Molecular Characterization and Transmission Dynamics of Animal Viruses in Tsushima leopard cat (*Prionailurus bengalensis euptilura*) with special reference to Feline leukemia virus and *Felis catus* gammaherpesvirus 1

(ツシマヤマネコにおける猫白血病ウイルスと猫ガンマヘルペスウイルス 1 型の感染に関する研究)

A DISSERTATION

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DECLARATION

I hereby certify that I have written this dissertation independently and that I have not used other than the cited sources. This dissertation has not been submitted for any other degree or purposes.

March 2019, Yamaguchi University ....

Isaac Josred Makundi

PREFACE

This dissertation discusses molecular epidemiological studies of Feline leukemia virus (FeLV) and *Felis catus* gammaherpesvirus 1 (FcaGHV1) in Tsushima leopard cat (TLC) *Prionailurus bengalensis euptilurus* and domestic cats on Tsushima Island, Nagasaki, Japan. Furthermore, I investigated FcaGHV1 DNA in feline lymphoma/leukemia tissues that were analyzed for B-lymphocyte and or T-lymphocyte clonal growth by molecular techniques.

The FeLV study focuses on identification of FeLV in TLC and domestic cats and I utilized in vitro infection assay to investigate the potential of cross-species FeLV transmission. This study also analyzed FeLV infections distribution and transmission dynamics on Tsushima.

The FcaGHV1 study also identify and determine the FcaGHV1 infection status in both cats population. It further elaborates on distribution and transmission dynamics of FcaGHV1 infections on Tsushima.

This dissertation is organized in five main sections. The first section is general introduction whereby I introduced the study area, TLCs, and reported diseases of TLCs. I also introduced FeLV and its clinical significance and provided the current status of FeLV infections in Japan. Lastly, I described about gammaherpesviruses and FcaGHV1. Molecular epidemiology of FcaGHV1 infections and the current status of FcaGHV1 infections in Japan.

The second section of this dissertation comprised of first study which is FeLV investigation on Tsushima. This second section is titled as chapter one and it is made of several subsections related to first study including abstract, introduction, materials and methods, results and discussion part.

The third section is also titled as chapter two and it include second study which is FcaGHV1 identification in TLCs. Similarly, as second section it also comprised of its own subsections related to FcaGHV1 study including abstract, introduction, materials and methods, results and discussion.

The fourth section of this dissertation describe about FcaGHV1 investigation in feline lymphoma/leukemia tissues. This section is titled as chapter three and it has its own subsections in the same format as described in preceding sections.

The fifth section of this dissertation comprised of general discussion and conclusion. This section discusses all results for each individual study/project. The significance of each study and general conclusion is found here.

The organization of this dissertation will enable the reader to follow each individual project from its start until its end without confusion. So, the reader would have an easier job to understand the whole concept of each study. In many cases, the introduction, materials and methods, results or discussion texts might resemble the ones occurring in the published papers. Several tables and figures are the ones found in the manuscripts.

Taken together, I believe the experiments and findings reported in this dissertation would add to our understanding of the FeLV and FcaGHV1 infections in TLCs, distribution and transmission dynamics of these infections which are essential to foster the ongoing management strategies of TLCs.

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ABSTRACT

Infectious diseases spill-over from domestic animals to wildlife or vice versa may significantly impair health and production of the affected animal. For the small population of endangered animal species the risk of infectious diseases may accelerate its extinction. Increased human activities such as agriculture, deforestation, development projects including roads construction have caused not only loss of habitat to wildlife but also enhances the contact between the wildlife and domestic animals. This interaction between wildlife and domestic animals has been the main pathway of disease transmission.

Tsushima leopard cat (TLC) is a small wild cat inhabiting Tsushima Island, Japan. TLC is classified as a critically endangered species due to drastic decrease of its population caused by several factors including habitat loss (deforestation), road kills, and infectious diseases. Several diseases and pathogens have been reported to infect TLCs.

In chapter one, I studied Feline leukemia virus (FeLV) infections in domestic cats and TLCs on Tsushima Island. Although FeLV is the most pathogenic infectious disease in cats, there was no epidemiological study conducted to investigate FeLV infections in this region. The prevalence of FeLV in domestic cats was 6.4% based on FeLV antigen p27. FeLV was not detected in TLC by either FeLV antigen p27 or PCR. To determine whether FeLV could potentially infects TLCs, I infected primary skin fibroblasts from TLCs with FeLV-A and FeLV-B strains. The TLC fibroblasts were susceptible to both viral strains indicating that FeLV replicate in TLC cells. These results suggest that there is high probability of cross-species transmission of FeLV infections from domestic cats to TLCs.

Sequence and phylogenetic analyses of FeLV strains in domestic cats on Tsushima revealed that all FeLV isolates belong to genotype I clade 3. The viruses in genotype I clade 3 were prevalent

and widespread on Kyushu, Japan based on previous studies of FeLV epidemiology. Furthermore, FeLVs strains on Tsushima were clearly separated into two areas, genotype I clade 3–1 in Kamijima and genotype I clade 3–2 in Shimojima according to geographical regions. The source of FeLV infections on Tsushima could be explained by how does the Tsushima is connected with other regions. Sea routes link the ports of Hitakatsu and Izuhara from the side of Tsushima and port of Hakata in Fukuoka. Air transportation connects Tsushima airport and Fukuoka and Nagasaki airports. This connection method between Tsushima and Fukuoka and or Nagasaki may probably be the main source of FeLV on Tsushima.

In second chapter, I explained about identification of *Felis catus* gammaherpesvirus 1 (FcaGHV1) in TLCs on Tsushima Island. Previous epidemiological data suggests that territorial aggression and fighting are commonly modes of FcaGHV1 and Feline Immunodeficiency Virus (FIV) transmission. Previous reports detected FIV in TLCs and its prevalence was significantly higher in domestic cats on Tsushima compared to other regions of Japan. I developed new FcaGHV1 virus-specific nested PCR system to detect FcaGHV1 in TLCs. FcaGHV1 DNA was detected in 3 out of 89 TLCs investigated. For the purpose of TLCs management and determining the source of FcaGHV1 infection in TLCs, I tested domestic cats on Tsushima and I found 28 out of 215 were positive for FcaGHV1 DNA.

Sequence and phylogenetic analyses revealed that FcaGHV1 strains in TLCs and domestic cats were of the same identity. On nucleotide sequence alignments, all three positive TLCs had similar nucleotide sequences forming one FcaGHV1 pattern which was also shared by domestic cats. Two other different patterns of FcaGHV1 strains were found only in domestic cats. The three patterns of FcaGHV1 strains were classified based on nucleotide polymorphisms. These results demonstrated

that domestic cats on Tsushima harbor all three patterns of FcaGHV1 strains probably due to the fact that domestic cat is the natural host of this virus. The probability of FcaGHV1 transmission from domestic cats to TLCs is supported by the following findings; first, FcaGHV1 was originally isolated from domestic cats, the high frequency of FcaGHV1 DNA detection in domestic cats than in TLCs suggest that the infections is endemic in domestic cats, and lastly, TLCs and domestic cats FcaGHV1 strains formed one genetic cluster on phylogenetic analyses.

The third chapter of this dissertation was the study about FcaGHV1 DNA detection in feline lymphoma/leukemia tissues that were submitted for investigation of B- or T-lymphocyte clonal growth. FcaGHV1 is a panlymphotropic gammaherpesvirus. Feline lymphoma remains to be the most common malignancy of domestic cats. Studies engaged to explore the association between lymphoma and various etiologies specifically FcaGHV1, are of significant importance for the welfare of domestic cats. I found no significant differences in FcaGHV1 DNA detection between lymphoma/leukemia tissues of B-cell/T-cell type and non B-cell/T-cell type clonality matched for age and sex. FcaGHV1 DNA was detected in feline blood, lymph node, effusions, biopsies, spleen, intestine and peritoneal masses. These results suggest that FcaGHV1 DNA is exclusively distributed in lymphoma/leukemia tissue irrespective of their clonal growth. Cats aged over 5 years and co-infected with retroviruses, particularly FIV, were found to be the risk factors for FcaGHV1 infection.

1.0. GENERAL INTRODUCTION

1.1. The Tsushima Island

Tsushima (708.6 km^2; 34°05′–34°42′N, 120°10′–129°30′E) is an island of the Japanese archipelago situated approximately 50 km from the Busan, Korean Peninsula and 138 km from Kyushu Island, Japanese mainland (Figure 1) (Oh D et al., 2014). The Tsushima Island is off the western coast of Japan and classified as part of Nagasaki Prefecture. The name Tsushima generally refers to over 100 smaller islands collectively. Administratively, the Tsushima is subdivided into two main islands; "North" known as Kamijima and "South" known as Shimojima. The modern city of Tsushima was established on March, 2004 following the merge of six boroughs on Tsushima Island. The six boroughs included Izuhara, and Mitsushima (from Shimojima), and Mine, Toyotama, Kamiagata, and Kamitsushima (from Kamijima).

1.2. The Tsushima leopard cat

The Tsushima leopard cat (TLC; *Prionailurus bengalensis euptilurus*) is the indigenous wild cat inhabiting the Tsushima Island. TLC is a small-sized felids with weights of 4–4.5 kg for males and 3–3.5 kg for females (Saitoh et al., 2015). They have long, fat tails, longer trunks and shorter-than-typical legs when compared to the domestic cats and their body color can range from chestnut-brown to cream peppered with indistinct brown spots. On the back of their rounded ears there is a white spot and a clear brown-white striped pattern on their foreheads (Figure 2).

The TLC was first recorded in science as a distinct species of leopard cat by a British zoologist, Thomas in 1908 and subsequently classified as a sub-species of the Amur leopard cat (leopard cats of Eurasia) that isolated for approximately 100,000 years (Murayama, 2008). The molecular phylogeny of TLC in comparison with other felines was first reported by Masuda and Yoshida (1995). In their

report they indicated that TLC has the same mitochondrial DNA (mtDNA) lineage as the Iriomote cat (*Prionailurus iriomotensis*) and leopard cat (*Prionailurus bengalensis*) (Figure 3). The phylogenetic understanding is the basis and the first stage to unveil the genetic diversity and evolution of endangered population for the purpose of conservation and management (Masuda et al., 1994).

The Tsushima leopard cat was designated as a Natural Monument of Japan in 1971 and a National Endangered Species in 1994. However, as of 2015 TLC was listed as Critically Endangered in the Red List of the Ministry of Environment, Japan. The Tsushima leopard cat was reported to inhabit all of the Ryukyu Islands with a population of 200 to 300 until the 1970's. Since then the number of TLCs continues to decline at the rate of about 10 % in every 10 years according to Japan Wildlife Research Center, 2005 (Saitoh et al., 2015). Road kills and habitat loss (deforestation) were reported to be the major factors accounting for the decline of TLCs population (Izawa et al., 2009a). A wide area of island is covered with forests. Forest protection and management are relatively weak on Tsushima; one-third of the forests are artificial plantations and most forests are privately owned without any regulation against deforestation. It is estimated that 59 TLCs were killed due to traffic accident from 2000 to 2013 (4.2 cats/year) on Tsushima Island (Saitoh et al., 2015). Several other potential threats for TLCs survival were identified including; diseases, inter-specific competition with carnivores, domestic cats (diseases, inter-specific competition, and hybridization), predation from dogs, introduced species, and lack of awareness from local people (Murayama, 2008).

1.3. Diseases of Tsushima leopard cats

TLCs have also been exposed to several diseases. Infectious diseases is another important potential factor limiting the survival of TLCs population. The interspecies transmission of diseases between domestic cats and TLCs have previously been reported (Hayama et al., 2010). Deforestation and increased human activities have eroded the natural habitats of TLCs accelerating the accidental contact with infectious domestic cats.

Feline immunodeficiency virus (FIV) is a retrovirus of the genus *Lentivirus* that was first isolated from the colony of domestic cats in USA in 1986 (Pedersen et al., 1987). FIV is known to be primarily transmitted from cat to cat via bite wounds during antagonistic or mating interactions. The infection in domestic cats is characterized by a long asymptomatic state, with progressive disruption of immune system and increased susceptibility to opportunistic infections leading to feline acquired immunodeficiency syndrome (Sellon and Hartmann, 2006). FIV was isolated from TLCs captured in 1996, with genetic analysis of the *env* gene sequences indicated that FIV from TLCs belongs to a similar cluster of subtype D FIV strain from domestic cats (Nishimura et al., 1999a). After this report, more FIV positive TLCs were identified in 2000 and 2002 (Hayama et al., 2010).

The ecologic surveys on Tsushima Island identified the presence of ticks and lice in TLCs suggesting the likelihood of arthropod-borne diseases (Tateno et al., 2013a). In their report, *Bartonella clarridgeiae*, *Ehrlichia canis* and *Anaplasma bovis* were identified in TLCs at the prevalence of 8%, 8% and 15% respectively. In another study, *Hepatozoon felis* infected TLCs were detected at the prevalence of 100% even though no obvious clinical signs of hepatozoonosis were seen (Tateno et al., 2013b).

