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Feline Immunodeficiency Virus in Domestic Cats on Tsushima Island and Its Potential Implications for the Endangered Tsushima Leopard Cat

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Abstract

The spillover of infectious diseases from domestic animal reservoirs poses a major threat to endangered wildlife. The Tsushima leopard cat (TLC; *Prionailurus bengalensis euptilurus*), a critically endangered felid endemic to Tsushima Island, Japan, faces potential exposure to pathogens maintained in sympatric domestic cats (*Felis catus*). To investigate feline immunodeficiency virus (FIV) circulation on Tsushima Island, we conducted a 15-year molecular epidemiological study of 720 domestic cats and available sequence-confirmed TLC cases. Phylogenetic analysis revealed that FIV sequences from two TLCs (Feu1, sampled in 1996, and Mt092000, sampled in 2000, and sequenced in this study) belonged to subtype D1 and formed a distinct, strongly supported monophyletic cluster (100% bootstrap support). Their close genetic relationship, despite the four-year sampling interval, was consistent with the possibility of viral persistence within the TLC population. However, the transmission history could not be conclusively determined because only two TLC sequences were available. Surveillance of sympatric domestic cats revealed high seroprevalence (25.0%), with peak positivity in Kamiagata, the primary TLC habitat. FIV subtype determination showed co-circulation of subtypes D1 (51.7%), A (40.6%), and B (7.7%). These findings highlight the high prevalence and genetic diversity of FIV in domestic cats on Tsushima Island and underscore the importance of continued surveillance to better understand its potential implications for the conservation of endangered Tsushima leopard cats.

Keywords: epidemiology; feline immunodeficiency virus; Tsushima leopard cat; Tsushima island; Japan

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1. Introduction

The spillover of pathogens from domestic animals to wildlife can cause devastating disease outbreaks that threaten wildlife populations [1–5]. Emerging infectious diseases can have particularly severe consequences for small, fragmented populations and may ultimately lead to the extinction of endangered species [5,6]. Understanding the factors driving interspecies pathogen transmission is therefore essential for developing effective conservation strategies to protect endangered wildlife.

The Tsushima leopard cat (TLC; *Prionailurus bengalensis euptilurus*) is an endangered subspecies of leopard cat endemic to Tsushima Island, Nagasaki Prefecture, Japan. Its population is estimated at approximately 100 individuals and has declined because of habitat loss, infectious diseases, and road traffic accidents [7–10].

Several infectious agents have been detected in TLCs, including feline immunodeficiency virus (FIV), feline foamy virus (FFV), *Felis catus* gammaherpesvirus 1 (FcaGHV1), and severe fever with thrombocytopenia syndrome virus, as well as bacterial and protozoal pathogens such as *Bartonella clarridgeiae*, *Anaplasma bovis*, and *Hepatozoon felis* [10–15]. These findings indicate that TLCs face substantial health threats from diverse infectious agents. Domestic cats are of particular concern because many on Tsushima Island roam freely and may come into contact with TLCs. In a previous FIV study, phylogenetic analysis of the viral env gene revealed that an FIV isolate from a TLC clustered with subtype D viruses from domestic cats, supporting the possibility of interspecies transmission from domestic cats to TLCs in the wild [11]. Similarly, FcaGHV1 sequences from domestic cats and TLCs formed a single phylogenetic cluster, suggesting that closely related viruses circulate in both host populations [14]. FFV has also been detected in both species, with sequences from TLCs clustering with those from domestic cats, further supporting viral circulation at the domestic cat–TLC interface [10]. These findings highlight domestic cats as a potential source of infectious pathogens for TLCs.

FIV is a *lentivirus* belonging to the family *Retroviridae* that infects members of the family Felidae [16,17] and shares several biological characteristics with human immunodeficiency virus [18,19]. Disease severity varies among felid species, with FIV infection generally associated with more severe disease in domestic cats (*Felis catus*) than in pumas, bobcats, leopards, Pallas's cats, and lions [18,20]. FIV is transmitted primarily through bite wounds sustained during aggressive encounters [21], although vertical transmission can also occur [22]. Clinical manifestations range from asymptomatic infection to lymphadenopathy, malaise, tumors, and neurological disorders [23,24]. Approximately half of FIV-infected domestic cats develop ulcerative and proliferative stomatitis, a painful condition that can lead to anorexia and emaciation [25].

Phylogenetically, FIV is classified into seven subtypes: A–F and U-NZenv. Globally, FIV prevalence ranges from 1% to 14% among clinically healthy cats and can reach 50% among sick cats [26,27]. In Japan, the overall prevalence has been reported to be 23.2% [28]. On Tsushima Island, a previous geospatial analysis reported FIV prevalences of 11.8% in domestic cats and 3.0% in TLCs [7]. The study also found a positive correlation between FIV infection in TLCs and the prevalence of FIV in nearby domestic cat populations. Because most domestic cats on the island roam freely, opportunities for contact with wildlife are increased, complicating disease control efforts [29].

Previous investigations of FIV on Tsushima Island have primarily focused on spatial patterns of prevalence, with limited characterization of the phylogenetic diversity of circulating viral subtypes, which is essential for understanding transmission dynamics. The only study to compare FIV subtypes between domestic cats and TLCs [11] included a relatively small sample ($n = 50$) and did not assess associated risk factors. These limitations highlight the need for updated, comprehensive data. To address these knowledge gaps, we conducted a 15-year epidemiological and molecular investigation of FIV in domestic cats and TLCs on Tsushima Island, Japan. Through the integration of prevalence data, risk factor analyses, and viral genotyping, this study provides updated insights into the epidemiology and genetic diversity of FIV and contributes information relevant to disease management and conservation of endangered TLCs.

2. Materials and Methods

2.1. Study Location

This study was conducted on Tsushima Island (34°05′–34°42′ N, 129°10′–129°30′ E), which is part of the Japanese archipelago and lies between the Korea Strait and the Tsushima Strait in Nagasaki Prefecture, Japan. Tsushima is administratively divided into two major landmasses: the northern region, Kamijima, and the southern region, Shimojima. Kamijima comprises four towns—Kamitsushima, Kamiagata, Mine, and Toyotama—whereas Shimojima includes two towns, Mitsushima and Izuhara (Figure 1A).

