One (TOYOBO, Osaka, Japan), 0.75 µl of each primer (10 pmol/µl), and 10 µl of distilled water. The amplification comprised 30 cycles, consisting of denaturation at 98°C for 10 sec, annealing at 60°C for 5 sec, and extension at 68°C for 1 min. PCRs were carried out in 200 µl thin-walled tubes using a C1000 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Amplicons with 15-nt overlapping sequences with pSP73 vector were cloned into pSP73 vector using NEBuilder HiFi DNA Assembly Cloning Kit (New England BioLabs Inc., Ipswich, MA, USA). Sanger sequencing was completed from both ends by a commercial sequencing service (Fasmac Co., Ltd., Tokyo, Japan).

Construction of an infectious molecular clone of FFV, strain NV138

To construct the infectious molecular clone from strain NV138, the 5′ half fragment was cloned into the 3′ half cloned-pSP73 vector; however, a 440-nt deletion in the *env* region was found by sequencing analysis. Thus, to recover the deletion and construct the clone with the complete genome of strain NV138, a fragment containing the region was newly amplified by PCR from the extracted DNA of the FFV (strain NV138)-infected CRFK cells and replaced with the corresponding region from the incomplete molecular clone (Fig. 1B). The PCR primers used to amplify the replaced fragments were 5′-AGGAGGCAATCCCAGAGGAGGAG-3′ (forward) and 5′- CATTCAGCTAGGACAGAGGATAC-3′ (reverse). The resultant clone, designated pLC960, was used to transfect CRFK cells using Avalanche everyday transfection reagent (APRO SCIENCE, Naruto, Japan). Three days after transfection, the culture supernatant was filtrated through a 0.45-µm filter unit (PALL, Tokyo, Japan) and used to inoculate CRFK cells in the presence of 8 µg/ml of polybrene (hexadimethrine bromide) (Sigma-Aldrich). One week after inoculation, the cells were fixed and stained with Hemacolor (Merck Millipore, Burlington, MA, USA). To confirm the presence of the FFV genome in the inoculated CRFK cells, genomic DNA was extracted from the inoculated cells using PureLink Genomic DNA Mini Kit (Invitrogen). Then, we conducted PCR targeting the *gag-pol* region. PCR primers used to amplify the *gag-pol* region of FFV were 5′-GGACTCCCAAGCCGATATTACCTG-3′ (forward) and 5′-CTCTGTAGTCCAATACCATTCTCC-3′ (reverse).

Growth kinetics of the infectious molecular clone-derived virus (LC960)

CRFK cells in 6-well plates (2.0×10^5 cells/well) were inoculated with LC960 (infectious molecular clone (pLC960)-derived virus), NV138 (the parental isolate), Coleman, and Sammy-1 at a multiplicity of infection (MOI) of 0.01. The viral titers in the culture mediums were measured 2, 4, 6, and 9 days after inoculation using FFG cells, as described previously [25]. The experiments were repeated three times independently.

Promoter assay of LTRs

To construct reporter plasmids to measure promoter activities of LTRs of LC960, Colman, and Sammy-1, each entire LTR sequence was inserted into pGL3-Basic-Vector (Promega) to create pGL3LC960LTR, pGL3ColmanLTR, and pGL3Sammy-1LTR, respectively. To construct expression plasmids of Tas of LC960, Colman, and Sammy-1, each *tas* sequence was inserted into pcDNA3.1 Myc-His plasmid (Invitrogen) to form pcDNALC960Tas, pcDNAColmanTas, and pcDNASammy-1Tas, respectively.