Surveillance of diseases and pathogens in domestic dogs and cats may indirectly help to clarify their transmission routes and interrelationship between wildcats and domestic animals. Jikuya et al., (2017) reported that the prevalence of *H. felis* was 2.4% in domestic cats on Tsushima Island. The phylogenetic analysis revealed that the *H. felis* detected in domestic cats was closely related to the *H. felis* previously isolated from TLCs suggesting the likelihood of interspecies transmission. On the other hand, hemoplasma were also reported in domestic cats. *Mycoplasma hemofelis*, *Candidatus* Mycoplasma haemominutum and *Candidatus* Mycoplasma turicensis were reported in domestic cats on Tsushima Island at the prevalence of 6.1%, 20.7% and 2.4% respectively (Jikuya et al., 2017). The natural mode of transmission of hemoplasma infection is not completely understood although direct contact (aggressive interactions) and vectors are possibilities. In conclusion, Jikuya et al., (2017) stated that the differences in hemoplasma species detected and their prevalence suggest that direct and frequent interspecies transmission of hemoplasma was unlikely to occur on Tsushima Island.

In addition to protozoans and hemoplasma, several species of helminths were also recovered from TLCs. Three species of trematodes, one species of cestode and nine species of nematodes were found to infect TLCs. The trematodes were *Pharyngostomum cordatum*, *Paragonimus species* and species of Dicrocoeliidae. The only cestode isolated was *Spirometra erinacei*. The nematodes recovered were *Arthrostoma hunanensis*, *Uncinaria felidis, Uncinaria species, Ancylostoma tubaeforme, Molineus springsmithi, Toxocara cati*, *Capillaria aerophila*, *Capillaria felis-cati* and *Capillaria species* (Yasuda et al., 1993). The *P. cordatum* was believed to be widespread in TLCs due to wide distribution of its intermediate hosts such as freshwater snails (*Polypylis hemisphaerula*), frogs, and snakes. *Spirometra*

erinacei, was the only cestode isolated from TLCs even though it is a very common cestode in Japanese domestic cats. *Arthrostoma hunanensis*, a hookworm found in the bile duct was first reported in TLCs (Yasuda et al., 1993).

Ebstein anomaly is a rare congenital heart disease first discovered in 11-month old TLC bred in the Kyoto City Zoo (Shimamura et al., 2017). The anomaly has been known to occur in domestic dogs, meerkat, pygmy goat, and lion. The authors reported that the TLC Echocardiography revealed a dilated right atrium and ventricle with an enlarged tricuspid valve annulus and apical displacement of the tricuspid valve leaflets. In their conclusion, the authors mentioned that the current significance of this anomaly for captive TLC breeding programs remains to be determined however, at the moment the present TLC with congenital heart disease should not be bred (Shimamura et al., 2017).

1.4. Feline Leukemia Virus

1.4.1. Feline Leukemia Virus (FeLV) genome

FeLV is an exogenous *Gammaretrovirus* approximately 8.4-kb in size containing two reading frames, one for *gag* and *pol* genes and a second *env* gene (Figure 4). *Gag* encodes group-specific capsid antigens, *pol* encodes protease, integrase, and reverse transcriptase (RT) enzymes, and *env* encodes the envelope proteins (Coffin et al., 1992). The 5′ and 3′ sequences of the coding regions contain promoters and enhancers known as long terminal repeats (LTRs).

1.4.2. Clinical significance of Feline leukemia virus infection

Clinical signs associated with FeLV infection are variable and they are determined by a combination of viral and host factors. These differences in the clinical course of infection can be

associated with characteristics of the virus itself, such as the subgroup known as subgroup-specific clinical phenotypes (Chiu et al., 2018). The course of FeLV infection may generally follow either of two pathways. Majority of exposed cat recovers and becomes immune. On the other hand, some of infected cats enters persistent infection featured by viraemia and associated with increased likelihood of developing severe and ultimately fatal disease (Willett et al., 2013). The diseases associated with persistent FeLV infection are primarily disorders of haematopoiesis such as lymphoma, myelodysplastic disorders, myeloid leukemia, aplastic anemia, and immune suppression (Hisasue et al., 2009; Rohn et al., 1994; Tzavaras et al., 1990).

1.4.3. Feline leukemia virus infection status in Japan

Before 2010, detection of FeLV infections in domestic cats were mainly reported as seroprevalence studies in Japan. In Tokyo area, 5.8% of FeLV-positive cats were found based on ELISA with majority of them showing variety of clinical disorders, most frequently renal diseases and anemia (Ishida et al., 1981). Maruyama et al., (2003) reported the national wide seroprevalence of 2.9% with infection rates being higher in outdoor and older age cats.

The development and use of new methods has enabled not only the studies of FeLV infections but also detailed phylogenetic and structural diversity of FeLV in Japan. A molecular-based epidemiological survey of FeLV infection covering the whole area of Japan has reported the prevalence of 12.2% based on PCR FeLV *gag* gene detection (Watanabe et al., 2013). Furthermore, the authors in their report classified FeLV into three distinct genetic clusters, termed as Genotypes I, II, and III based on FeLV *env* gene (Figure 5). Genotypes I and II were distributed within Japan with Genotype I being the major genetic cluster further sub-classified into Clades 1 to 7. Genotype III

represented FeLV samples sourced outside of Japan. In another study, authors characterized the FeLV *gag* gene from Japanese isolates and identified recombination between endogenous and exogenous FeLV *gag* gene sequences. Pattern of recombination revealed that each recombinant was generated *de novo* and then transmitted (mainly horizontally) among cats (Kawamura et al., 2015).

1.5. Gammaherpesviruses

Gammaherpesviruses (GHVs) are the members of herpesviruses family. GHVs are enveloped, with icosahedral, spherical to pleomorphic, and round geometries. Genomes are linear and non-segmented, around 170kb in length. GHVs belong to four separate genera namely: Lymphocryptovirus, Rhadinovirus, Macavirus and Percavirus (Figure 6). GHVs replicate and persist in lymphoid cells but some are capable of undergoing lytic replication in epithelial or fibroblast cells (Sattler et al., 2016). Therefore, GHVs have been known to be associated with the development of lymphoproliferative disorders, lymphomas, and other nonlymphoid cancers. Majority of known GHVs such as Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) persist in an individual after the viruses undergone latency in B lymphocytes (Barton et al., 2011).

GHVs infect a wide range of vertebrates, including humans and other mammals. Host immunity plays a significant role in control of GHVs infections, however GHVs limit this control through multiple mechanisms of immune evasion (Means et al., 2007). Furthermore, GHVs infections may become apparent in non-adapted susceptible host.

1.5.1. *Felis catus* Gammaherpesvirus 1

Felis catus Gammaherpesvirus 1 (FcaGHV1) is a new identified member of genus *Percavirus*, subfamily *Gammaherpesvirinae*, family *Herpesviridae* (Troyer et al., 2014). The FcaGHV1 was isolated

from a 9-year-old male cat with intestinal T cell lymphoma. The virus has the genome length of about 121-kb (Troyer et al., 2015). In humans, infection by EBV and KSHV have been responsible for over 50% cases of HIV-associated lymphomas (Pinzone et al., 2015). Understanding the pathogenic potential of FcaGHV1 as a causal factor in feline immunodeficiency virus (FIV)-associated lymphoma is currently under active investigation. To begin with accumulating the evidence for establishing the association between FcaGHV1 and FIV-associated lymphoma, Aghazadeth et al., (2018) detected FcaGHV1 transcripts (at low copy number) in 50% of FIV-associated lymphomas investigated. In their conclusion, the authors emphasized on a subset of intestinal T-cell tumors, large granular lymphocyte lymphoma as the target for the future investigations of the pathogenic potential of FcaGHV1.

1.5.2. Prevalence of FcaGHV1 infections

FcaGHV1 DNA has been detected in domestic cats from countries on most continents including Europe, Oceania, North and South America and Asia (Beatty et al., 2014; Ertl et al., 2015; Tateno et al., 2017; Kurissio et al., 2018). The prevalence of FcaGHV1 infection varies from one geographical location to another however, majority of previous studies reported the rates between 9.6% and 23.6% based on whole blood nested PCR and or quantitative real-time PCR. Previous studies have identified several risk factors for FcaGHV1 infection including adult and male status, geographical location, health status (sick) and co-pathogens such as retroviruses and haemoplasma (Beatty et al., 2014; Ertl et al., 2015; Stutzman-Rodriguez et al., 2016; McLuckie et al., 2016a; McLuckie et al., 2017; Tateno et al., 2017; Kurissio et al., 2018). Aggressive encounters such as fighting are suggested to be the major common method of FcaGHV1 transmission.

1.5.3. FcaGHV1 infection status in Japan

To date, FcaGHV1 survey in Japan is reported by one study (Tateno et al., 2017). FcaGHV1 DNA was detected by nested PCR in 23 out of 1738 domestic cats, with an overall prevalence of 1.3%. The reported prevalence is lower than the prevalence's documented from other similar studies elsewhere. Sequence alignment and BLAST analysis revealed that all the sequences reported in Japan were highly similar (99.9%) to FcaGHV1 isolates from the United States of America. Older age (over 5 years old) and FIV infection were significant risk factors for FcaGHV1 infection. Majority of positive cats were located in the western part of Japan (Figure 7).

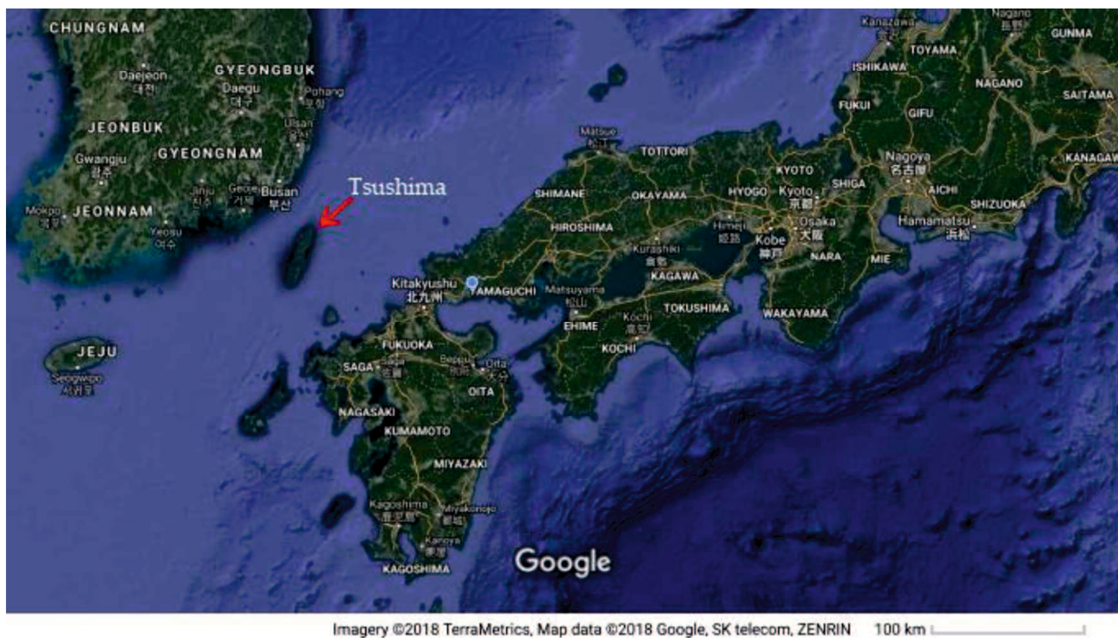
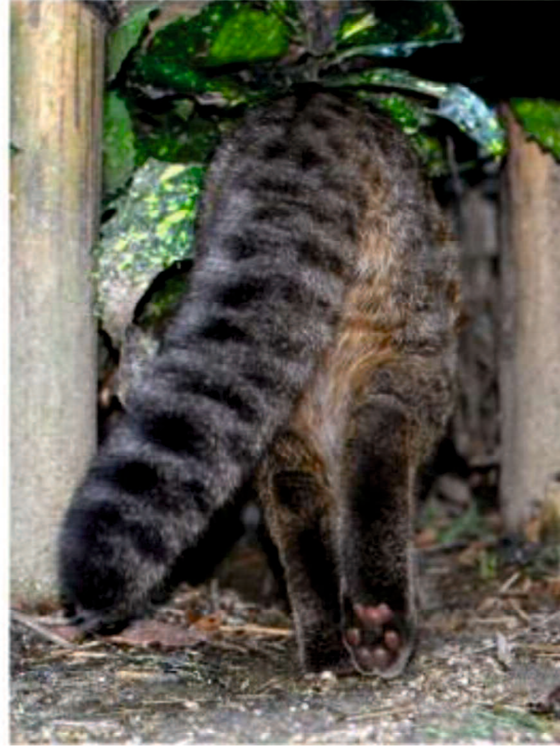


Figure 1. The location of Tsushima Island, Google Earth (2018)



(a) **(b)**

Figure 2: A photo of Tsushima Leopard cat (*Prionailurus bengalensis euptilurus*) front and rear view. The photos were cited from Pontafon–Fukuoka Zoo, Japan (a) and Hatena blog–Tsushima Island, Japan