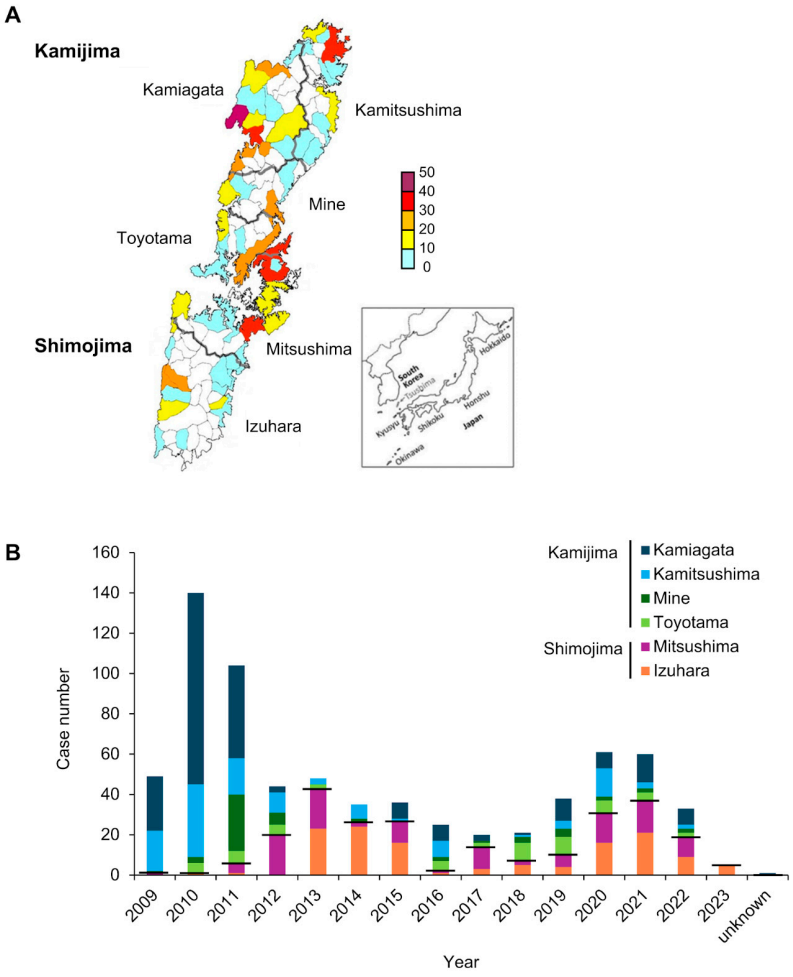


Figure 1. Spatial and temporal distribution of domestic cat sampling on Tsushima Island. (**A**) Geographic map of Tsushima Island showing the northern region (Kamijima), comprising four administrative towns (Kamitsushima, Kamiagata, Mine, and Toyotama), and the southern region (Shimojima), comprising two towns (Mitsushima and Izuhara). All town names are labeled in English. The map also shows Tsushima Island's location in the Japanese archipelago between Kyushu and the Korean Peninsula. (**B**) Bar chart showing the annual number of domestic cats sampled from 2009 to 2023. 2015 indicates the boundary between Kamijima and Shimojima. All samples were screened for antibodies against FIV using a commercial test kit (IDEXX Laboratories Inc., Westbrook, ME, USA) according to the manufacturer's instructions.

2.2. Sample Collection

Archived blood samples from domestic cats collected from 2009 to 2023 through the Tsushima Animal Medical Center, a nonprofit veterinary clinic, were analyzed in this study. Samples for whole-blood collection were collected in EDTA- or heparin-coated tubes. After routine diagnostic processing at the clinic, the specimens were retained as archived samples

for use in this study. Aliquots of approximately 500 µL were transferred into Eppendorf tubes, transported frozen to our laboratory, and stored at −20 °C to −30 °C until analysis. Additionally, these samples were utilized for viral genetic testing. Cats were presented to the clinic for various reasons, including routine health examinations, medical conditions, and, most commonly, surgical procedures such as spaying and neutering. The sampled population included indoor-only cats, free-roaming cats, and cats kept under mixed indoor–outdoor conditions. For each cat, the sampling location, sex, and age were recorded. Staff at the Tsushima Wildlife Conservation Center, a rescue and management facility operated by Japan's Ministry of the Environment for endangered species, collected and archived blood and tissue samples from TLCs. Most samples were obtained from individuals that died as a result of road traffic accidents, whereas a smaller number were collected from rescued animals admitted to the center for veterinary care following such accidents, including TLC Mt092000. From 1999 to 2023, samples from 91 TLCs were archived and used for FIV surveillance. All available serum samples were screened for antibodies against FIV using a commercial test kit (IDEXX Laboratories Inc., Westbrook, ME, USA) according to the manufacturer's instructions. Frozen whole-blood samples were transported to the laboratory for DNA extraction and subsequent genetic analyses.

2.3. DNA Extraction and Nested PCR

Total DNA was extracted from whole blood using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop instrument (Bio-Rad Laboratories, Carlsbad, CA, USA), and the extracted DNA was stored at −30 °C until use. The V3–V5 region of the FIV env gene was amplified using two primer pairs. The outer primer pair (Fe-835S-GAGTAGATACWTGGTTRCAAG and Fe-866R-CATCCTAATTCTTGCATGC) was expected to yield a 1193 bp amplicon, whereas the inner primer pair (Fe-836S-CAAAATGTGGATGGTGGAAYC and Fe-867R-ACCATTCCWATAGCAGTRGC) was expected to produce an 859 bp fragment corresponding to the V3–V5 env region, as assessed by 1% agarose gel electrophoresis [30]. The total volume of the reaction mixture used for each nested polymerase chain reaction (PCR) was 25.0 µL and contained 12.5 µL of KOD One™ PCR Master Mix (TOYOBO Bio Inc., Osaka, Japan), 8.5 µL of ultra-pure PCR-grade water, and 1.5 µL each of the forward and reverse primers. The first-round PCR consisted of 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 15 s, and extension at 68 °C for 30 s. The second-round PCR also consisted of 30 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 15 s, and extension at 68 °C for 30 s.

2.4. Multiple Sequence Alignment and Phylogenetic Analysis

DNA sequencing was performed using both direct sequencing of PCR products and sequencing of the cloned PCR products generated by insertion into the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Sequencing was performed bidirectionally by Fasmac Co., Ltd. (Kanagawa, Japan). Sequence visualization and analysis were performed using UGENE version 45.0 (Novosibirsk, Russia). GENETYX Ver. 8.0.0 (Nihon Server Corporation, Tokyo, Japan) and the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST—https://www.ncbi.nlm.nih.gov (accessed on 8 September 2026)) were used to compare the 859 bp sequences with other FIV env sequences. The V3–V5 env nucleotide sequences obtained from the study samples and reference FIV isolates retrieved from GenBank were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) [31]. The reference sequences included Petaluma FIV14 (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), AIC01 (AB010396), MIE24 (AB515132), FKS14 (AB515002), IBG1 (AB515047), ISK5 (AB515057), NGT9 (AB515160), TYM14 (AB515262), SAP01–SAP03 (AB010402–AB010404), PTH-BM3 (AB010401), TAU01 (AB010405), OKA01 (AB010400), KUM01–KUM02 (AB010398–AB010399), and the TLC clone from 1996 Feu1 (AB021100), as well as 10 FIV sequences from domestic cats on Tsushima Island (TSU101, TSU102, TSU104, TSU107, TSU109, TSU116, TSU202, TSU210, TSU215, and TSU226; GenBank accession numbers AB021109–AB02118). Phylogenetic relationships were inferred using the neighbor-joining method [32]. The tree was drawn to scale, with branch lengths representing the evolutionary distances used to infer the phylogeny. Evolutionary distances were calculated using the Kimura two-parameter model [33] and expressed as the number of base substitutions per site. Rate variation among sites was modeled using a gamma distribution with a shape parameter of 1. The analysis included 189 nucleotide sequences. Positions with less than 95% site coverage were excluded, allowing fewer than 5% alignment gaps, missing data, or ambiguous bases at each position (partial deletion). The final dataset comprised 618 nucleotide positions. All evolutionary analyses were performed using Molecular Evolutionary Genetics Analysis version X (MEGA X Version 10.2.6) [34].