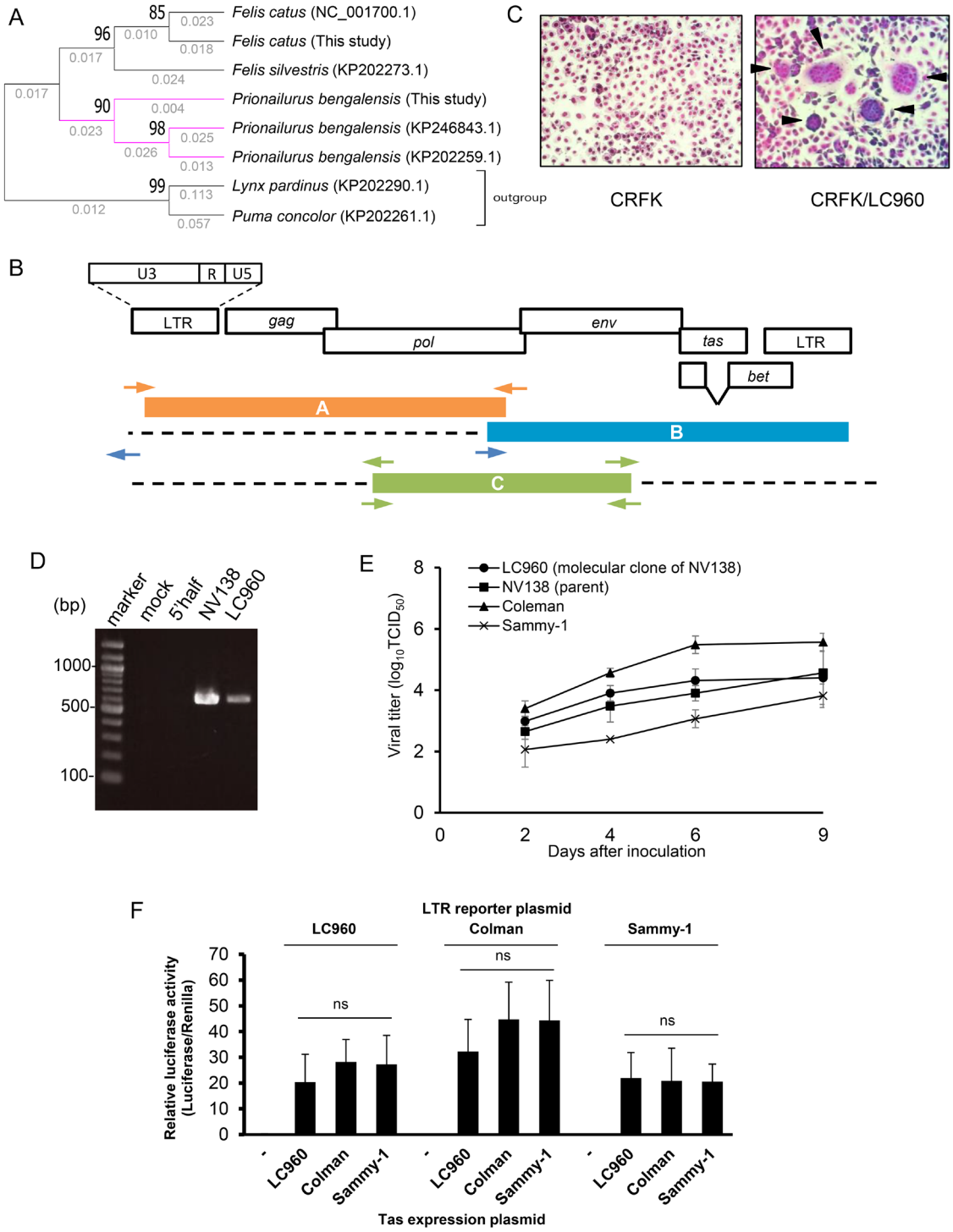

Fig. 1. Construction and characterization of the infectious molecular clone pLC960. (**A**) Analysis of mitochondrial DNA sequences. A maximum likelihood tree of D-loop sequences of mitochondrial sequences of Felidae. The tree was constructed using MEGA-X with bootstraps of 1,000 replicates with substitution models, HKY+G. Accession numbers for mitochondrial sequences are indicated in parentheses. Nodes are labeled with bootstrap support values. (**B**) Cloning strategy and primer positions for the infectious molecular clone pLC960. First, we constructed a plasmid cloned into the 3′ half of the feline foamy virus (FFV) genome <B>. A 5′ half fragment of the FFV genome <A> was inserted into the plasmid <B>. Later, a deletion was found in <B>, so we replaced a part of it with a new fragment <C> in order to recover the deletion and create a complete molecular clone. (**C**) Cytopathic effects induced by FFV derived from pLC960. (**D**) Detection of viral DNA in CRFK cells inoculated with the pLC960-derived virus by PCR. As a negative control, the supernatant of CRFK cells transfected with the 5′-half fragment of pLC960 was supplied to recipient CRFK cells, and we confirmed the absence of plasmid contamination. NV138 (parental isolate)-infected CRFK cells were used as a positive control. (**E**) Growth kinetics of the FFV isolates LC960, NV138, Sammy-1, and Coleman. Values are the means ± standard errors of data from three independent experiments. (**F**) Tas activity on the LTR promoters of the three FFV isolates LC960, Colman, and Sammy-1. Values are the means ± standard errors of data obtained from three independent experiments in triplicate. By one-way ANOVA with Tukey's multiple comparison tests, P=0.182 (LC960), 0.0942 (Colman), 0.928 (Sammy-1); ns, not significant.