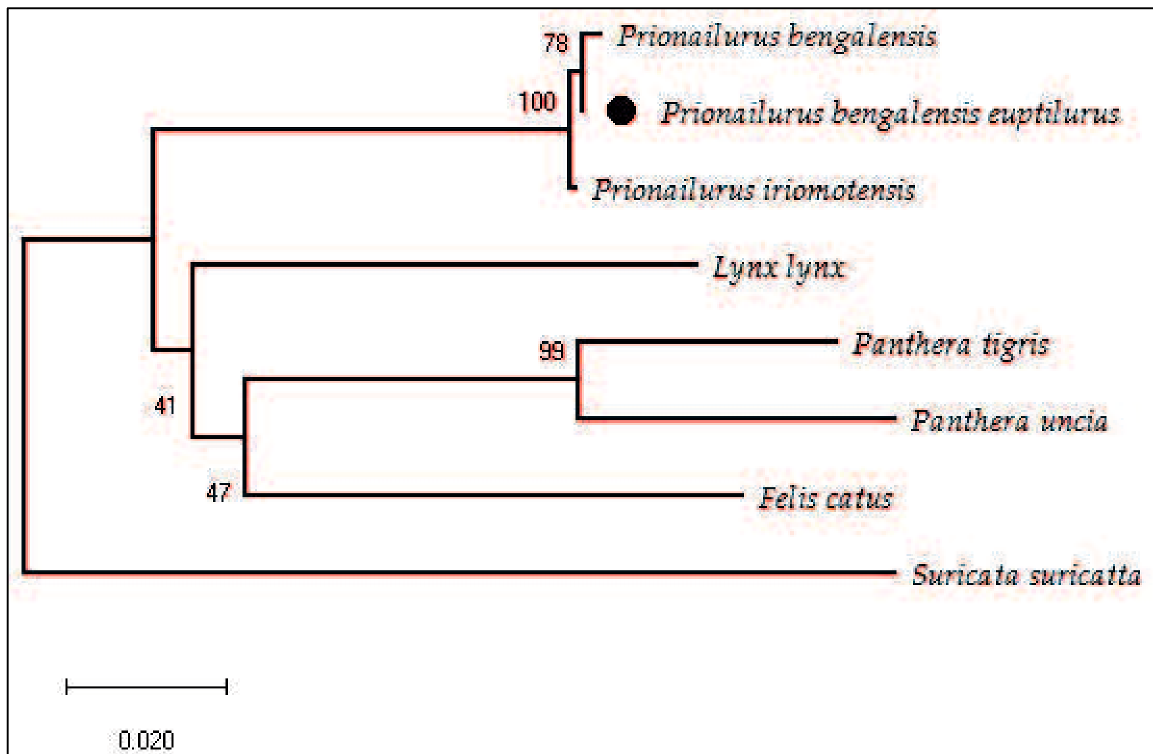


Figure 3: Neighbor-joining phylogenetic relationship of TLC and other felines based on cytochrome b nucleotide alignments. The tree showed that the TLC (indicated with black dot) and Iriomote cat clustered with a high confidence (100% bootstrap value). The GenBank accession numbers of the sequences used in the phylogenetic tree are *Prionailurus bengalensis euptilurus* (D49449), *Prionailurus bengalensis* (D28901), *Prionailurus iriomotensis* (D28900), *Lynx lynx* (D28902), *Panthera tigris* (D28905), *Panthera uncia* (D28904), *Felis catus* (D28903), and *Suricata suricatta* (D28906) which was used as an outgroup. The scale bar indicates an evolutionary distance of 0.02 substitution per site.

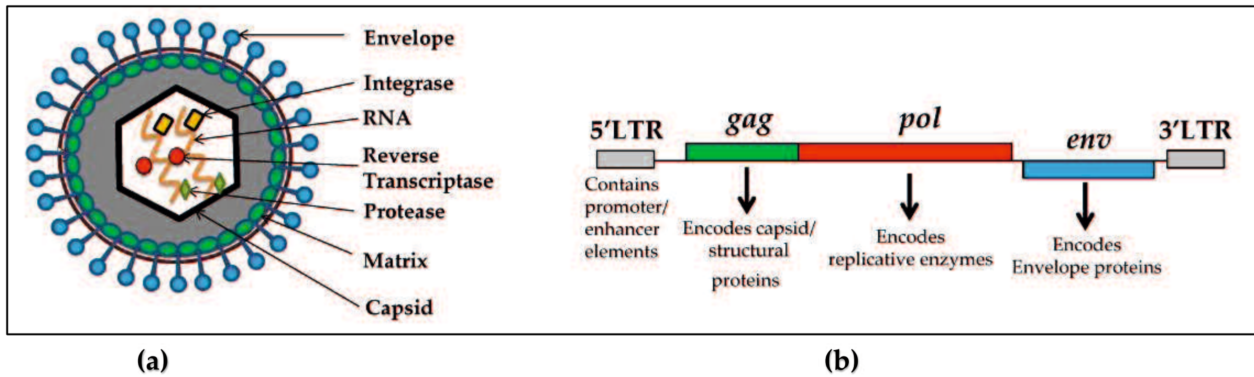


Figure 4: Genomic map of Feline leukemia virus showing its organizational structure. Schematic structure of FeLV (a) and Organizational structure of FeLV provirus (b).

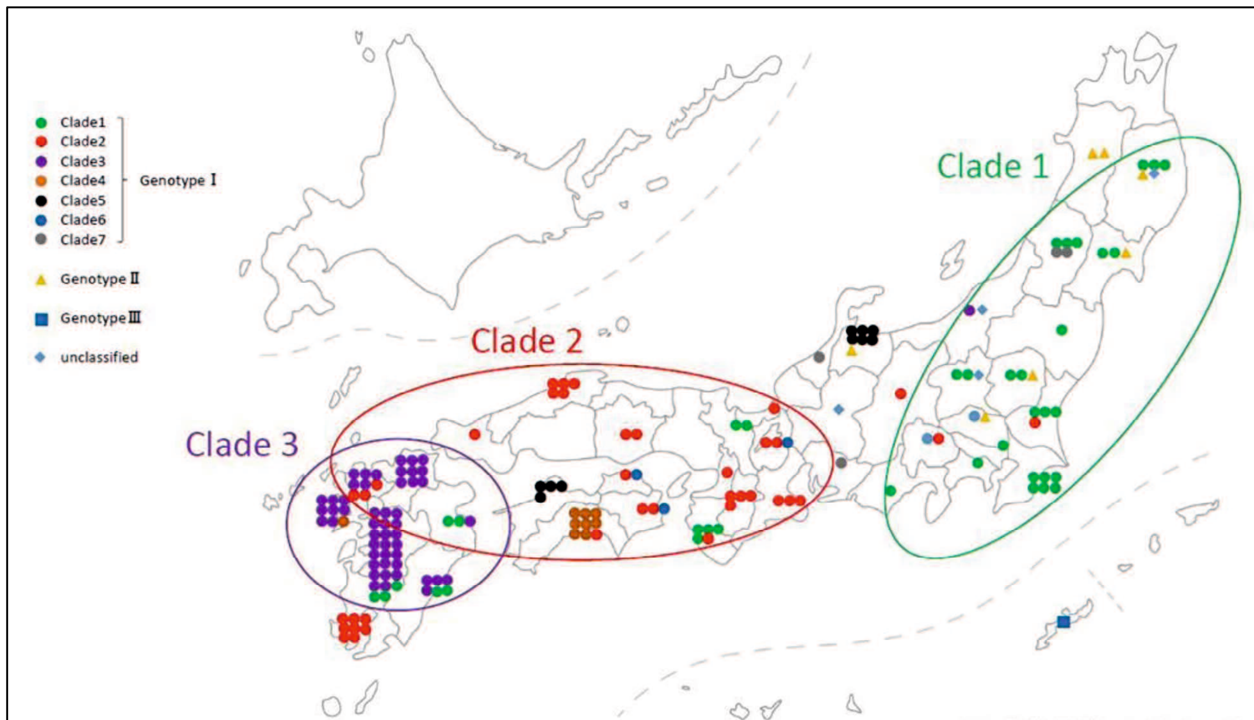


Figure 5: Geographic map of Japan showing the distribution of FeLV genotypic clusters (Genotypes I, II, and III) and the seven clades of genotype I (Watanabe et al., 2013).

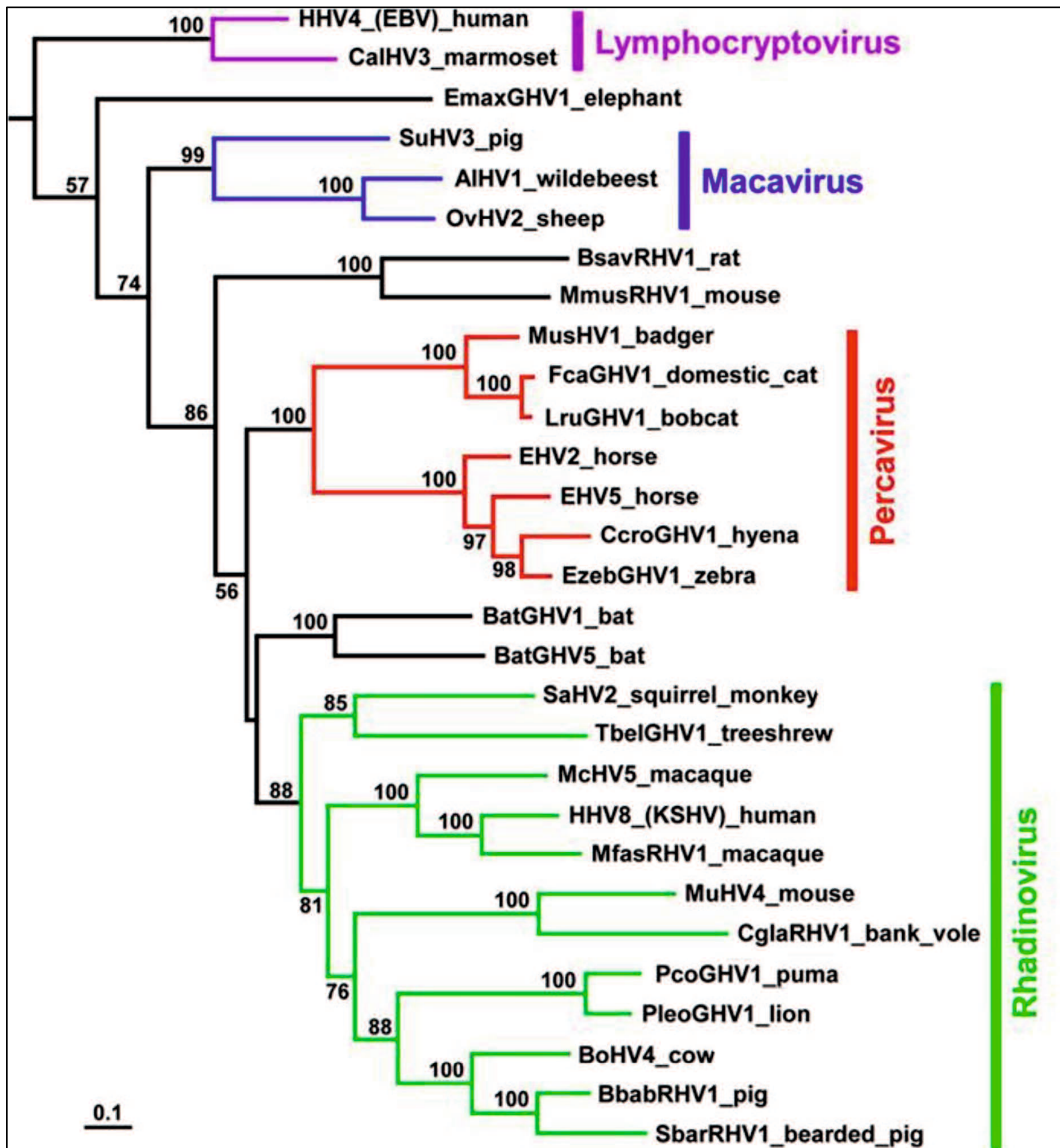


Figure 6: Maximum-likelihood phylogenetic analysis of gammaherpesviruses using concatenated *DNA polymerase* and *glycoprotein B* amino acids alignments (Troyer et al., 2014).

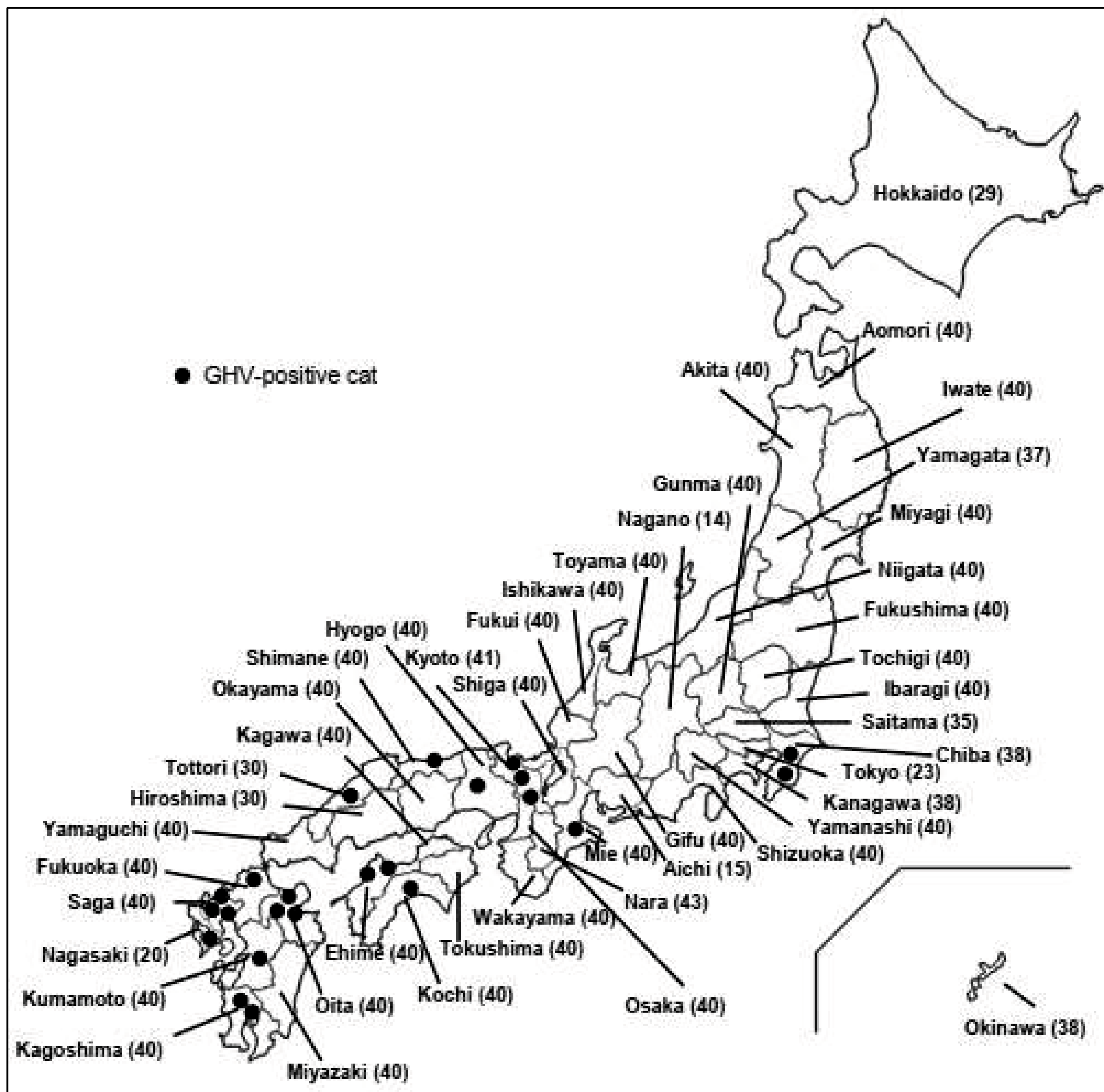


Figure 7: Map of Japan showing the distribution of gammaherpesvirus-positive cats. Positive cats are represented by black dots and the number in brackets indicate the number of samples collected in each prefecture (Tateno et al., 2017).