2.5. Statistical Analysis

Statistical analyses were performed using R version 4.5.1 (R Core Team, 2025; R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA). Associations between FIV seropositivity and demographic and environmental variables, including sex, age group, housing conditions, and administrative town, were assessed using Pearson's chi-square test or Fisher's exact test, as appropriate based on the expected cell counts. Crude odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using separate univariable logistic regression models for each predictor. All tests were two-tailed, and p-values < 0.05 were considered statistically significant.

2.6. Nucleotide Sequence Accession Numbers

The V3–V5 regions of the FIV *env* gene sequenced in this study were deposited in the DNA Data Bank of Japan and GenBank databases under accession numbers LC800888–LC800959 and LC805774–LC805845.

3. Results

3.1. FIV Prevalence and Demographics

A total of 720 domestic cat blood samples were collected between 2009 and 2023, primarily during trap-neuter-return (TNR) programs. The demographic characteristics and geographical distribution of the sampled cats are summarized in Table 1, Table S1, and Figure 1. Of these, 65.4% (471/720) were from Kamijima and 34.6% (249/720) from Shimojima. Overall, 45.6% (328/720) of the sampled cats were male, and 54.4% (392/720) were female. The ages of 62.9% (453/720) of the cats were unknown, whereas the recorded ages of the remaining cats ranged from <1 to >10 years. Regarding living conditions, 83.2% (599/720) of the cats lived outdoors, 4.3% (31/720) indoors, and 12.5% (90/720) in mixed indoor–outdoor environments. Collectively, these characteristics indicate that the sampled population consisted predominantly of free-roaming cats.

Table 1. Profile of cats by region.

		Kamijima			Shimojima			Total	FIV+ (%)
		Kamiagata	Kamitsushima	Mine	Toyotama	Mitsushima	Izuhara		
Age (Years)	<1	28	16	15	13	10	23	105	6 (5.7)
	1	11	11	8	7	11	14	62	7 (11.3)
	2	12	2	2	2	6	11	35	10 (28.6)
	3	5	2	3	3	10	3	26	9 (34.6)
	4	2	5	1	0	1	4	13	4 (30.8)
	5	2	1	2	1	3	2	11	4 (36.4)
	6	1	0	0	0	0	0	1	0 (0.0)
	7	0	1	1	0	0	1	3	1 (33.3)
	8	0	0	2	1	0	0	3	0 (0.0)
	≥10	2	3	1	2	0	0	8	2 (25.0)
	unknown	170	86	21	26	79	71	453	137 (30.2)
	total	233	127	56	55	120	129	720	180 (25.0)
Sex	Male	100	67	22	18	61	60	328	119 (36.3)
	Female	133	60	34	37	59	69	392	61 (15.6)
	total	233	127	56	55	120	129	720	180 (25.0)
Environment	Outdoor	197	104	45	43	107	103	599	159 (26.5)
	Mixed [#]	29	18	5	9	9	20	90	13 (14.4)
	Indoor	7	5	6	3	4	6	31	8 (25.8)
	total	233	127	56	55	120	129	720	180 (25.0)

[#] Indicates cats kept both indoors and outdoors.

FIV antibody testing revealed an overall seroprevalence of 25.0% (180/720). FIV-positive cats were distributed across the towns of Tsushima Island, with Kamiagata recording the highest number of cases (Figure 2A). The annual FIV seroprevalence varied substantially across the years, ranging from 6.8 to 65.0%, with the highest prevalence found in 2017. No positive cases were detected in 2023; however, the sample size for that year was markedly small. The seroprevalence also varied geographically among towns (Figure 2B).

Kamiagata consistently exhibited elevated seroprevalence, exceeding 30–80% in several years. Kamitsushima and Toyotama showed moderate but fluctuating seroprevalence, whereas Mine generally exhibited low seroprevalence, with occasional spikes associated with very small sample sizes. On Shimojima, Mitsushima showed highly variable annual seroprevalence, largely attributable to small annual sample sizes, whereas Izuhara exhibited moderate seroprevalence, with occasional high-prevalence years (Figure 2B, Table S2). Overall, these findings indicate pronounced inter-annual variability rather than stable patterns of seroprevalence across towns.

Male cats had a significantly higher seropositivity rate (36.3%, 119/328) than female cats (15.6%, 61/392; chi-square test: $\chi^2 = 39.55$, $p < 0.001$). The odds of FIV seropositivity were 3.08 times higher in males than in females (OR, 3.08; 95% CI, 2.13–4.47). Age-stratified analysis of cats with known ages ($n = 267$) demonstrated a strong age-dependent increase in seropositivity. Adult cats (≥2 years old) had a seropositivity rate of 30.0% (30/100), compared with 7.8% (13/167) in young cats (<2 years old; chi-square test: $\chi^2 = 21.23$, $p < 0.001$). The odds of seropositivity were 5.08 times higher in adult cats than in young cats (OR, 5.08; 95% CI, 2.49–10.36). The rate of FIV seropositivity also increased with age across individual age groups: 5.7% (6/105) in cats <1 year old, 11.3% (7/62) at 1 year, 28.6% (10/35) at 2 years, 34.6% (9/26) at 3 years, and 36.4% (4/11) at 5 years. Cats of unknown age had a seropositivity rate of 30.2% (137/453), comparable to that of the adult group. Seropositivity rates also varied by housing conditions: 26.5% (159/599) in completely outdoor cats, 14.4% (13/90) in cats with mixed indoor-outdoor access, and 25.8% (8/31) in strictly indoor cats. The overall seroprevalence did not differ significantly between Kamijima (25.1%, 118/471) and Shimojima (24.9%, 62/249; $\chi^2 = 0.00$, $p = 1.00$).