To investigate the *trans*-activity of Tas proteins of LC960, Colman, and Sammy-1 on the promoter activities of LTR, LTR reporter plasmid (250 ng), Tas expression plasmid (400 ng), and pRL-TK (25 ng) were used to co-transfect CRFK cells using Avalanche everyday transfection reagent. Forty-eight hours after transfection, the luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) with Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany), according to the manufacturer's instructions. We used pRL-TK (Promega) containing the Renilla luciferase reporter gene under the control of herpes simplex virus 1 thymidine kinase promoter as an internal control. The experiments were performed in triplicate and repeated three times independently.

Mitochondrial DNA analysis

Genomic DNA containing mtDNA was isolated using PureLink Genomic DNA Mini Kit from PBMCs of the leopard cat, from which NV138 was isolated. Then, the partial mitochondrial D-loop sequence was amplified by PCR using the primers with the 15-nt overlapping sequences with pSP73 vector 5′-TCGAGCTCGGTACCCGGGACATCTCGATGGACTAATGA-3′ (forward) and 5′-CTCTAGAGGATCCCCGTCCTGTGGAACAATAGG-3′ (reverse). The fragments were cloned into pSP73 and sequenced. We designed the forward primer from the consensus D-loop sequence. We used a primer, termed JHmtR3 as the reverse primer from the study of Grahn *et al.* [6].

Phylogenetic analyses

Mitochondrial D-loop sequences and the nucleotide sequences of *pol*, *gag*, LTR, and *env* were aligned using the MUSCLE program, and the best-fit substitution models were inferred in MEGA X [16]. MEGA X was also utilized for maximum-likelihood (ML) tree construction. The reliability of the branching orders was evaluated with 1,000 bootstrap replicates. Accession numbers of sequences of FFVs used for the phylogenetic analyses are indicated in Table 1.

Recombination analyses of env gene

The potential recombination events in *env* regions were detected using RDP4 software (version 4 of recombination detection program) [20]. The program statistically identifies recombination events from a given set of aligned nucleotide sequences using multiple methods. We analyzed a dataset of aligned *env* genes using RDP, Chimera, Bootscan, 3Seq, GENECONV, MaxChi, and SiScan methods. We tested and refined preliminary recombination hypotheses following the manual. Accession numbers of sequences used for the recombination analyses are indicated in Table 1.

Comparison of Env and Bet proteins of FFV isolates

The datasets of amino acid sequences of the surface unit (SU) of Env and Bet were aligned using the MUSCLE program in MEGA-X [16]. The amino acid identities were calculated using Ident and Sim programs [29].

RESULTS

Construction of an infectious molecular clone of FFV derived from a leopard cat

First, to confirm that PBMCs used for the isolation of strain NV138 in our previous study [22, 23] were derived from leopard cats, we sequenced the mitochondrial DNA D-loop region of PBMCs and aligned with those of *Felis catus, Prionailurus*

Table 1. The list of Accession No. of reference sequences. Feline foamy virus strains sequenced in this study were indicated by asterisks