CHAPTER ONE

2.0. Epidemiologic Survey of Feline Leukemia Virus in Domestic cats on Tsushima Island, Japan: Management Strategy for Tsushima Leopard Cats

The work described in this chapter has been published as follows:

Makundi I, Koshida Y, Kuse K, Hiratsuka T, Ito J, Baba T, Watanabe S, Kawamura M, Odahara Y, Miyake A, Yamamoto H, Kuniyoshi S, Onuma M, Nishigaki K. Epidemiologic survey of feline leukemia virus in domestic cats on Tsushima Island, Japan: Management strategy for Tsushima leopard cats. *J Vet Diagn Investig* 2017; **29**: 889–895.

2.1. Abstract

The Tsushima leopard cat (TLC) *Prionailurus bengalensis euptilurus*, a subspecies of *P. bengalensis*, is designated a National Natural Monument of Japan, and lives only on Tsushima Island, Nagasaki Prefecture, Japan. TLCs are threatened by various infectious diseases. Feline leukemia virus (FeLV) causes a serious infectious disease with a poor prognosis in cats. Therefore, the transmission of FeLV from Tsushima domestic cats (TDCs) to TLCs may threaten the TLC population. I investigated the FeLV infection status of both TDCs and TLCs on Tsushima Island by screening blood samples for FeLV p27 antigen and using PCR to amplify the full-length FeLV *env* gene. The prevalence of FeLV was 6.4% in TDCs and 0% in TLCs. I also demonstrated that the virus can replicate in the cells of TLCs, suggesting its potential cross-species transmission. The viruses in TDCs were classified as genotype I/clade 3, which is prevalent on a nearby island, based on previous studies of FeLV genotypes and FeLV epidemiology. The FeLV viruses identified on Tsushima Island can be further divided into 2 lineages within genotype I/clade 3, which are geographically separated in Kamijima and Shimojima, indicating that FeLV may have been transmitted to Tsushima Island at least twice. Monitoring FeLV infection in the TDC and TLC populations is highly recommended as part of the TLC surveillance and management strategy.

2.2. Introduction

The Tsushima leopard cat (TLC; *Prionailurus bengalensis euptilurus*; family *Felidae*) is only found on Tsushima Island, Nagasaki, Japan. Tsushima Island is part of the Japanese archipelago, situated in the north of the Tsushima Strait between the Japanese mainland and the Korean Peninsula (Figure 8, box). TLC is considered a subspecies of *P. bengalensis*, which migrated from the Eurasian continent to the Japanese archipelago when the archipelago was part of the continent. Most TLCs live in Kamijima (north Tsushima), but a few live in Shimojima (south Tsushima). Kamijima is composed of 4 boroughs—Kamitsushima, Kamiagata, Mine, and Toyotama—and Shimojima is made up of two boroughs— Mitsushima and Mine (Figure 8). TLC was designated a National Natural Monument of Japan in 1971 and an endangered species in 1994. In 2015, TLC was specified a critically endangered species on the Japanese Red List. Several factors increase the extinction risk for endangered species, including habitat loss, overexploitation, and infectious diseases (Izawa, 2009b).

The interspecies transmission of disease causing organisms from feral domestic cats to TLCs has previously been reported. For example, TLCs have been shown to be infected with *Bartonella clarridgeiae*, *Anaplasma bovis*, and *Hepatozoon felis*, with prevalences of 8%, 15%, and 100%, respectively (Tateno et al., 2013a; Tateno et al., 2013b). TLCs have also been infected by feline immunodeficiency virus (FIV), derived from Tsushima domestic cats (Nishimura et al., 1999a; Hayama et al., 2010). Species *Feline leukemia virus* (FeLV; family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Gammaretrovirus*) is transmitted horizontally among domestic cats. FeLV can cause lymphoma, leukemia, myelodysplastic syndrome, aplastic anemia, and immunodeficiency in domestic cats, and FeLV-infected cats have a poor prognosis (Hartmann, 2012). FeLV has been reported to infect wildlife (Rasheed and Gardner, 1981; Briggs and Ott, 1986; Daniels et al., 1999; Sleeman et al., 2001; Ostrowski

et al., 2003; Brown et al., 2008; Cunningham et al., 2008; Guimaraes et al., 2009; Meli et al., 2009; Tangsudjai et al., 2010).

I investigated FeLV infection in domestic cats and TLCs on Tsushima Island. Furthermore, I used cell-culture infection assay to determine the potential cross-species transmission of FeLV from domestic cats to TLCs. There have been no reports of the incidence of FeLV infection in this region. Understanding the FeLV infection status of domestic cats on Tsushima Island is a necessary part of the current ongoing strategy for the surveillance and management of TLCs.

2.3. Materials and Methods

2.3.1. Ethical approval

Animal studies were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

2.3.2. Samples and DNA Extraction

A total of 438 blood samples from domestic cats were collected through the Tsushima Animal Medical Center, a nonprofit animal hospital on the island. Domestic cats living indoors and outdoors were brought by their owners to the center between 2009 and 2015. Chromosomal DNA was extracted using commercial kits (Dr. GenTLE System, Takara Bio, Kyoto, Japan; DNAzol reagent, Life Technologies Japan, Tokyo, Japan) according to manufacturer recommendations.

For investigation of FeLV infection in TLCs, blood, spleen and kidney samples (90 TLCs in total) were collected between 1999 and 2014. The majority of TLCs had been hit by vehicles (road kills).

2.3.3. Screening for FeLV infections

Blood samples were tested for FeLV infections by SNAP FeLV/FIV combo kit, IDEXX Laboratories, Westbrook, ME. By using chromosomal DNA, PCR was used to confirm all doubtful results (Kawamura et al., 2015).

2.3.4. PCR and cloning the FeLV *env* gene

To determine the FeLV genotypes in the FeLV-positive domestic cats, PCR was used. PCR amplified the full-length FeLV *env* (~1.9 kb) gene from the regions of the *pol* and *LTR* sequences conserved in FeLV strains using specific primers (Watanabe et al., 2013). The list of primers used here are shown in Table 1.

Table 1: PCR primers used in the FeLV investigation on Tsushima Island.

Primer	Primer sequence (5′→3′)	Amplicon size (gene)
Fe-44S	CATCGAGATGGAAGGTCC	1.9kb (*env*)
Fe50R	CATGGTTGGTCTGGACGTATTG	
Fe-23S	CAGCAGAAGTTTCAAGGCCACT	2.4kb (*gag*)
Fe-48R	CYGTGGCTCCTTGCACC	
MY-1F	GAGGAGGAGAACTTCTACCAGCA	0.45kb (*c-myc*)
MY-2R	CTGCAGGTACAAGCTGGAGGT	

PCR was performed in 25 μL reactions with KOD FX Neo DNA polymerase (TOYOBO CO., LTD, Osaka, Japan). The PCR cycling conditions were: predenaturation at 98 °C for 2 min; 30 cycles of denaturation at 98 °C for 10 s and extension at 68 °C for 1 min; followed by a final extension at 68 °C for 2 min. For samples that were not amplified using the conditions described above, PCR was performed with the following cycling conditions: 30 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s, and extension at 68 °C for 1 min. The PCR products were cloned into pBlueScript II KS (−) (Agilent Technologies, Santa Clara CA, USA), pCR4Blunt-TOPO (Invitrogen, Carlsbad, CA, USA), or pCR-Blunt (Invitrogen), and the sequences were analyzed by using BioEdit and GeneTyx programs.

2.3.5. Phylogenetic Analyses

The sequence data obtained in this study were included in a multiple sequence alignment based on the epidemiological survey of Japanese FeLV previously conducted (Watanabe et al., 2013). The subsequent phylogenetic analysis was based on the almost-complete surface and transmembrane regions of the FeLV *env* gene (FeLV-A 61E [Donahue, 1988]; positions 6080–7885).

To detect any recombination between endogenous FeLV and exogenous FeLV, a SimPlot analysis (ver.3.5.1) was performed. Clones that were the result of recombination between endogenous FeLV and exogenous FeLV, such as FeLV-B, were excluded from the phylogenetic analysis (Watanabe et al., 2013). Phylogenetic trees were constructed using the maximum likelihood method (Felsenstein, 1981) with the best-fit model (TN93+G and GTR+G) and 1000 bootstrap replicates in MEGA5 (Kumar et al., 2008; Tamura et al., 2011). The sequence of an endogenous FeLV (accession number AY364318) was used as the outgroup to root the trees. Feline leukemia virus (FeLV) *env* sequences used in the phylogenetic analysis are found in the supplementary Table 1 (ST1).

2.3.6. Feline leukemia Viruses preparation for Infection Assay

293Lac cells (Miyake et al., 2016) carrying the LacZ-encoding retroviral vector (Kuse et al., 2016) were infected with FeLV-A/clone 33 (Nishigaki et al., 2002) or FeLV-B/Gardner–Arnstein (Nunberg et al., 1984). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal calf serum (FCS, MP Bio-medicals, Illkirch Cedex, France). The viral supernatants were collected, filtered through a membrane with 0.45 μm pores, and stored at −80 °C until analysis.

2.3.7. Infection Assay and determination of viral infectivity

Primary skin fibroblasts from TLCs, maintained in DMEM supplemented with 10% FCS, were infected with each viral supernatant (FeLV-A and -B) in the presence of Polybrene (10 μg/ml) for ~48 h. The cells were collected by centrifugation, washed, and stored at −20 °C before analysis with PCR. The infected cells, prepared in six- and 24-well culture plates, were stained with 5-bromo-4chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Viral infectivity was calculated by counting the blue-stained nuclei under a microscope, as previously described (Anai et al., 2012).

2.3.8. PCR detection of FeLV in TLC skin fibroblasts

Genomic DNA was isolated from infected TLC fibroblast cells using DNAzol® Reagent (Life Technologies Japan Ltd., Tokyo, Japan), according to the manufacturer's instructions. The PCR primers to amplify the full-length FeLV *gag* gene were used (Kawamura et al., 2015). The names of primers were: Fe-23S and Fe-48R (Table 1). The PCR was performed in 25 μL reactions with KOD FX Neo DNA polymerase. The PCR cycling conditions were: predenaturation at 94 °C for 2 min; 30 cycles of denaturation at 98 °C for 10 s and extension at 68 °C for 1 min; followed by a final extension at 68 °C for 3 min. The PCR primers used to amplify the *c-myc* exon 2 region were MY-1F and MY-2R (Table 1).

2.3.9. Infection Assay and Immunostaining

To assess the viral replication in TLC cells, the supernatants of 293T cells infected with FeLV-A/clone 33 or FeLV-B/Gardner–Arnstein were used to infect skin fibroblast cells from TLCs. Briefly, skin fibroblast cells from TLCs, maintained in DMEM supplemented with 10% FCS, were infected with each viral supernatant (FeLV-A and -B) in the presence of Polybrene (10 μg/ml) for ~48 h. The infected cells were then collected and prepared in 24-well culture plate and maintained further for 7 days. The infected cells were washed by Phosphate Buffered Salts (PBS) and the FeLV envelope glycoprotein, gp70 was determined on day 7 post-infection. Immunostaining was carried out using anti-FeLV-gp70 antibody (FeLV gp85/70 (C11D8), Santa Cruz Biotechnology, Dallas, TX. The secondary antibody was anti-Mouse Immunoglobulin conjugated with horseradish peroxidase (anti-Mouse IgG, HRP-linked Antibody #7076s), Cell Signaling Technology. Detection of HRP activity was done by Peroxidase stain DAB kit (Brown stain), Nacalai Tesque, Kyoto, Japan.

2.4. Results

2.4.1. Prevalence of FeLV infections in domestic cats

FeLV antigen was detected in 28 of 438 domestic cats (Table 2), with a prevalence of 3.1% (10 of 327) in the Kamijima region and 16.2% (18 of 111) in the Shimojima region. The overall prevalence was therefore 6.4%.