However, seroprevalence differed significantly among the six administrative towns ($\chi^2 = 11.62$, $p = 0.0404$). Seropositivity rates ranged from 16.1% in Mine to 31.8% in

Kamiagata, with intermediate rates in Izuhara (26.4%), Mitsushima (23.3%), Toyotama (20.0%), and Kamitsushima (18.9%).

Of the 91 TLCs screened for FIV antibodies, two (2.2%) tested positive. Sequence data suitable for phylogenetic analysis were available from one FIV-positive TLC (Mt092000). This sequence was analyzed together with the previously reported TLC sequence Feu1 retrieved from GenBank.

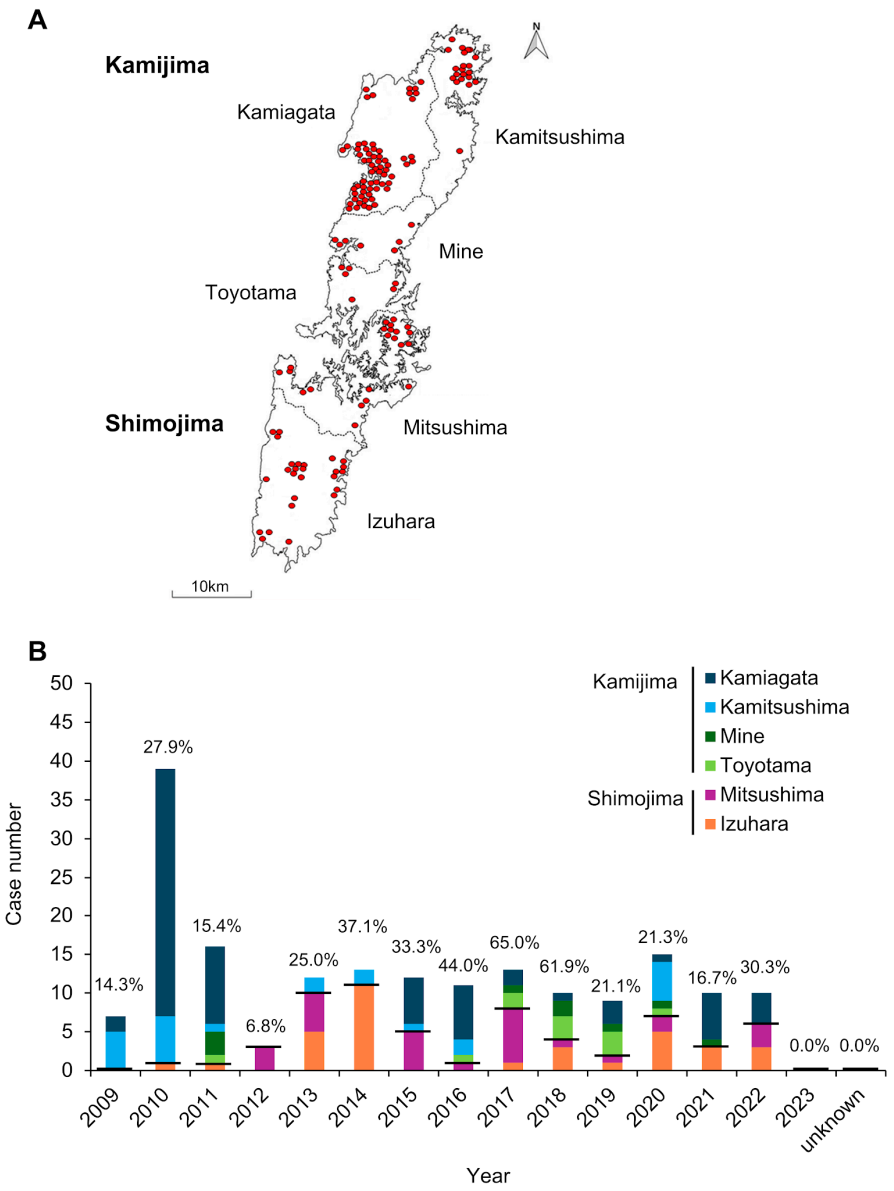


Figure 2. Spatial and temporal distribution of feline immunodeficiency virus (FIV)-positive domestic cat samples on Tsushima Island. (**A**) Geographic map showing the sampling locations of 180 domestic cats that tested positive for FIV antibodies using an FIV antibody test kit. The red dots indicate the location of FIV seropositive samples (**B**) Bar chart showing the annual number of FIV-positive domestic cat samples collected from 2009 to 2023. % indicates the FIV seropositivity per year; 2015 is the boundary between Kamijima and Shimojima.

3.2. Phylogenetic Analysis Based on the FIV Env Region

Of the 180 domestic cat samples that tested positive in the antibody screening, 143 were subsequently subjected to sequence analysis. The remaining 37 samples failed to yield any reliable nucleotide sequence data, despite repeated sequencing attempts, and were therefore excluded from the genetic analyses. In addition, one FIV-positive TLC sample

(Mt092000), originally sampled in 2000, was available for molecular analysis and was therefore included in the study. A phylogenetic tree was constructed using all 144 FIV-positive samples from this study (143 domestic cats and one TLC) and 45 reference sequences, including the TLC sequence Feu1, retrieved from GenBank. Phylogenetic analysis revealed that domestic cats on Tsushima Island were infected with FIV subtypes A, B, and D1. The TLC sample belonged to subtype D1. Within subtype A, three samples from Mitsushima formed a distinct subcluster supported by high bootstrap values (97–99%) (Figure 3). Of the 143 domestic cat sequences, 40.6% (58/143) clustered within FIV subtype A, 7.7% (11/143) within subtype B, and 51.7% (74/143) within subtype D1 (Table 2). The TLC sequence clustered with several domestic cat sequences within subtype D1, indicating that this endangered wild felid harbored the same viral subtype as most infected domestic cats.



Figure 3. Neighbor-joining phylogenetic tree of 189 FIV V3–V5 env sequences. The phylogenetic tree was constructed using the neighbor-joining method in MEGA X, with 1000 bootstrap replicates. Sequences prefixed with "TSM" represent domestic cat samples analyzed in this study, whereas Mt092000 represents a sequence from a Tsushima leopard cat (TLC). All other sequences are reference strains. Bootstrap values of ≥70% are shown at major nodes. The scale bar (0.05) indicates the number of nucleotide substitutions per site.

Further phylogenetic analysis revealed substantial intra-genotypic diversity within the three FIV subtypes identified in domestic cats on Tsushima Island. Within subtype A (58 sequences), a distinct subcluster comprising three samples collected in Mitsushima in 2013 was supported by high bootstrap values (97–100%) (Figure S1A). This lineage was not detected in other sampling years or regions. In Izuhara, two subtype A lineages

were identified: one was detected repeatedly from 2013 to 2022, whereas the other was observed only in 2014 and was not detected in subsequent years (Figure S1B,E). In northern and central Kamijima, a lineage detected in Kamiagata from 2011 to 2017 clustered with sequences from Mine collected through 2021 (Figure S1D). In addition, a lineage detected in Kamiagata in 2020–2021 clustered closely with sequences from Mine (Figure S1C). Another widely distributed lineage included samples from four administrative towns spanning the island from north to south: Kamiagata, Izuhara, Mitsushima, and Toyotama (Figure S1E). Subtype A sequences from Toyotama were distributed across four distinct clusters, with two samples collected in 2017 (TSM110 and TSM109) forming a strongly supported pair (bootstrap value, 99%) (Figure S1E).