Isolate	Host	Collection site	Accession No.
*LC960 (NV138)	Leopard cat	Vietnam	MW349910
*SV201	Leopard cat	Vietnam	MW349911
*VN114	Domestic cat	Vietnam	MW349912
*VN119	Domestic cat	Vietnam	MW349913
*VN150	Domestic cat	Vietnam	MW349914
*Sammy-1	Domestic cat	Japan	MW349915
S7801	Domestic cat	Japan	AB052796
Coleman	Domestic cat	USA	AB052797
F17 (Chatul-2)	Domestic cat	USA	AJ564745
X1041DC	Domestic cat	USA	MH633417
X1324DC	Domestic cat	USA	MH633430
FUV	Domestic cat	Australia	Y08851
Baj	Domestic cat	Australia	MH633336, MH633265
Betsie	Domestic cat	Australia	MH633337, MH633266
pc-X102	Puma	USA	KC292054
pc-X103	Puma	USA	KC292055

bengalensis, and other Felids (Supplementary Data 1). D-loop sequences are functional nucleotide sequences for the duplication of mitochondrial DNA. In the phylogenetic analysis, the obtained sequence and the leopard cat's reference D-loop sequence (accession number: NC_028301) were classified into the same clade distinct from the clade of domestic cats, indicating that the FFV strain NV138 was isolated from leopard cats (Fig. 1A). We then tried to construct an infectious molecular clone from strain NV138. In the initial construction, we identified a 440-nt deletion in the *env* region. Therefore, a partial *env* region was replaced with a new fragment to recover the deletion and create the infectious molecular clone with the complete FFV genome (Fig. 1B). We designated the resultant plasmid as pLC960 (accession number: MW349910). To confirm the infectivity of pLC960-derived virus, pLC960 was used to transfect CRFK cells. Three days after transfection, the culture supernatant was recovered and used to inoculate naive CRFK cells. As a result, severe CPE was observed four days after inoculation (Fig. 1C), and a DNA fragment of the *gag-pol* region was detected by PCR in the cells inoculated with the supernatant from cells transfected with pLC960 (Fig. 1D).

Biological characterization of LC960

To compare the growth kinetics of the clone-derived virus with the parental virus and other FFV isolates, CRFK cells were inoculated with the isolates; LC960 (clone-derived), NV138 (the parental isolate), Coleman, and Sammy-1. The viral titers of the culture supernatants were measured at 2, 4, 6, and 9 days after inoculation. The growth kinetics of LC960 were similar to those of the parental NV138 isolate, and faster than those of Sammy-1 and slower than those of Coleman (Fig. 1E). Because Tas is a transactivater, it affects the efficiency of viral replication and may be involved in the efficiency of FFV replication in each species. Thus, we compared Tas activities on the promoter activity of LTRs of the three isolates by dual luciferase reporter assay. No significant difference in Tas activity in the presence of homologous or heterologous LTR was observed (Fig. 1F).

Phylogenetic analyses of FFVs

To infer the phylogenetic relationship of NV138 and SV201, rooted ML phylogenetic trees were constructed from aligned nucleotide sequences of *pol*, *gag*, and LTR (the entire U3-R-U5 region) (Fig. 2A–C, Supplementary Data 2–4). In the phylogenetic tree of *pol*, FFV isolates from leopard cats, NV138 and SV201, were the most closely related to each other (Fig. 2A). In the phylogenetic trees of *pol* and *gag*, the FFV isolates in Vietnam, NV138, SV201, VN114, and VN119, including domestic cats and leopard cats, were clustered into a single clade. Intriguingly, F17 isolate from the USA was also included in the clade (Fig. 2A and 2B). On the other hand, the phylogenetic tree based on LTR sequences showed relatively low bootstrap values (Fig. 2C), thus it was difficult to make a phylogenetic discussion.

Recombination and phylogenic analysis of env gene

To assess the recombination events in *env* genes, we tried to detect recombination events using the RDP4 program. We detected several recombination events and two recombination hotspots, where the recombination breakpoints were densely located (Fig. 3A and 3B). The two hotspots reside at both ends of the Env SU domain, nucleotide position: 343–567 and 1,678–1,755 from the 5′-terminus of *env* (Fig. 3B). Based on these hotspots, we divided the *env* into two regions, the conserved region (nucleotide positions: 1–342 and 1,756–2,946, and amino acid positions: 1–114 and 584–982) and variable region (nucleotide position: 568–1,677; amino acid position: 190–559). We constructed phylogenetic trees from aligned sequences of the two regions and the entire *env* region (Fig. 3C–E, Supplementary Data 5–7). The entire *env* region and the variable region were divided into two clades regardless of the geographic areas they were derived from (Fig. 3C and 3E). On the other hand, the phylogenetic tree based on the conserved region reflected the geographical distance (Fig. 3D).