Table 2: Detection of feline leukemia virus in various regions of Tsushima Island.

	Kamijima				**Shimojima**		
	Kamitsushima	**Kamiagata**	**Mine**	**Toyotama**	**Mitsushima**	**Izuhara**	**Total**
n	94	178	37	18	48	63	438
Antigen positive	4	4	2	0	3	15	28 (6.4%)

Age, sex, and other basic background data of the FeLV-positive cats are given in Table 3. Most of the FeLV-positive cats were strays or had access to the outdoors.

Table 3: Age, sex, and other basic background data for FeLV-positive cats on Tsushima Island.

	Sex	Age	Region	Breeding environment	FeLV antigen	Accession	FeLV*
1	Neutered	5 y	Mitsushima	In- and outdoor	+		
2	Male	5 y	Izuhara	Domestic cat, outdoor	+	AB970836	TD61
3	Male	Unknown	Kamitsushima	Road kill	+	AB970837	TD73
4	Neutered	Unknown	Kamiagata	Stray cat	+	AB970838	TD80
5	Neutered	Unknown	Kamitsushima	Stray cat	+	AB970839	TD101
6	Male	1 y, 8 mo	Kamitsushima	Domestic cat, outdoor	+	AB970840	TD111
7	Spayed	Unknown	Kamiagata	Stray cat	+	AB970841	TD138
8	Neutered	Unknown	Kamiagata	Stray cat	+	AB970842	TD168
9	Spayed	6 mo	Kamiagata	Stray cat	+		
10	Spayed	3 y	Mitsushima	Domestic cat, outdoor	+	AB970843	TD204
11	Spayed	Unknown	Izuhara	Stray cat	+	AB970844	TD233
12	Spayed	Unknown	Mine	Domestic cat, outdoor	+		
13	Spayed	1 y, 9 mo	Mitsushima	Domestic cat, outdoor	+	AB970845	TD325
14	Spayed	4 y	Kamitsushima	Domestic cat, outdoor	+	AB970846	TD388
15	Neutered	Unknown	Izuhara	Stray cat	+		
16	Spayed	Unknown	Izuhara	Stray cat	+		
17	Spayed	Unknown	Izuhara	Stray cat	+		
18	Spayed	6 mo	Mine	Domestic cat, indoor	+		
19	Female	3 y	Izuhara	Domestic cat, outdoor	+	LC144878	TD427
20	Neutered	Unknown	Izuhara	Domestic cat, outdoor	+	LC144879	TD430
21	Neutered	Unknown	Izuhara	Domestic cat, outdoor	+	LC144880	TD431
22	Neutered	Unknown	Izuhara	Domestic cat, outdoor	+	LC144881	TD433
23	Spayed	Unknown	Izuhara	Domestic cat, outdoor	+		
24	Spayed	Unknown	Izuhara	Domestic cat, outdoor	+	LC144882	TD437
25	Neutered	Unknown	Izuhara	Domestic cat, outdoor	+	LC144883	TD439
26	Spayed	Unknown	Izuhara	Domestic cat, outdoor	+		
27	Spayed	Unknown	Izuhara	Domestic cat, outdoor	+	LC144884	TD443
28	Neutered	Unknown	Izuhara	Domestic cat, outdoor	+	LC144885	TD449

* Each FeLV clone was isolated from a FeLV-positive cat.

2.4.2. PCR Amplification of FeLV *env* gene

The *env* gene was amplified in 19 of the 28 FeLV-positive domestic cats (Table 3).

2.4.3. Phylogenetic analysis of FeLV *env* gene

A phylogenetic analysis of the *env* gene classified the FeLV isolates found on Tsushima Island as genotype I/clade 3 (GI/3; Figure 9), and the isolates were closely related to the prevalent strains on

nearby Kyushu, the third largest island of Japan and the most southwesterly of the 4 main Japanese islands.

I next constructed another phylogenetic tree using the GI/3 strains that are prevalent in Kyushu. I found that the 7 isolates from the Kamijima area of Tsushima Island were closely related to the epidemic strain in Saga and Fukuoka Prefectures, whereas the 12 isolates from the Shimojima area of Tsushima Island were closely related to the epidemic strain in Nagasaki and Fukuoka Prefectures (Figure 10). These results indicate that the viral strains on Tsushima Island form two lineages (clade 3-1 and clade 3-2 within FeLV GI/3) and that these two lineages are geographically separated in Kamijima and Shimojima, respectively.

2.4.4. Nucleotide sequence accession numbers

The nucleotide sequences reported in this study are available in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: AB970836–AB970846 and LC144878–LC144885.

2.4.5. Prevalence of FeLV infections in TLCs

No FeLV antigen or *gag* or *env* gene was detected in any TLC sample with the commercial kit or PCR.

2.4.6. FeLV infection of TLC fibroblast cells

Following infection, TLC fibroblast cells were susceptible to both viral strains at relatively similar high viral titer (Figure 11a).

PCR primers Fe-23S and Fe-48R amplified a fragment of approximately 2.4 kb (i.e., the entire FeLV *gag* gene) when the genetic material from the TLC skin fibroblasts was infected with FeLV-A/clone 33 or FeLV-B/Gardner–Arnstein virus (Figure 11b). The 2.4-kb fragment was not amplified

from the genomic DNA of uninfected TLC skin fibroblasts (Figure 11b; first lane). DNA sequencing confirmed that this primer pair amplified the exogenous FeLV *gag* sequence.

2.4.7. Detection of FeLV glycoprotein, gp70 on TLC cells by Immunostaining

The FeLV gp70 was detected on day 7 post-infection by immune staining, indicating that both FeLV-A and FeLV-B replicate in TLC skin fibroblast cells (Figure 12).

2.5. Discussion

The FeLV infections identified in domestic cats on Tsushima Island were all FeLV GI/3, which is widespread on Kyushu in Japan, indicating that the FeLV strains on Tsushima Island may have originated in Kyushu. The FeLV strains on Tsushima Island were clearly separated into 2 areas, Kamijima and Shimojima. GI/3-1 was found in Kamijima and GI/3-2 in Shimojima, suggesting that FeLV has been transmitted to Tsushima Island at least twice in the past. After the arrival of FeLV on Tsushima Island, the virus may have spread in each area. However, the exact time of arrival and the origins of these FeLV strains are unknown. Transportation to Tsushima Island is by ship and airplane. Air transport moves between Tsushima airport and Fukuoka and Nagasaki airports, and sea routes link the ports of Hitakatsu and Izuhara on Tsushima to the port of Hakata in Fukuoka. The viruses isolated from some cats on Tsushima Island are of the same genotype and clade as those observed in the nearby Kyushu region, which together with the available means of transportation, suggests the transmission of FeLV between Kyushu and Tsushima Island.

The FeLV strains currently present on Tsushima Island are of two types, located in different geographic regions. This observation extends our understanding of the epidemiology of FeLV infection on Tsushima Island. In particular, our analysis of the FeLV genotypes suggests that the virus

originated outside the island. It is important to continue monitoring FeLV infections and to determine the viral genotypes.

The possibility of FeLV transmission from South Korea must also be considered because ferries operate between the two regions. Although the transportation of pets and live animals via ferry is subject to restrictions, such as the international certification of animal movements, the possibility of FeLV transmission cannot be ruled out. To date, the molecular epidemiology and characterization of FeLV in South Korea have not been reported.

I demonstrated in this study that FeLV can replicate in TLC cells, suggesting potential cross-species transmission. I used the prototype FeLV-A for the cell-culture infection assay because this virus has been detected in all naturally infected cats and because FeLV-A is considered the most transmissible form of FeLV (Hartmann, 2012). In contrast, FeLV-B displays a broader host range in vitro (Jarrett et al., 1973). I also investigated FeLV infections (based on antigen and PCR detection) in TLCs in our laboratory, but found no positive cases. However, the maintenance of this virus in domestic cats significantly endangers the health and survival of TLCs on the island.

The close proximity of households with domestic animals to the local wildlife can facilitate the transmission of disease both from domestic animals to wildlife and vice versa. Domestic dogs and cats are known to be susceptible to many infectious agents, including canine distemper virus (CDV), canine parvovirus, *Echinococcus granulosus*, *Toxoplasma gondii*, and rabies virus. In 1994, a CDV epidemic in Tanzania's Serengeti ecosystem not only caused the death of ~30% of Serengeti lions, but also affected other free-ranging canids and felids. The domestic dogs of local villages living near the National Park were implicated as the source of this deadly virus (Roelke-Parker et al., 1996).

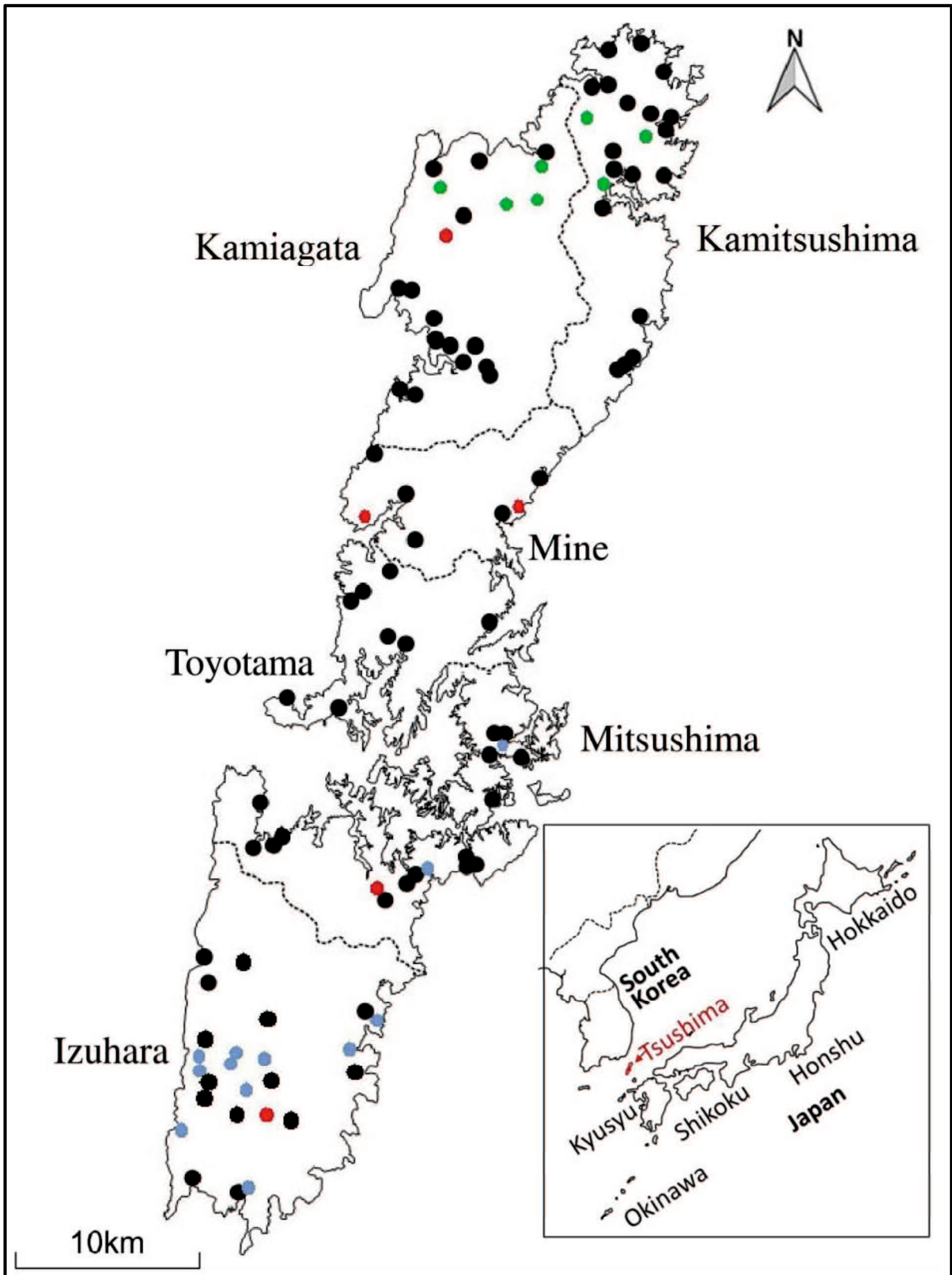

N
Kamiagata
Kamitsushima
Mine
Toyotama
Mitsushima
Izuhara
10km
South Korea
Tsusbima
Hokkaido
Honshu
Kyusyu
Shikoku
Japan
Okinawa

Figure 8: Schematic map of Tsushima Island and sites of blood sampling from domestic cats (shown in black dots). Sites of detection of FeLV-positive cats with undetermined FeLV genotypes are shown in red dots; sites of cats with FeLV genotype I/clade 3-1 are shown in green; sites of cats with FeLV genotype I/clade 3-2 are shown in blue. Location of Tsushima Island is shown in the box.