Table 2. FIV Subtypes in Domestic cats on Tsushima Island (Location).

	A	B	D1	Total
Kamijima				
Kamiagata	23	0	41	64
Kamitsushima	0	2	17	19
Mine	5	0	2	7
Toyotama	4	0	2	6
Shimojima				
Mitsushima	15 *	2	4	21
Izuhara	11	7	8	26
Total	58	11	74	143

* Includes three phylogenetically distinct subtype A samples. Subtype information was available for 143 of 180 FIV-seropositive domestic cats.

Phylogenetic analysis of subtype B ($n = 11$) revealed distinct clustering patterns depending on the geographic region and sampling period. During the early survey period, two samples collected in Kamitsushima, northern Kamijima, in 2010 (TSM38 and TSM40) formed an independent, strongly supported monophyletic cluster (bootstrap value, 100%) (Figure S2). This lineage was not detected elsewhere on Kamijima or on the rest of the island after 2010. In contrast, subtype B sequences from Shimojima (Izuhara and Mitsushima) were detected repeatedly from 2011 to 2022. The Kamitsushima and Shimojima sequences formed genetically divergent clusters (Figure S2). FIV subtype D1 exhibited greater phylogenetic structuring, with multiple clusters distributed across towns and sampling years. The primary cluster comprised a lineage detected over 14 years (2009–2022) across four towns spanning northern and southern Tsushima Island: Kamiagata, Izuhara, Toyotama, and Mitsushima (Figure S3A). A second cluster was restricted to Kamiagata in 2009–2011 and was not detected in subsequent sampling periods (Figure S3B). The third cluster included sequences from Kamiagata, Mine, and Kamitsushima collected from 2009 to 2021 (Figure S3C); the fourth included sequences from Kamiagata and Kamitsushima collected from 2009 to 2022 (Figure S3D); and the fifth comprised sequences from Mine, Toyotama, Kamitsushima, and Kamiagata (Figure S3E). Each cluster also contained sequences from domestic cats analyzed in an earlier study that grouped phylogenetically with the sequences in this study (Figure 4A). Collectively, these findings demonstrate substantial intra-genotypic diversity among FIV subtypes on Tsushima Island.

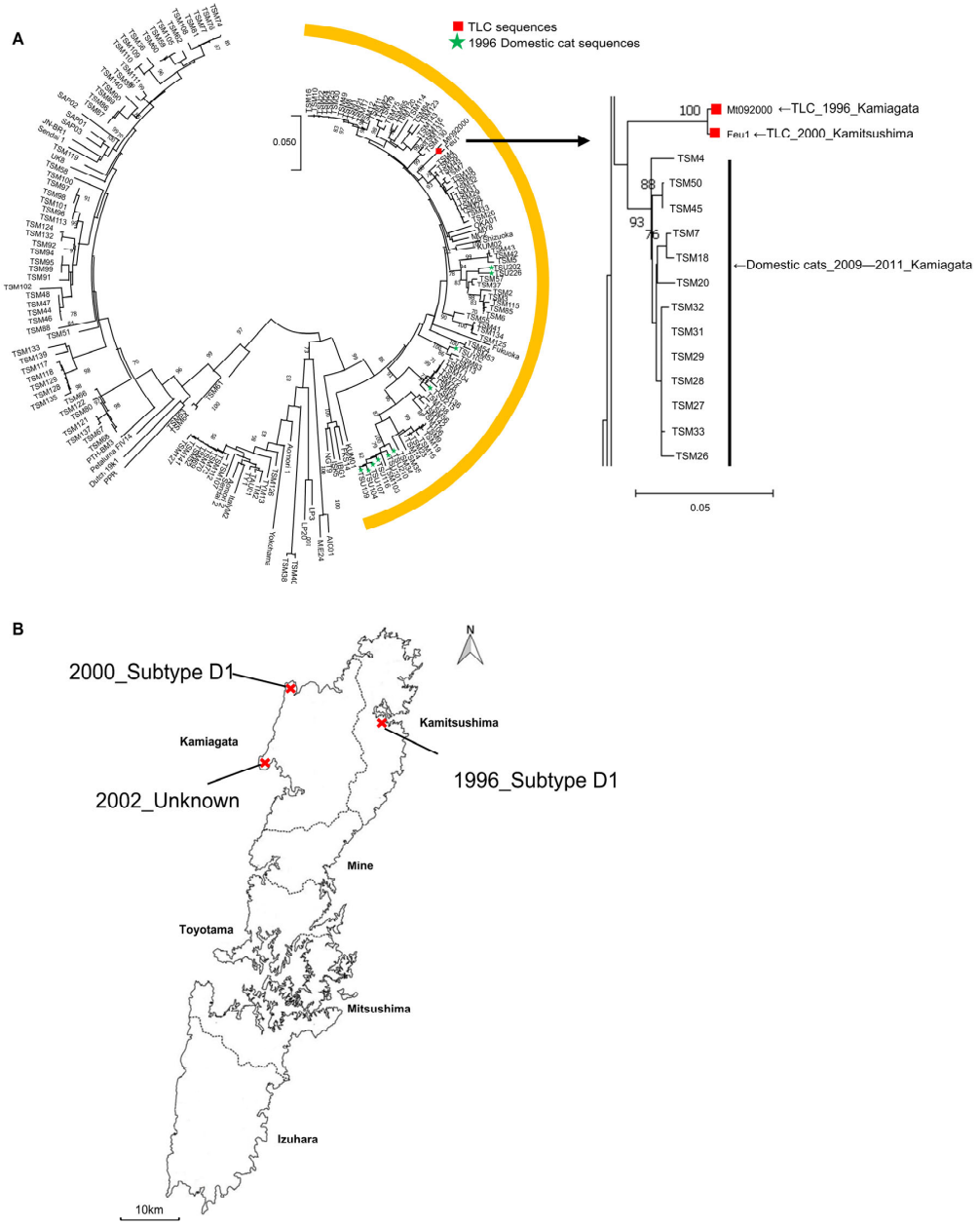


Figure 4. Phylogenetic relationships and geographic distribution of FIV-positive Tsushima leopard cats (TLCs) and related domestic cat sequences on Tsushima Island. (**A**) Two FIV subtype D1 sequences obtained from TLCs were analyzed phylogenetically alongside related domestic cat sequences. The two TLC sequences formed a closely related cluster with high bootstrap support and clustered with domestic cat samples collected in Kamiagata between 2009 and 2011, indicating close genetic relatedness among the viral sequences from the two host species. (**B**) Geographic map showing the locations of TLCs sampled in 1996, 2000, and 2002, all of which tested positive for FIV antibodies. Only the TLCs sampled in 1996 and 2000 were included in the phylogenetic analysis presented in this study.