To examine whether FFVs from leopard cats have specific adaptive substitutions not found in FFVs from domestic cats, amino acid identities of Env were calculated for FFVs from leopard cats (NV138 and SV201) and closely related isolates from domestic cats (VN114, VN119, and F17). While the identity of SU amino acid sequences of Env protein was also found to be high (99.31%) between the two FFVs from leopard cats, the maximum identity between FFVs from a leopard cat and a domestic cat was also high (99.54%). Moreover, there were no amino acid substitutions specific to FFVs of leopard cats in Env (Fig. 4A).

Sequence analysis of Bet protein

To examine whether Bet of FFV from leopard cats contributes to adaptive evolution, the sequence identity of Bet protein was calculated for FFVs from the leopard cat (strains NV138 and SV201) and closely related isolates (strains VN114, VN119, VN150, and F17). The identity of amino acid sequences of Bet was 97.61% between two FFV strains from leopard cats, while the maximum identity between FFVs from a leopard cat and a domestic cat was 97.61%. There were no amino acid substitutions specific to FFVs from leopard cats (Fig. 4B). In Bet proteins of bovine, equine, simian, primate FV, and FFV, highly conserved motifs (motifs 1 to 4) and low conserved motifs (motifs 5 and 6) were previously described [19]. High conserved motifs are conserved except for motif 2, where two substitutions were observed. In low conserved motifs, a few substitutions were observed in strain NV138 (Fig. 4B).

DISCUSSION

Here, we constructed an infectious molecular clone, termed pLC960, from an FFV isolate of a leopard cat and sequenced the complete genome of two FFV isolates from leopard cats. We found that replication of the pLC960-derived virus in CRFK cells was faster than the Sammy-1 isolate but slower than the Coleman isolate (Fig. 1E). These data indicate the successful construction