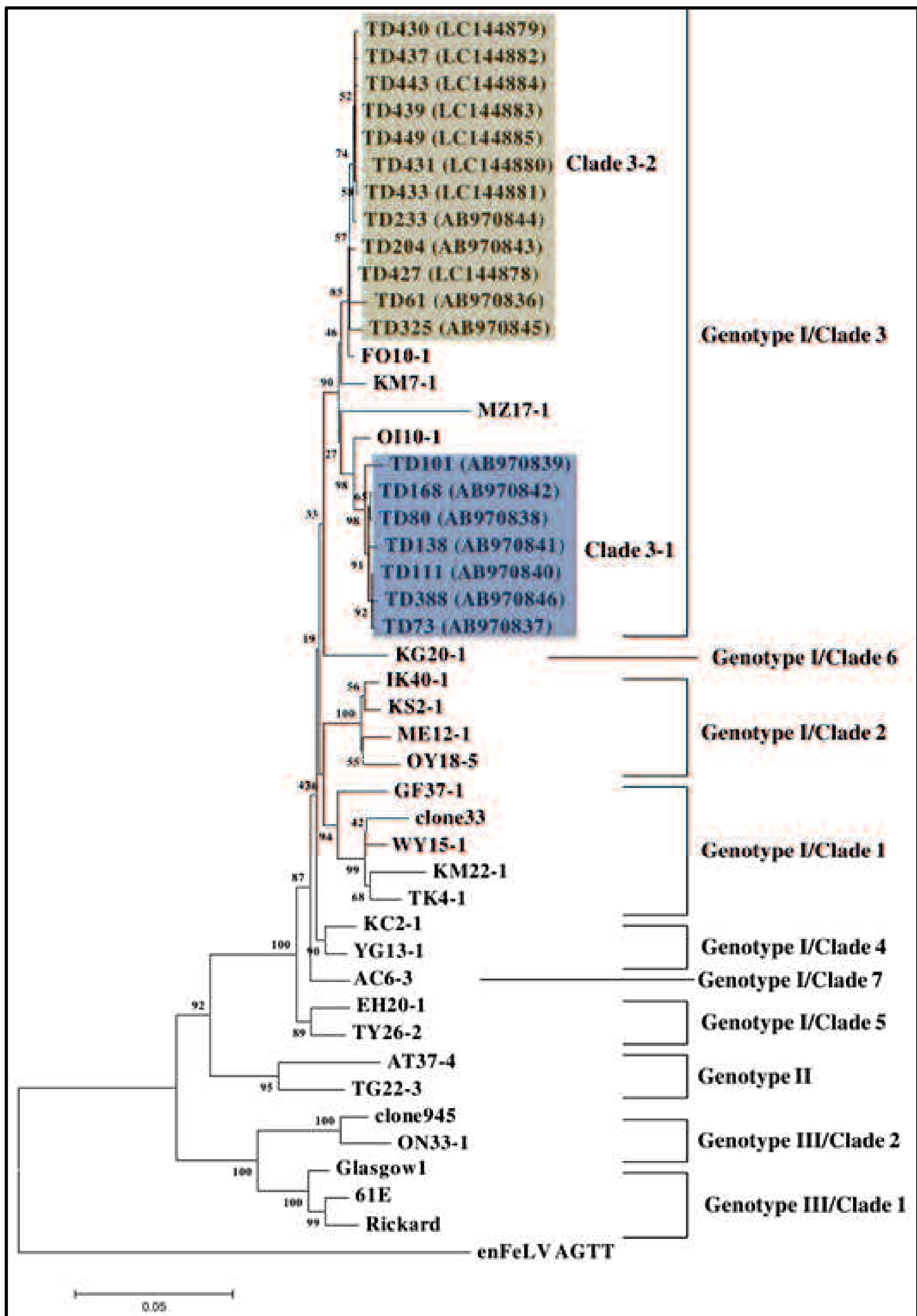

TD430 (LC144879)
TD437 (LC144882)
TD443 (LC144884)
TD439 (LC144883)
TD449 (LC144885)
TD431 (LC144880)
TD433 (LC144881)
TD233 (AB970844)
TD204 (AB970843)
TD427 (LC144878)
TD61 (AB970836)
TD325 (AB970845)
Clade 3-2
FO10-1
KM7-1
MZ17-1
OI10-1
TD101 (AB970839)
TD168 (AB970842)
TD80 (AB970838)
TD138 (AB970841)
TD111 (AB970840)
TD388 (AB970846)
TD73 (AB970837)
Clade 3-1
Genotype I/Clade 3
KG20-1
Genotype I/Clade 6
IK40-1
KS2-1
ME12-1
OY18-5
Genotype I/Clade 2
GF37-1
clone33
WY15-1
KM22-1
TK4-1
Genotype I/Clade 1
KC2-1
YG13-1
Genotype I/Clade 4
AC6-3
Genotype I/Clade 7
EH20-1
TY26-2
Genotype I/Clade 5
AT37-4
TG22-3
Genotype II
clone945
ON33-1
Genotype III/Clade 2
Glasgow1
61E
Rickard
Genotype III/Clade 1
enFeLV AGTT
0.05

Figure 9: Phylogenetic analysis of isolated FeLV env clones (with gene accession) detected in this study. FeLV clones detected are shown in highlighted boxes. Maximum likelihood tree was constructed from a phylogenetic analysis of near-full-length feline leukemia virus (FeLV) env nucleotide. The first 2 uppercase letters in the name of each viral clone indicate the prefecture in which the sample was collected. Located on Honshu are: AT, Akita; IK, Ibaraki; TG, Tochigi; TK, Tokyo; NG, Nigata; TY, Toyama; AC, Aichi; GF, Gifu; ME, Mie; NR, Nara; WY, Wakayama; OY, Okayama; and YG, Yamaguchi. Located on Shikoku are: KG, Kagawa; EH, Ehime; and KC, Kochi. Located on Kyushu are: FO, Fukuoka; SA, Saga; OI, Oita; NS, Nagasaki; KM, Kumamoto; MZ, Miyazaki; and KS, Kagoshima. ON, Okinawa.

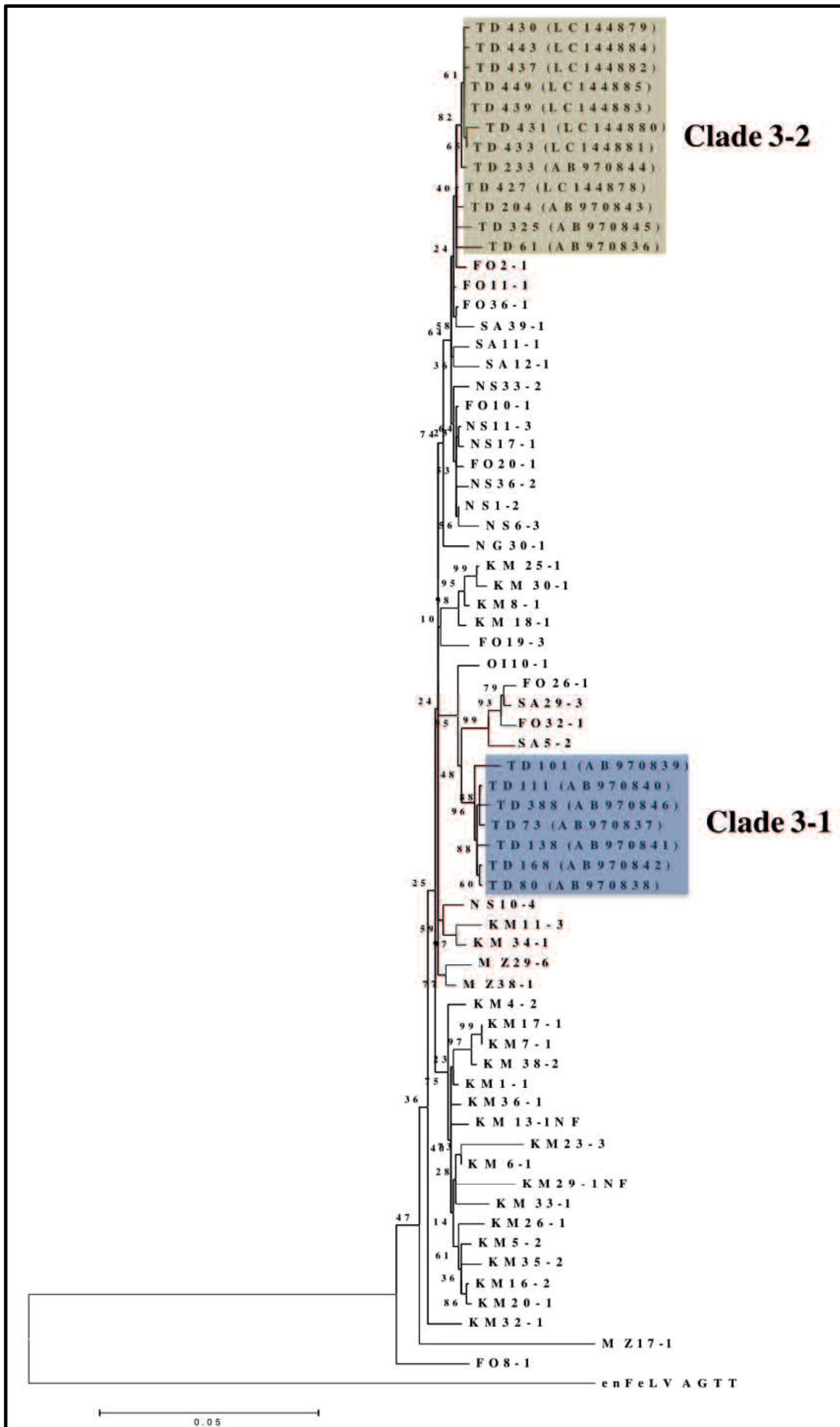
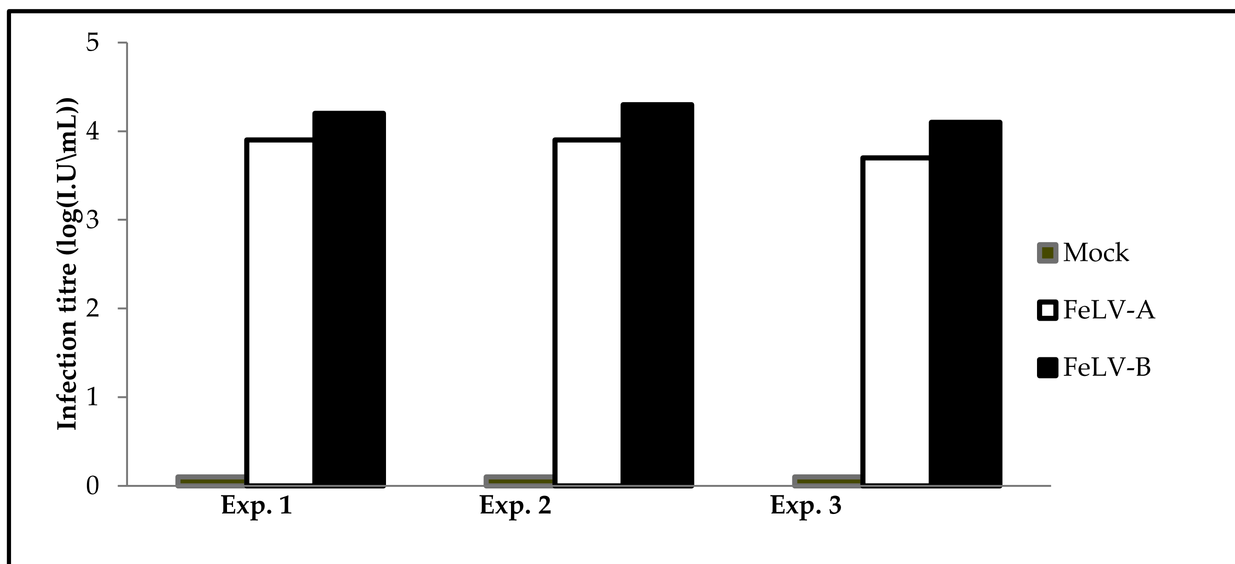
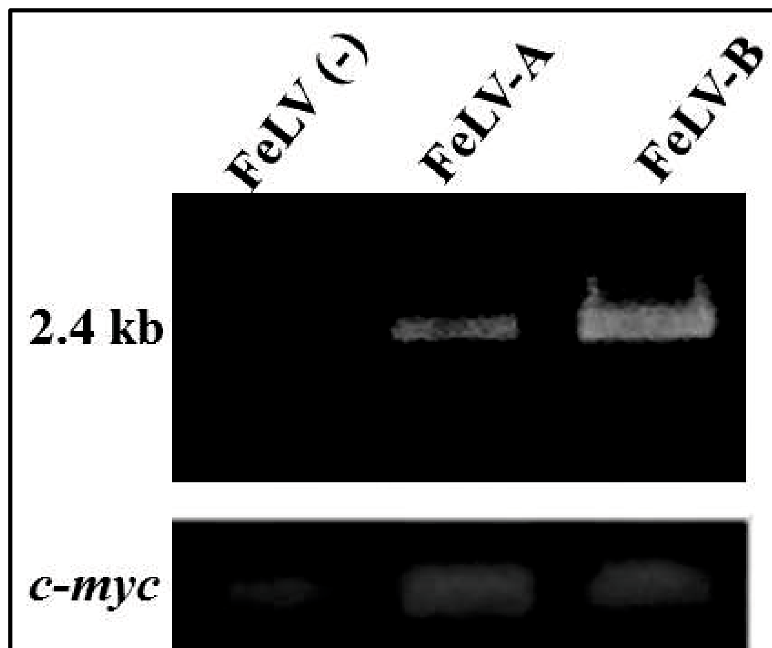

TD430 (LC144879)
TD443 (LC144884)
TD437 (LC144882)
TD449 (LC144885)
TD439 (LC144883)
TD431 (LC144880)
TD433 (LC144881)
TD233 (AB970844)
TD427 (LC144878)
TD204 (AB970843)
TD325 (AB970845)
TD61 (AB970836)
Clade 3-2
FO2-1
FO11-1
FO36-1
SA39-1
SA11-1
SA12-1
NS33-2
FO10-1
NS11-3
NS17-1
FO20-1
NS36-2
NS1-2
NS6-3
NG30-1
KM25-1
KM30-1
KM8-1
KM18-1
FO19-3
OI10-1
FO26-1
SA29-3
FO32-1
SA5-2
TD101 (AB970839)
TD111 (AB970840)
TD388 (AB970846)
TD73 (AB970837)
TD138 (AB970841)
TD168 (AB970842)
TD80 (AB970838)
Clade 3-1
NS10-4
KM11-3
KM34-1
MZ29-6
MZ38-1
KM4-2
KM17-1
KM7-1
KM38-2
KM1-1
KM36-1
KM13-1NF
KM23-3
KM6-1
KM29-1NF
KM33-1
KM26-1
KM5-2
KM35-2
KM16-2
KM20-1
KM32-1
MZ17-1
FO8-1
enFeLV AGTT
0.05

Figure 10: Phylogenetic analysis of FeLV genotype I/ clade 3 *env* sequences. The maximum likelihood tree was constructed from a phylogenetic analysis of the near-full-length env gene sequences of FeLV genotype I/clade 3. The first 2 uppercase letters in the name of each viral clone indicate the prefecture in which the sample was collected. Located on Honshu are: AT, Akita; IK, Ibaraki; TG, Tochigi; TK, Tokyo; NG, Nigata; TY, Toyama; AC, Aichi; GF, Gifu; ME, Mie; NR, Nara; WY, Wakayama; OY, Okayama; and YG, Yamaguchi. Located on Shikoku are: KG, Kagawa; EH, Ehime; and KC, Kochi. Located on Kyushu are: FO, Fukuoka; SA, Saga; OI, Oita; NS, Nagasaki; KM, Kumamoto; MZ, Miyazaki; and KS, Kagoshima. ON, Okinawa. FeLV genotype I/clade 3-1 and FeLV genotype I/clade 3-2 are shown in the figure.