3.3. Molecular Phylogenetic Characteristics of FIV in TLCs

Comparative phylogenetic analyses were performed to examine the relationships between FIV sequences obtained from endangered TLCs (*Prionailurus bengalensis euptilurus*) and subtype D1 strains from domestic cats on Tsushima Island. The leopard cat samples were collected in Kamitsushima in 1996 (Feu1) and Kamiagata in 2000 (Mt092000). In the present study, the FIV sequence was determined for the strain isolated from the FIV-seropositive TLC rescued in 2000 (Mt092000). The sequence obtained from the leopard cat captured in

1996 (Feu1), along with reference sequences from domestic cats, was retrieved from public sequence databases for comparative analysis. The expanded phylogenetic tree showed that the two leopard cat sequences (Feu1 and Mt092000) formed a distinct monophyletic cluster supported by a bootstrap value of 100%. This cluster was phylogenetically separate from the main domestic cat subtype D1 cluster from Kamiagata, which included strains TSM4, TSM7, TSM18, TSM20, and TSM26–29, among others, collected between 2009 and 2011. Instead, the leopard cat cluster branched from the base of the main domestic cat lineage, forming a sister group. The leopard cat strains also exhibited shared derived mutations that distinguished them from the contemporary domestic cat sequences (Figure 4, Table S4).

3.4. Spatial-Temporal Distribution of FIV Subtypes in Domestic Cats

The spatiotemporal distribution of FIV subtypes across Tsushima Island is shown in Figure 5A,B. On Kamijima, subtypes A, B, and D1 accounted for 33.3% (32/96), 2.1% (2/96), and 64.6% (62/96) of cases, respectively (Table 2). In Kamiagata, subtype A was detected predominantly in the southeastern region, whereas subtype D1 was distributed across the central, western, and northern regions, with several cases occurring within 1 km of one another. In Kamitsushima, subtypes B and D1 were concentrated in the northern region. In Mine and Toyotama, subtypes A and D1 were detected in multiple villages across both the western and eastern regions.

On Shimojima, subtypes A, B, and D1 accounted for 55.3% (26/47), 19.1% (9/47), and 25.5% (12/47) of cases, respectively (Table 2). In Mitsushima, subtype A was detected mainly in the northern region, whereas the two subtype B cases occurred in the northern and southern regions, and subtype D1 was detected in the southeastern region. In Izuhara, subtype A was distributed across the central, eastern, and northwestern regions; subtype B was concentrated in the southeastern region; and subtype D1 was detected in the central and eastern regions. Overall, subtype A was widely distributed across the central portions of both Kamijima and Shimojima. Subtype B showed a more localized distribution, occurring primarily in northern Kamijima and southern Shimojima. Subtype D1 had the broadest distribution, particularly across northern Kamijima.

From 2009 to 2022, the distribution of FIV subtypes A, B, and D1 across Tsushima Island varied over time and by location (Figure 5B, Table S3). D1 was the most prevalent subtype in the early study period. Kamiagata accounted for 93.8% (30/32) of all FIV infections detected in 2009–2010, after which its frequency substantially declined. In Kamitsushima, subtype D1 accounted for 80.0% (8/10) of all FIV infections detected in the town in 2009–2010 and 100% of infections detected in 2013–2014 (4/4) and 2015–2016 (3/3). Thereafter, its frequency declined markedly, while only sporadic cases were observed in other towns. Subtype A showed greater temporal variability and a broader geographic distribution. In Kamiagata, subtype A was detected repeatedly, with the highest frequency in 2015–2016, accounting for 91.7% (11/12) of all FIV infections in the town. In Izuhara, it represented 53.3% (8/15) of all FIV infections in the town during 2013–2014 and remained present during subsequent sampling periods. In Mitsushima, it accounted for 80.0% (4/5) of FIV infections in the town during 2013–2014 and 100% (6/6) in 2015–2016. In contrast, Toyotama and Mine had only intermittent, low-level detections, whereas no subtype A cases were recorded in Kamitsushima throughout the study period. Subtype B remained uncommon across all towns and sampling periods. It was detected sporadically in Izuhara, Mitsushima, and Kamitsushima, and was not detected in Kamiagata, Toyotama, or Mine. Overall, subtype D1 predominated early in the study period and subsequently declined, whereas subtype A persisted at moderate but fluctuating levels across multiple towns (Figure 5B, Table S3).

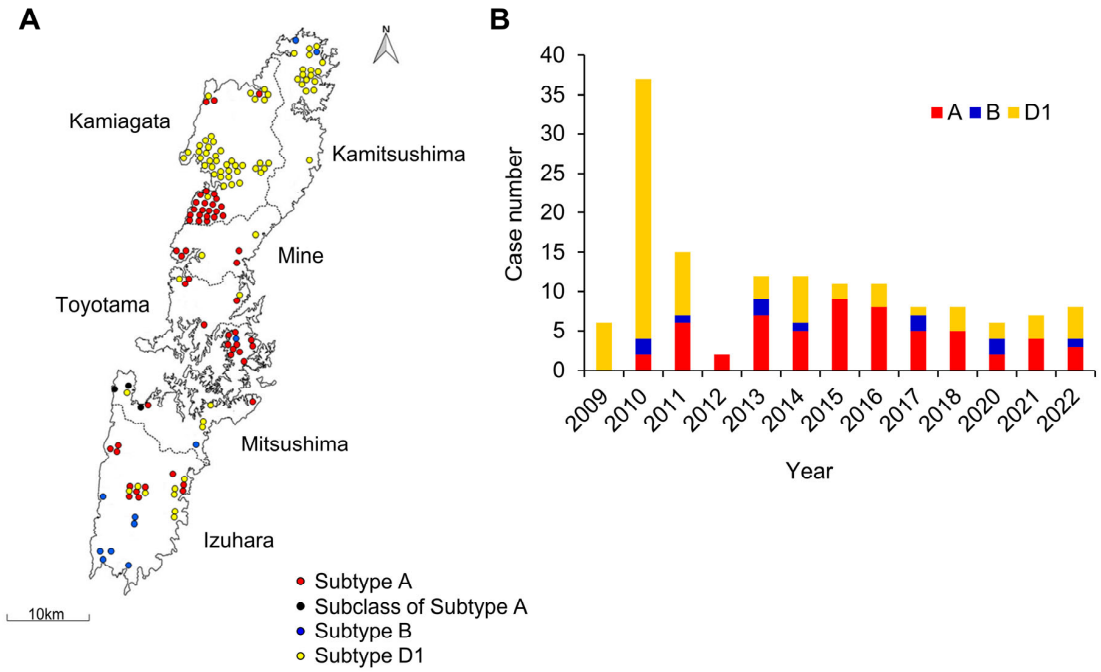


Figure 5. Spatial and temporal distribution of FIV subtypes A, B, and D1 in domestic cats on Tsushima Island, 2009–2022. (**A**) Geographic distribution of 143 genotyped FIV-positive domestic cat samples classified into three FIV subtypes (A, B, and D1) and mapped by town across Tsushima Island. (**B**) Stacked bar chart showing the annual numbers of FIV-positive samples classified by subtype. The *x*-axis represents the sampling year.