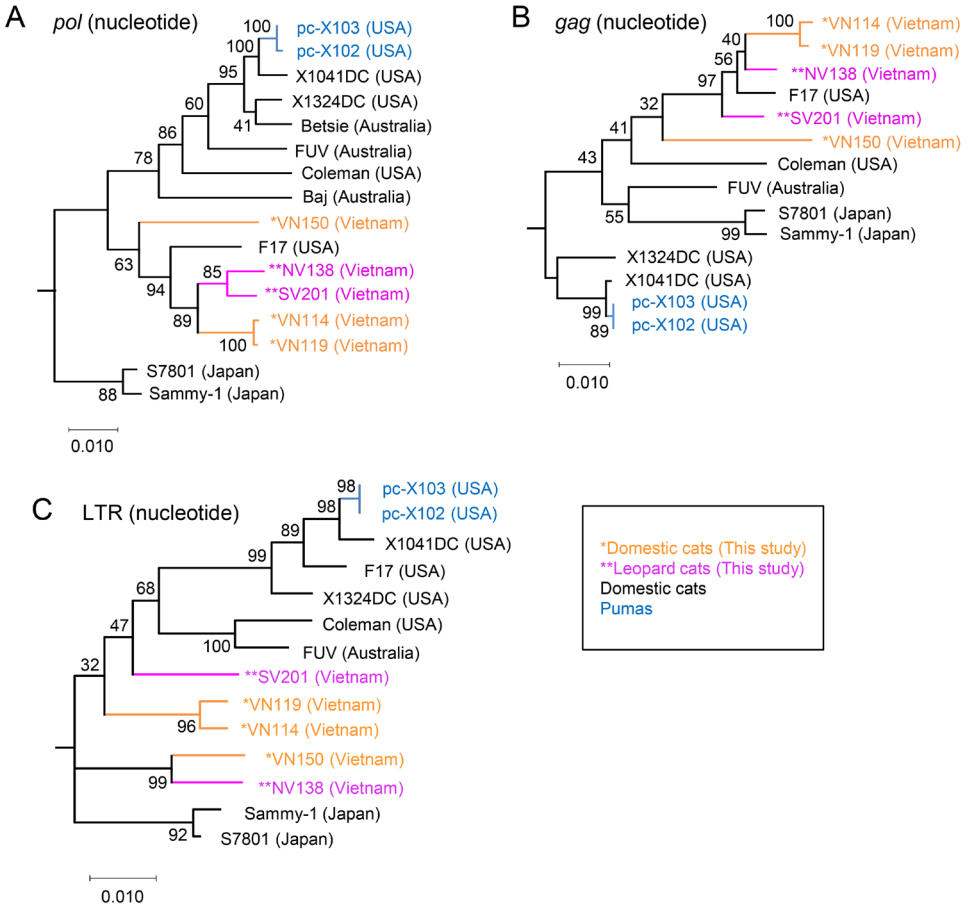

Fig. 2. Phylogenetic analyses of feline foamy viruses derived from leopard cats and domestic cats in Vietnam. Trees based on (**A**) nucleotide sequence of *pol*, (**B**) nucleotide sequence of *gag*, and (**C**) nucleotide sequence of LTR of FVs from domestic cats, leopard cats, and other mammals are shown. The GenBank accession number for each virus isolate is also shown in Table 1. The rooted maximum likelihood phylogenetic trees were constructed using MEGA-X with bootstraps of 1,000 replicates with the substitution models, (**A**) HKY+G, (**B**) GTR+G, and (**C**) T92+G, respectively. The root of trees was determined using SFV (MF472626), bovine foamy virus (BFV) (NC_001831), and equine foamy virus (EFV) (NC_001831) as sequences in the outgroup. Nodes are labeled with bootstrap support values. The isolates sequenced in this study are indicated by single and double asterisks for isolates from domestic cats and leopard cats, respectively.

of an infectious clone and revealed that FFVs from leopard cats can replicate as efficiently as FFVs from domestic cats in CRFK cells, derived from domestic cats. Also, the *trans*-activity of Tas protein from the leopard cat was comparable with that of Tas of those from domestic cats (Fig. 1F). Therefore, the interactions between LTR and Tas may not have changed through interspecies transmission.

Phylogenetic analysis using *pol* sequences showed that FFVs from leopard cats are closely related to those from domestic cats in Vietnam, suggesting the recent transmission of FFVs from domestic cats to leopard cats, as previously reported (Fig. 2A and 2B) [24]. Intriguingly, FFVs isolated in Vietnam were closely related to F17 isolated in the USA (Fig. 2A and 2B). Therefore, it is suggested that the cross-continent movement of domestic cats brought FFVs into Vietnam from the USA or vice versa. The two isolates derived from leopard cats were closely related to each other despite their collection sites being far apart in northern and southern Vietnam (Fig. 2A and 2B). FFVs may be prevalent in the leopard cat population of Vietnam.

The SU domain of Env protein is necessary for the interaction with the host receptor and determination of the host specificity. We showed that frequent recombination occurs in the SU domain of FFVs (Fig. 3A–C). This result was similar to those of previous studies demonstrating frequent recombination events in SFVs or FFVs [1, 15]. The result also showed that recombination in the SU domain could have caused significant changes of the amino acid sequence in the variable region. However, the sequences of the variable region of FFVs from leopard cats were closely related to the sequences from domestic cats which were classified into the same subtype (Fig. 3E). Therefore, it cannot be concluded that evolution of the SU domain by recombination caused interspecies transmission.

Bet protein should have co-evolved with host species because the protein inhibits host restriction factors, APOBEC3s [2, 10]. Bet protein of FFVs from leopard cats and domestic cats are very similar. Among Bet proteins, highly conserved motifs (motifs 1 to 4) and low conserved motifs (motifs 5 and 6) among FVs were reported, and motifs 1, 2, and 3 were important for binding of