(a)



(b)

Figure 11: Feline leukemia virus infection of TLC fibroblast cells. **(a)** FeLV-A/clone 33 and FeLV-B/Gardner–Arnstein viral strains were used to infect TLC skin fibroblasts. TLC skin fibroblasts were pre-infected with no virus (mock; gray), FeLV-A/clone 33 (white), or FeLV-B/Gardner–Arnstein (black). X-Gal–positive cells were counted as infectious units (IU) at 48 h post-infection. The graph shows the infectious titer, calculated from 3 independent experiments. **(b)** FeLV proviral gag gene was amplified from chromosomal DNA of infected TLC skin fibroblasts. The expected amplicon size of 2.4 kb is shown. c-myc was amplified as the positive control.

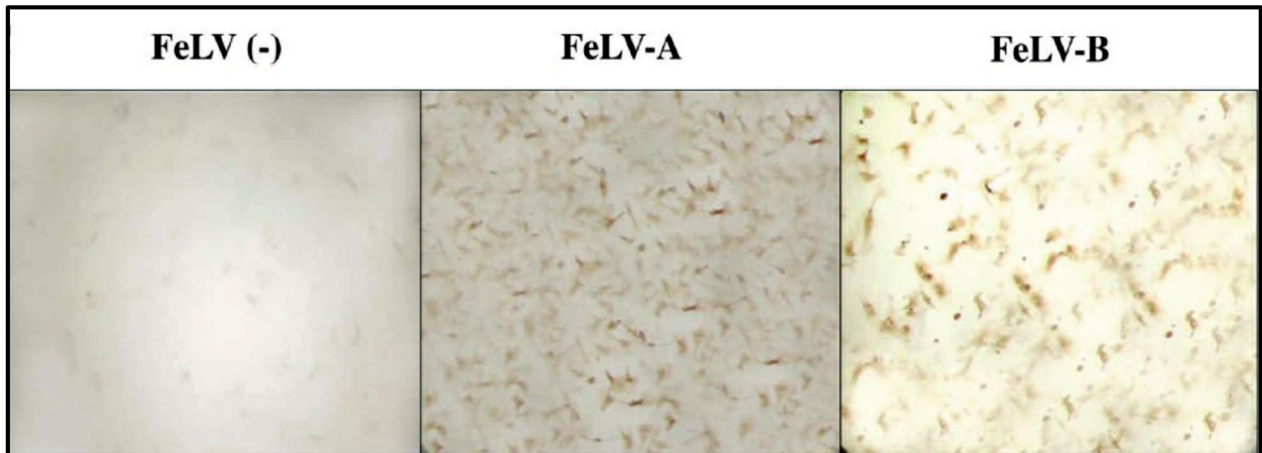


Figure 12: Immunostaining of FeLV envelope glycoprotein, gp70 on TLC cells infected with FeLV-A or FeLV-B. Cells infected with FeLV-A, FeLV-B or mock were stained at day 7 post-infection.

CHAPTER TWO

3.0. Identification of *Felis catus* Gammaherpesvirus 1 in Tsushima Leopard Cats (*Prionailurus bengalensis euptilurus*) on Tsushima Island, Japan

The work described in this chapter has been published as follows:

Makundi I, Koshida Y, Endo Y, Nishigaki K. Identification of *Felis catus* Gammaherpesvirus 1 in Tsushima Leopard Cats (*Prionailurus bengalensis euptilurus*) on Tsushima Island, Japan. *Viruses* 2018; **10**: 378.

3.1. Abstract

Felis catus gammaherpesvirus 1 (FcaGHV1) is a widely endemic infection of domestic cats. Current epidemiological data identify domestic cats as the sole natural host for FcaGHV1. The Tsushima leopard cat (TLC; *Prionailurus bengalensis euptilurus)* is a critically endangered species that lives only on Tsushima Island, Nagasaki, Japan. Nested PCR was used to test the blood or spleen of 89 TLCs for FcaGHV1 DNA; three (3.37%; 95% CI, 0.70–9.54) were positive. For TLC management purposes, I also screened domestic cats and the virus was detected in 13.02% (95% CI, 8.83–18.27) of 215 cats. Regarding phylogeny, the partial sequences of FcaGHV1 from domestic cats and TLCs formed one cluster, indicating that similar strains circulate in both populations. In domestic cats, we found no significant difference in FcaGHV1 detection in feline immunodeficiency virus-infected (p = 0.080) or feline leukemia virus-infected (p = 0.163) cats, but males were significantly more likely to be FcaGHV1 positive (odds ratio, 5.86; 95% CI, 2.27–15.14) than females. The higher frequency of FcaGHV1 detection in domestic cats than TLCs, and the location of the viral DNA sequences from both cats within the same genetic cluster suggests that virus transmission from domestic cats to TLCs is likely.

3.2. Introduction

The Tsushima leopard cat (TLC: *Prionailurus bengalensis euptilurus*; family Felidae) is a small wild cat inhabiting Tsushima Island, Nagasaki, Japan. Most TLCs live in Kamijima but a few live in Shimojima. The TLC has been designated a critically endangered species, and several management strategies have been implemented to maintain the species (Mitani et al., 2009; Tateno et al., 2013b). Interspecies transmission of several diseases from free-ranging domestic cats to TLCs have been reported (Nishimura et al., 1999a; Hayama et al., 2010; Tateno et al., 2013a). Feline immunodeficiency virus (FIV) isolated from a wild TLC shared *env* gene sequences with FIV isolated from domestic cats (Nishimura et al., 1999a). In addition, a study that examined FIV infection risk in TLCs using Geographical Information System (GIS) data found that TLCs living in areas densely populated with domestic cats were at higher risk of infection than those from areas with fewer domestic cats (Hayama et al., 2010). I recently reported the prevalence of feline leukemia virus (FeLV) infection in domestic cats on Tsushima Island. I did not detect FeLV in TLCs; however, I demonstrated that the virus could replicate in their cells (Makundi et al., 2017).

Gammaherpesvirus (GHV) infection is typically characterized by an extended period of viral latency in the host that persists for the host's lifetime (Speck and Ganem, 2010; Barton et al., 2011). There is cumulative evidence of GHV infection in several felid species (Kruger et al., 2000; Ehlers et al., 2008; Troyer et al., 2014; Lozano et al., 2015), suggesting that many animal species are infected with one or more GHVs (Ackermann, 2006). For example, two GHV strains, LruGHV1 and LruGHV2, have been isolated from bobcats (*Lynx rufus*). In addition, two different GHV strains, PcoGHV1 and LruGHV1, have been isolated from pumas (*Puma concolor*) (Lozano et al., 2015).

Felis catus gammaherpesvirus 1 (FcaGHV1) commonly infects domestic cats and has a worldwide distribution (Beatty et al, 2014; Ertl et al., 2015; Tateno et al., 2017; Kurissio et al., 2018). To date, FcaGHV1 DNA has not been detected in other feline species; therefore, the host range of FcaGHV1 is currently unknown (McLuckie et al., 2018). Previous reports support the pathogenic potential of FcaGHV1 infection as FcaGHV1-positive cats were at least twice more likely to be in ill-health than healthy on physical examination (Beatty et al., 2014; Tateno et al., 2017). The disease outcome and risks associated with the transmission of FcaGHV1 from domestic cats to TLCs is currently unknown. Therefore, monitoring the TLC population for infectious diseases is highly recommended as part of a surveillance and management strategy since measures required to control disease in wild populations can be challenging (Makundi et al., 2017). The expansion of human habitats facilitates the spill-over of feline pathogens from domestic cats into wildlife populations; thus, determination of the pathogenic potential of FcaGHV1 is a priority for feline, human and wildlife health (Beatty et al., 2014). Previous studies have identified several risk factors for FcaGHV1 infection including adult and male status, geographical location, health status (sick) and co-pathogens (Beatty et al., 2014; Ertl et al., 2015; McLuckie et al., 2016a; Stutzman-Rodriguez et al., 2016; McLuckie et al., 2017; Tateno et al., 2017; Kurissio et al., 2018).

The TLC usually has a large home range during breeding season and thus the possibility of contact between TLCs and free-roaming domestic cats is reasonably high (Hayama et al., 2010). The prevalence of FIV in up to 27% of the domestic cats on Tsushima Island (for example, in Kamiagata) is higher than in other regions of Japan (Hayama et al., 2010). Previous epidemiological data suggests that territorial aggression and fighting are the most common modes of FcaGHV1 and FIV transmission. Based on this association between FcaGHV1 and FIV, and on the previously reported detection of FIV in TLCs, I hypothesized that TLCs are similarly at a high risk of GHV infection.

3.3. Materials and Methods

3.3.1. Ethics Statement

This study was approved by the Institutional Animal Care and Use Committee of Yamaguchi University (identification code 2017/315, approved on 9 May 2017). Animal studies were conducted following the guidelines for the Care and Use of the Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

3.3.2. Study Area

Tsushima Island is part of the Japanese archipelago. It is situated in the northern Tsushima Strait between Japan and the Korean Peninsula. Tsushima Island actually comprises two main islands: Kamijima (north Tsushima) and Shimojima (south Tsushima). Kamijima is composed of four boroughs (Kamitsushima, Kamiagata, Mine and Toyotama) while Shimojima has two boroughs (Mitsushima and Izuhara) as shown in Figure 13.

3.3.3. Sample Collection and DNA Preparation

Blood and spleen samples were collected from TLCs between 1999 and 2017. The majority of these were from animals killed by vehicles. In total, 89 TLCs (60 blood and 29 spleen) samples were available for this study (Makundi et al., 2017). Blood samples from domestic cats were donated by the Tsushima Animal Medical Center, a nonprofit animal hospital on the island. The domestic cats included both indoor-only and free-roaming cats brought by their owners to the center between 2009 and 2016 (Makundi et al., 2017).

Blood samples were screened for FeLV and FIV infection using the SNAP FeLV/FIV Combo Kit (IDEXX Laboratories Inc., Westbrook, ME, USA). Chromosomal DNA from whole blood samples was purified using the Dr. GenTLE System (Takara Bio Inc., Kyoto, Japan) and DNAzol reagent

(Life Technologies Japan, Tokyo, Japan) according to the manufacturer's instructions. DNA from spleen was extracted using commercial kit (DNeasy® Blood & Tissue Kit, QIAGEN, Hilden, Germany).

3.3.4. Feline Glyceraldehyde-3-phosphate dehydrogenase (FeGAPDH) PCR

To confirm the presence of amplifiable template DNA, a conventional PCR for feline glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was performed as previously described (Beatty et al., 2014). Briefly, primers GAPfwd and GAPrev, designed to amplify an 80-bp product, were used. Reaction mixtures for PCR contained 0.5 μM of each primer, 2.5 mM of dNTPs, 2.5 units of TaKaRa Ex Taq DNA polymerase, and template containing up to 100 ng of DNA in a total volume of 25 μL. PCR conditions were pre-denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s and a final extension at 72°C for 5 min. Electrophoresis was performed on 2.0% agarose gels in 1% Tris-acetate buffer containing 0.5 μg ethidium bromide/mL and the 80-bp product was visualized in 89 samples of TLC and 215 samples of domestic cats under ultraviolet illumination.