The distribution of FIV subtypes by age, sex, and environment is summarized in Table 3. Most cases involved cats of unknown age; among cats of known age, cases were concentrated in younger animals, particularly those aged 1–5 years. Male cats showed higher positivity across all subtypes, accounting for 62.2–72.2% of infections. Cats with a history of outdoor exposure comprised nearly all FIV-positive cases (137/143), including 128 cats who lived exclusively outdoors and nine cats from mixed indoor-outdoor environments. These patterns highlighted the association between FIV infection, male sex, and outdoor exposure.

Table 3. FIV Subtypes in Domestic Cats on Tsushima Island. (Age, Gender, Environment).

		Subtype			Total
		A	**B**	**D1**	
Age (Years)	<1	2	1	1	4
	1	2	2	3	7
	2	4	1	3	8
	3	3 ‡	0	3	6
	4	0	0	4	4
	5	0	0	3	3
	6	0	0	0	0
	7	0	0	0	0
	8	0	0	0	0
	>10	2	0	1	3
	unknown	45 ‡	7	56	108
	total	58	11	74	143

Table 3. *Cont.*

		Subtype			Total
		A	B	D1	
Sex	Male	39 *	8	46	93
	Female	19	3	28	50
	total	58	11	74	143
Environment	Outdoor	53 *	7	68	128
	Mixed #	2	4	3	9
	Indoor	3	0	3	6
	total	58	11	74	143

# Indicates cats kept both indoors and outdoors. * Includes three phylogenetically distinct subtype A samples. ‡ Includes three phylogenetically distinct subtype A samples: one in the 3-yr group and two in the unknown-age group. Subtype information was available for 143 of 180 FIV-seropositive domestic cats.

4. Discussion

This study provides updated epidemiological insights into FIV infection among domestic cats on Tsushima Island and documents the continued presence of FIV subtype D1 in the TLC. The overall FIV prevalence of 25.0% among domestic cats is comparable to that reported in a 2010 nationwide survey, which found a prevalence of 23.2% among owned cats in Japan [28]. However, the prevalence observed on Tsushima Island is markedly higher than that reported in North America (2.5–7.5%), Europe (3.2–8.3%), and Australia (15%) [35–41]. Although the reasons for these marked variations in FIV infection rates across regions currently remain unknown, the high seroprevalence observed in the present study is nevertheless consistent with the endemic FIV circulation on Tsushima Island. FIV vaccination is not administered to domestic cats on Tsushima Island; therefore, all seropositive cases detected herein reflected natural viral infections rather than vaccine-induced antibodies. Over the study period, the total number of animals sampled gradually decreased, possibly reflecting the long-term effects of the trap-neuter-return program initiated in 2009, which focused primarily on the spaying and castration of free-roaming cats. The larger numbers of FIV-positive cases detected in 2010 and 2011 likely reflect the more intensive sampling conducted during these years. In contrast, the high positivity rates observed between 2013 and 2018 should be interpreted with caution as they were based on relatively few samples. Under such conditions, a small number of positive cases can substantially influence prevalence estimates, suggesting that the observed fluctuations were more likely due to sampling variations than genuine changes in FIV prevalence. Kamijima initially accounted for a large proportion of samples and FIV-positive cases, but both numbers declined consistently over the sampling period. This trend may reflect the clinic's greater accessibility to pet owners and stray cats compared with Shimojima, where sample numbers remained consistently low, potentially contributing to inflated FIV positivity rates.

A major factor contributing to the high prevalence is the predominance of outdoor cat management on the island, with 83.2% of sampled cats kept outdoors. This finding is consistent with a previous study documenting similar outdoor cat management practices on Tsushima Island [7]. Outdoor cats have greater opportunities for contact with potentially infected animals and engage more frequently in aggressive encounters, facilitating viral transmission. Although no significant difference in FIV prevalence was detected between indoor and outdoor cats in this study, this finding may reflect the small proportion of indoor-only cats and the resulting limited statistical power. These results are consistent with the endemic circulation of FIV in the island's domestic cat population. More male cats were FIV-positive than female cats, consistent with previous studies attributing higher infection rates in males to increased aggression and fighting behavior [42–44]. These findings highlight

the need to strengthen public awareness, expand vaccination programs to include FIV, and promote indoor cat management to reduce transmission risk.

Three FIV subtypes (A, B, and D1) were detected among domestic cats on Tsushima Island. Previous studies have documented subtypes A, B, C, and D circulating in Japan, with subtype B historically reported as the most common nationwide [28,45]. In contrast, our findings identify subtype D1 as the dominant subtype on Tsushima Island, followed by subtypes A and B. Nationally, subtype B accounts for approximately 42% of infections, whereas it represented only 7.7% of cases in our study. This discrepancy likely reflects regional variation and differences in sampling scope, as the present study focused exclusively on Tsushima Island, where a high prevalence of FIV subtype D has previously been reported [11]. Although FIV subtype D is divided into D1 and D2, with D2 previously confirmed in the Hokuriku region [28], our results indicate that only subtype D1 was detected among domestic cats on Tsushima Island. Notably, subtype D1 accounted for 64.6% of cases in Kamijima, the primary habitat of the TLC, demonstrating substantial spatial overlap between the dominant domestic cat subtype and the endangered wild felid population. Previous studies have also reported subtype C in Japan, particularly in the Chubu and Kinki regions and Aichi Prefecture [28,30]. However, this subtype was not detected in our dataset, further highlighting regional heterogeneity in FIV subtype distribution across Japan. Phylogenetic analysis showed that FIV circulating among domestic cats on Tsushima Island comprises three subtypes (A, B, and D1), each exhibiting notable intra-genotypic diversity. Subtype A formed several well-supported clusters, some of which persisted across multiple towns and sampling years, indicating the long-term maintenance of specific viral lineages within the island's cat population. In contrast, only one cluster was detected in 2014 and was not observed in the subsequent sampling periods. These findings suggest that persistent lineages can coexist with lineages detected only transiently within the same geographical area. Subtype B showed more limited diversity, forming two small clusters that were geographically restricted but persisted over time. Subtype D1 exhibited the greatest phylogenetic structuring, with multiple clusters spanning several towns and sampling years, suggesting the long-term circulation of related viral lineages across multiple cat populations. Notably, one lineage was detected across both the northern and southern regions of the island during much of the study period, while the other was restricted to the early years of sampling. In addition, several distinct lineages were repeatedly detected in northern and central Kamijima, including areas overlapping with the primary habitat of Tsushima leopard cats. The clustering of sequences collected in 1996 with more recent samples further supports the long-term persistence of FIV lineages on the island. Collectively, these patterns suggest that FIV subtypes on Tsushima Island are maintained through repeated local transmission events and the movement of domestic cats between towns. Such movement may have been facilitated by human-mediated activities, including the transportation and relocation of cats between different areas of the island, resulting in shared viral lineages and sustained circulation over several decades.