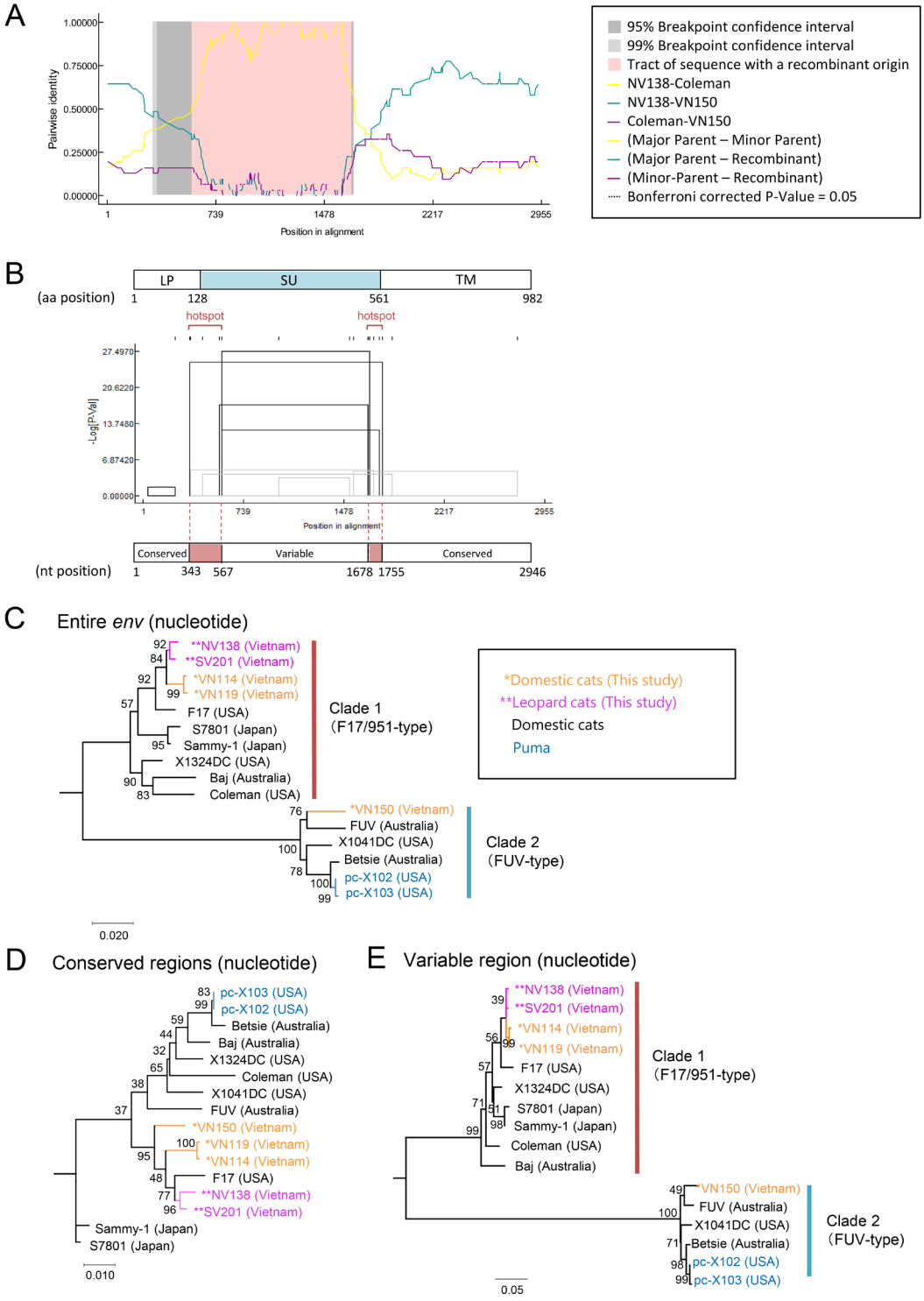

Fig. 3. Recombination analysis of *env*. (**A**) One of the graphical results of recombination analysis using RDP. The plot shows pairwise identity between the recombinant (VN150) and its major and minor parent sequences (NV138 and Coleman, respectively). (**B**) Schematic outline of the Env domain structure (top), the recombination event map (middle), and schematic outline of recombination hotspots, in conserved and variable regions (bottom). In the recombination map, the plot shows estimated positions of recombination breakpoints and *P*-values associated with the detected recombination signals. Whereas gray lines indicate that parental sequences were more distantly related, black lines indicate that they were more closely related. The tick marks above the plot indicate individual recombination breakpoints inferred. From the results, two recombination hotspots were found: the first region is between 343 and 567-nt (5′ recombination hotspot), and the second one is between 1,678 and 1,755-nt (3′ recombination hotspot) of the alignment. (**C**–**E**) Maximum likelihood trees of *env* genes of (**C**) the entire *env* sequence, (**D**) conserved region, and (**E**) variable region. Phylogenetic trees were constructed using MEGA-X with bootstraps of 1,000 replicates with the substitution models, (**C**) GTR+G, (**D**) T92+G, and (**E**) T92+G+I, respectively. Nodes are labeled with bootstrap support values. The isolates sequenced in this study are indicated by a single asterisk and double asterisks for isolates from domestic cats and leopard cats, respectively. LP, leader peptide; SU, surface unit Env; TM, transmembrane Env; RBD, receptor-binding domain.

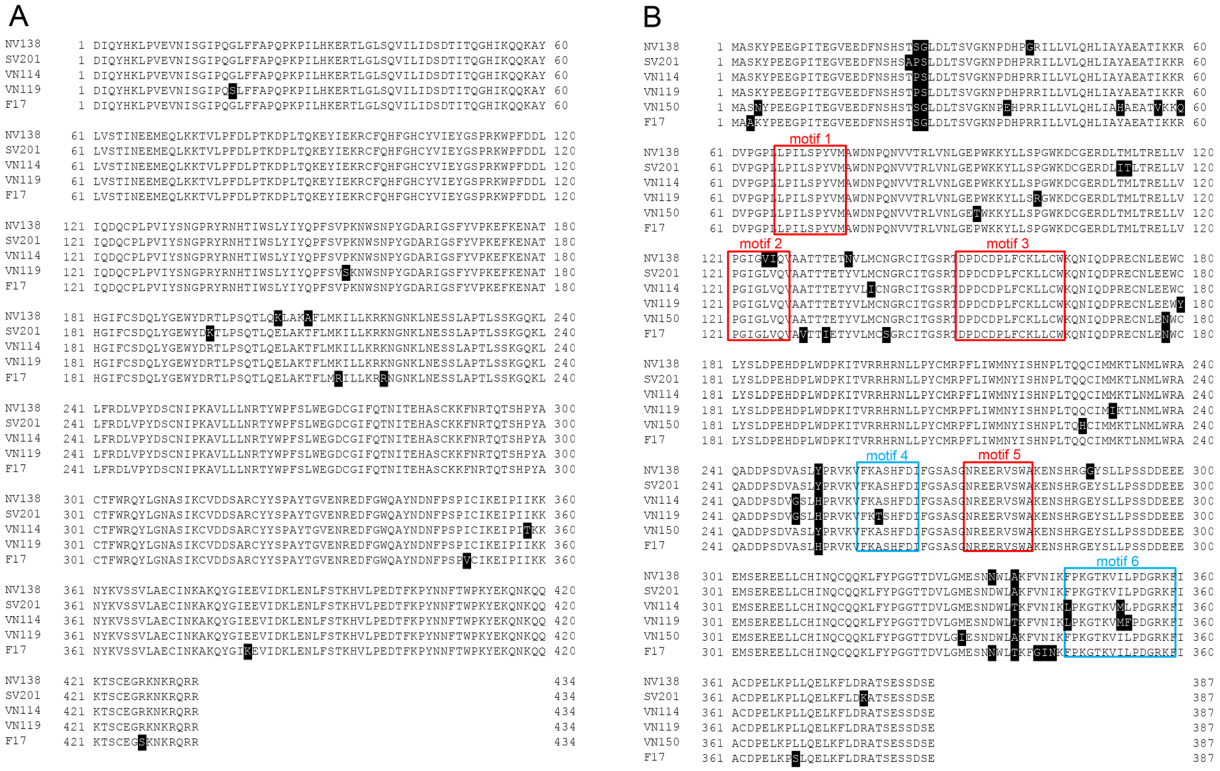

Fig. 4. The alignments of amino acid sequences of the surface unit (SU) of Env (**A**) and Bet (**B**) proteins. Conserved motifs among foamy viruses which were reported by Lukic *et al.* are marked by red (highly conserved) or blue (poorly conserved) boxes [18].

Bet to APOBEC3s of domestic cats [19]. Two substitutions, L125V and V126I, were specifically observed in motif 2 of NV138. Whether these mutations modify Bet functions should be confirmed experimentally in the future.

In conclusion, we successfully generated an infectious molecular clone of FFV isolated from a leopard cat in Vietnam. We then performed biological and genetical analyses of this isolate and other FFV isolates. Our results revealed no traces indicating that FFVs have evolved to adapt to leopard cats, suggesting the transmission from domestic cats to leopard cats without specific viral adaptation in Vietnam. Our data suggest that wild cats have the potential to become new hosts of FFVs from domestic cats.

CONFLICT OF INTEREST. The authors declare that there are no conflicts of interest.

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