Phylogenetic analysis of FIV sequences from confirmed cases in endangered TLCs (Feu1, sampled in 1996 [11] and Mt092000, sampled in 2000 and also reported at a conference [46] showed that both belonged to subtype D1 and formed distinct, well-supported monophyletic clusters. Their close genetic relationship, despite being sampled four years apart, was consistent with the possibility of viral persistence within the TLC population. As only two TLC sequences were available for analysis, the direction, timing, and frequency of the transmission events could not be conclusively determined. Nevertheless, the shared subtype between domestic cats and TLCs is consistent with the hypothesis that cross-species transmission occurred at some point in the history of FIV on Tsushima Island. Since 1999, samples from 91 TLCs have been screened for FIV antibodies. Only two samples

collected in 2000 and 2002 tested positive for FIV antibodies, corresponding to an apparent prevalence of 2.2% (Table S4). This contrasts with the 25.0% seroprevalence observed in domestic cats on Tsushima Island from 2009 to 2023 and indicates that FIV infection has been detected infrequently in sampled TLCs. Our previous work on Tsushima Island also documented cross-species transmission of Felis catus gammaherpesvirus 1 from domestic cats to TLCs [14], further emphasizing the vulnerability of TLCs to pathogens maintained in domestic animal populations.

The primary limitation of this study is its reliance on a single archival TLC sample and one previously published TLC sequence. However, given the strict protection status of the TLC and the risks associated with capturing and sedating wild individuals, opportunities for invasive sampling are extremely limited. Archival samples therefore provide valuable insights into historical spillover events. Furthermore, FIV genotyping of the two TLC cases available to date could not conclusively determine the origin of the virus. Additionally, the retrospective study design introduces spatial and temporal sampling biases, as the number of archived samples available for analysis varies among years and localities. Consequently, the observed distribution and apparent persistence of intra-genotypic lineages may reflect the localized collection patterns, rather than the full epidemiological diversity of FIV circulating on Tsushima Island.

5. Conclusions

In conclusion, mitigating infectious disease risks to the TLC while improving domestic cat health across the island requires an integrated One Health strategy. Strengthening domestic cat source control through strict indoor housing and expanded TNR programs could reduce free-roaming cat density, limit territorial aggression, and prevent feeding-driven aggregations. Quarantine and other preventive measures, including pre-entry viral screening of imported cats, widespread adoption of core and feline leukemia virus vaccination, and routine PCR and serological surveillance, could reduce pathogen introduction and enable early detection. For wild leopard cats, rigorous quarantine and PCR testing of rescued individuals, evaluation of field vaccination against highly lethal pathogens such as feline panleukopenia virus, and strict biosecurity in rehabilitation and breeding facilities are essential to prevent inadvertent transmission. Effective implementation will require coordinated governance involving the Ministry of the Environment, Nagasaki Prefecture, Tsushima City, veterinary associations, and local communities to sustain public engagement and promote responsible pet ownership.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v18111282/s1, Figure S1: Phylogenetic clustering of feline immunodeficiency virus (FIV) subtype A sequences from domestic cats on Tsushima Island. Fifty-eight FIV subtype A sequences from domestic cats on Tsushima Island were analyzed using the neighbor-joining method, revealing multiple well-supported clusters within subtype A. Panels A–E highlight distinct intra-genotypic lineages identified across different towns and sampling years, illustrating the genetic diversity and spatial distribution of subtype A on the island. The dark teal triangle indicates a Toyotama-derived FIV sequence representing the fourth distinct subtype A cluster identified within Toyotama Town; Figure S2: Phylogenetic clustering of FIV subtype B sequences from domestic cats on Tsushima Island. Eleven FIV subtype B sequences from domestic cats on Tsushima Island were analyzed phylogenetically, revealing two distinct clusters corresponding to samples from Kamijima and Shimojima. These clusters indicate geographically structured intra-genotypic diversity within subtype B on the island; Figure S3: Phylogenetic clustering of FIV subtype D1 sequences from domestic cats on Tsushima Island. Seventy-four FIV subtype D1 sequences from domestic cats on Tsushima Island were analyzed phylogenetically, revealing multiple well-supported clusters across different towns and sampling years. Panels A–E highlight distinct intra-genotypic lineages, several of which persisted over extended periods and were shared among cats sampled in Kamiagata, Kamitsushima,

Mine, Toyotama, Mitsushima, and Izuhara. These patterns indicate substantial genetic structuring and long-term circulation of subtype D1 within the island's domestic cat population; Table S1: Detailed regional counts of FIV-positive cases in domestic cats by age, sex, and environmental exposure. Table S2: Annual number of cat samples and FIV-seropositive cases by region. Table S3: FIV Subtypes in Domestic Cats on Tsushima Island. (Year vs. Location) Table S4: Profile of FIV-seropositive Tsushima leopard cats.

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Institutional Review Board Statement: The experiments were approved by the Genetic Modification Safety Committee of Yamaguchi University (Protocol Code: J24025; Date of Approval: 6 October 2024). All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Japan's Ministry of Education, Culture, Sports, Science, and Technology.

Informed Consent Statement: Blood samples used in this study were originally collected by attending veterinarian (Dr. Yushi Koshida) as part of routine clinical diagnostic procedures. The blood samples were collected for the purpose of viral testing aimed at monitoring the cats' health status and were also utilized for viral genetic testing. No additional blood collection or animal handling was performed. Following completion of diagnostic testing, residual samples were voluntarily provided by the attending veterinarian and/or cat owners.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BLAST	Basic Local Alignment Search Tool
CI	Confidence interval
FcaGHV1	Felis catus gammaherpesvirus 1
FFV	Feline foamy virus
FIV	Feline immunodeficiency virus
MEGA X	Molecular Evolutionary Genetics Analysis Version X
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NCBI	National Center for Biotechnology Information
OR	Odds ratio
PCR	Polymerase chain reaction

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