3.3.5. PCR Amplification and Sequencing

Using Primer3 Input (primer3.ut.ee) I designed FcaGHV1-specific primers to amplify a portion of the conserved glycoprotein B (*gB*) gene based on the sequence from isolate KF840715 (Troyer et al., 2014). The method was optimized using samples confirmed by sequencing to amplify the target sequence. Nested PCR was performed using two primer pairs for the *gB* gene that amplify 715 and 580-bp gene products in the first and second rounds, respectively (Table 4). Second round reactions were employed to enhance the specificity and sensitivity of the PCR reaction. PCR was performed in 25 μL reactions using TaKaRa Ex Taq polymerase (Takara Bio Inc.). The first round PCR product (1 μL) was used as the template for the second round PCR. The PCR cycling conditions for

both reactions were: pre-denaturation at 94°C for 2 min followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s followed by a final extension at 72°C for 7 min. The identity of the PCR amplification products was further verified by electrophoresis through 1% agarose gels, purification with the FastGene Gel/PCR extraction kit (Nippon Genetics Co. Ltd., Tokyo, Japan), and the second PCR products were sequenced in both directions by Fasmac Co., Ltd., Kanagawa, Japan. Sequences were visualized and analyzed by BioEdit (Hall, 1999) and, after removal of the primer sequence, the 553-bp *gB* sequences were compared with other GHV *gB* sequences using GeneTyx (Software Development Co., Tokyo, Japan) and NCBI Blast programs.

Table 4: PCR primers for amplification of *Felis catus* gammaherpesvirus 1 *glycoprotein B* and *DNA polymerase* genes.

Name of Primer [a]	Sequence (5′ to 3′)	Target Gene	Product Size (bp)	Reference
FcaGHV1gB–1s	GACCTGCACCAGAGCATGAG	*gB*		
FcaGHV1gB–1as	AGGATCCCTGGCAGATTGGT		715	This study
FcaGHV1gB–2s	TGCACCAGAGCATGAGAGTT			
FcaGHV1gB–2as	TCCCCCGAGAGGGTTTTTGA		580	This study
FcaGHV1pol–1s	GGTGTTAATGGAAGCCCTGTG	*DNApol*		
FcaGHV1pol–1as	TTAGTCAGCCTTGGCATTGC		818	This study
FcaGHV1pol–2s	ATGGAAGCCCTGTGAAGTTT			
FcaGHV1pol–2as	CAGTGTCTCATTGCTTGCTGT		568	This study

[a] s = sense; as = antisense.

To further verify the detection of FcaGHV1 in TLCs, I amplified the second adjacent conserved gene, DNA polymerase (*DNApol*), by using PCR specific primers from the same KF840715 isolate (Table 4). A nested PCR using TaKaRa Ex Taq polymerase was carried out similarly as for detection of the *gB* gene; however, the annealing temperatures for the first- and second-round PCRs were 55°C and 60°C, respectively. Purification of the PCR products, sequencing and sequence analysis were conducted as for the *gB* gene.

3.3.6. Determination of PCR Sensitivity

To determine the sensitivity of our nested PCR for the detection of FcaGHV1 *gB*, I performed serial dilutions of DNA from the peripheral blood mononuclear cells (PBMCs) and spleen of TLCs and the PBMCs of domestic cats, as previously described (Sleeman et al., 2001). Six log serial dilutions of 1 μg of DNA were added to the first PCR reactions and 1 μL of a 1:10 dilution of the first-round product was used for each second round of PCR. Second-round amplification products were electrophoresed at 125 V for 30 min. through 1% agarose gels, stained with ethidium bromide, and visualized under UV transillumination.

3.3.7. Phylogenetic Analyses

The GHV partial *gB* and *DNApol* nucleotide sequences were aligned using ClustalW 1.6 and phylogenetic analysis was performed using MEGA6 (Tamura et al., 2013). Maximum likelihood (ML) phylogenetic analyses were conducted based on the Kimura 2-parameter model with all areas containing gaps being ignored. The betaherpesvirus human cytomegalovirus (HCMV; human herpesvirus 5{HHV5}) was used as an outgroup to root the tree. Bootstrap analysis was performed with 100 iterations to evaluate the stability of the tree.

3.3.8. Statistical Analyses

All statistical analyses were conducted using the Minitab Statistical program (Minitab version 18, Minitab Inc., Shanghai, China, 2018). The frequency of FcaGHV1 and its 95% exact confidence intervals were computed using the Maximum Likelihood (ML) estimation. I performed descriptive analyses and created contingency tables of categorical variables with the outcome FcaGHV1 infection in domestic cats. The associations between these variables and FcaGHV1 infection were initially evaluated by univariable analysis using Fisher's exact test and variables with p values < 0.2 were

included in the final multivariable analysis. Univariable and multivariable analyses were performed using the Minitab 18 logit function. Binary logistic regression and model fit was evaluated by Hosmer–Lemeshow goodness-of-fit test.

3.4. Results

3.4.1. Frequency and Distribution of FcaGHV1 on Tsushima Island

In total, 89 (blood and spleen) samples from TLCs and 215 blood samples from domestic cats were available for FcaGHV1 testing (Table 5). Three TLCs were positive for FcaGHV1, giving an overall frequency of FcaGHV1 detection in TLCs of 3.37% (95% CI, 0.70–9.54). These three positive TLCs originated from Kamijima and comprised two adult females and one adult male (Figure 13).

Table 5: *Felis catus* gammaherpesvirus 1 *glycoprotein B* detected in feline DNA samples by nested FcaGHV1 PCR.

Host Species	No. of Samples	No. of FcaGHV1 Positive	%Positive (95% CI)
Leopard cat	89	3	3.37 (0.70–9.54)
Domestic cat	215	28	13.02 (8.83–18.27)

For the purpose of TLC management and because the domestic cat is the primary natural host for FcaGHV1, I decided to investigate the infection status of FcaGHV1 in domestic cats. As shown in Table 5, the overall frequency of FcaGHV1 detection in domestic cats was 13.02% (95% CI, 8.83–18.27). The characteristics of the domestic cats tested in this study are presented in Table 6. The probability of FcaGHV1 infection in domestic cats did not significantly differ between the two test regions, Kamijima and Shimojima (Fisher's exact test, p = 1.0 and odds ratio, 0.94: 95% CI, 0.36–2.47). In Kamijima, the domestic cats that were positive for FcaGHV1 were mostly found in the western part (Kamiagata) of the island (Figure 13).

Table 6: Contingency table of categorical variables with the outcome FcaGHV1 *gB* detection in domestic cats.

Variables	Categories	FcaGHV1 Status		Total	% Positive
		Positive	Negative		
Sex	Male	22	72	94	23
	Female	6	115	121	5
Region	Kamijima	22	145	167	13
	Shimojima	6	42	48	12
FIV infection	Negative	11	107	118	9
	Positive	17	80	97	17
FeLV infection [1]	Negative	22	165	187	12
	Positive	6	22	28	21

[1] Cited from reference Makundi et al., 2017

3.4.2. Variations of the FcaGHV1 Sequence

Partial *gB* sequences (553 nucleotides) were used for analysis of sequence variations. Sequences from the 31 FcaGHV1-positive animals in this study (three TLCs and 28 domestic cats) contained nucleotide polymorphisms (NPs) at three different positions: 126, 323 and 420 of KF840715. The three TLC sequences were identical to published FcaGHV1 sequences (GenBank KF840715) at nucleotide 126 but with thymidine and adenine replacing cytosine and guanine at nucleotides 323 and 420, respectively. Furthermore, the three TLC sequences were 100% identical to 13 of the 28 sequences obtained from the domestic cats in our study. Similarly, in nine of the 28 domestic cat sequences thymidine replaced cytosine at nucleotide 126 of the published FcaGHV1 (KF840715) sequence. A similar change was present at nucleotide 323 in 13 of the 28 domestic cat sequences and in 17 of the 28 domestic cat sequences adenine replaced guanine at nucleotide 420. Overall, of the 31 sequences obtained in this study, 11 were 100% identical to FcaGHV1 *gB*, KF840715.

Using DNA polymerase gene amplification, I successfully confirmed the presence of FcaGHV1 in all three of the *gB*-positive TLCs. This PCR detection of FcaGHV1 *DNApol* yielded relatively more

non-specific amplification products compared with *gB* PCR. Despite this, I detected FcaGHV1 *DNApol* in 14 of the 28 domestic cats that were positive for the *gB* gene. Sequence analysis of 527 nucleotides revealed 99% homology with GenBank sequences KF840715 and KT595939. Comparison of the nucleotide sequences from the three positive TLCs revealed one nucleotide substitution at position 1219 of KF840715, in which thymidine was replaced by adenine.

3.4.3. Sex and FIV/FeLV Status as Risk Factors for FcaGHV1 Infection in Domestic Cats

Univariable analysis showed that males had a significantly higher probability of being FcaGHV1 positive (odds ratio, 5.86; 95% CI, 2.27–15.14) than females (Table 7). Similar results were obtained following subsequent multivariable analysis (Table 8). Neither FIV nor FeLV infection status was significantly associated with FcaGHV1 detection. The model fit in the multivariable analysis was sufficient (Hosmer–Lemeshow goodness-of-fit $p = 0.77$; $p > 0.05$ suggests an adequate model fit).

Table 7: Univariable logistic regression analyses to evaluate the association between explanatory variables and the outcome, FcaGHV1 detection in domestic cats.

Variables	Categories	b [1]	SE [2]	Odds-Ratio	95% CI	*p*-Value
Intercept	Male vs. female	−0.58	0.64			
Sex		1.77	0.48	5.86	2.27–15.14	<0.0001
Intercept	Kamijima vs. Shimojima	2.13	1.87			
Region		−0.06	0.49	0.94	0.36–2.47	0.903
Intercept	Positive vs. negative	−2.08	2.25			
FIV infection		0.73	0.41	2.07	0.92–4.66	0.080
Intercept	Positive vs. negative	−3.71	4.01			
FeLV infection		0.72	0.51	2.05	0.75–5.60	0.163

[1] Coefficient of variable estimate; [2] Standard error of the variable estimate; SE, Standard error; CI, Confidence interval.

Table 8: Multivariable logistic regression analyses to identify risk factors for FcaGHV1 detection in domestic cats.

Variables	Categories	b [1]	SE [2]	Odds-Ratio	95% CI	*p*-Value
Intercept		−11.02	5.87			
Sex	Male vs. female	1.64	0.49	5.17	1.95–13.70	0.001
FIV infection	Positive vs. negative	0.57	0.47	1.77	0.71–4.44	0.223
FeLV infection	Positive vs. negative	0.96	0.58	2.60	0.84–8.09	0.099

[1] Coefficient of variable estimate; [2] Standard error of the variable estimate; SE, Standard error; CI, Confidence interval.

3.4.4. Sensitivity of FcaGHV1 *gB* PCR

Second-round 580-bp amplicons were amplified from all dilutions (0.01 ng to 1 μg) of spleen and PBMC DNA. The PCR was able to detect virus to at least the 0.01 ng dilution (Figure 14).

3.4.5. Phylogenetic Analyses and Comparison with Other GHVs

I aligned the FcaGHV1 partial *gB* and *DNApol* sequences to sequences of previously reported viruses for phylogenetic analysis. All FcaGHV1 sequence data detected in the present study formed one cluster with other GHVs within the Percavirus genus (Figure 15a, b).

3.4.6. Nucleotide Sequence Accession Numbers

The partial FcaGHV1 nucleotide sequences obtained in this study have been deposited in the DDBJ, EMBL and GenBank databases under accession numbers LC331812–LC331842 for *gB* and LC384804–LC384820 for *DNApol.*

3.5. Discussion

This is the first study to report the detection of FcaGHV1 DNA in TLCs (*Prionailurus bengalensis euptilurus*). Detection of FcaGHV1 DNA in felids other than the domestic cat—its natural host—suggest that FcaGHV1 can be transmitted to other feline species. A qPCR assay was the first diagnostic tool for FcaGHV1 DNA detection since the discovery of the virus in domestic cats (Troyer et al., 2014). Even though the sensitivity of qPCR has not yet been fully evaluated and it is thought to usually underestimate the true prevalence of FcaGHV1 infection (Beatty et al., 2014), the majority of previous studies have used this assay. However, the recent development of new indirect ELISAs has enabled not only rapid diagnosis of FcaGHV1 infection but also more sensitive detection of viral exposure (Stutzman-Rodriguez et al., 2016).

FcaGHV1 DNA detection in TLCs and domestic cats on Tsushima Island provided additional data on the GHV distribution in Japan. Recently, a similar molecular epidemiological study was conducted in Japan to investigate the prevalence and risk factors of FcaGHV1 infection in domestic cats (Tateno et al., 2017). I decided to extend and include Tsushima Island in this survey of GHV infection because TLCs only live in this region and evidence of FcaGHV1 transmission from domestic cats to TLCs may affect the management strategies that are in place to protect this endangered species.

The frequency of FcaGHV1 detection in Tsushima domestic cats (13.02%) was similar to previously reported frequencies of between 9.6% and 23% in Singapore, Australia, Central Europe (Germany and Austria), the USA and Brazil (Troyer et al., 2014; Beatty et al., 2014; Ertl et al., 2015; Kurissio et al., 2018), and higher than the prevalence (1.3%) reported in other parts of Japan (Tateno et al., 2017). The prevalence and risk of FcaGHV1 infection varies among countries and geographical locations (Troyer et al., 2014; Stutzman-Rodriguez et al., 2016). Even though there

was no significant difference between the frequency of FcaGHV1 infection in Kamijima and Shimojima, FcaGHV1-positive domestic cats were predominantly found in the western zone of the island.

Being a male was found to be a strongly significant risk factor for FcaGHV1 infection—this supports the previous evidence that FcaGHV1 transmission corresponds with increased aggressive encounters (Beatty et al., 2014; Stutzman-Rodriguez et al., 2016; McLuckie et al., 2016a; Kurissio et al., 2018).

The observation that FeLV infection was not associated with an increased likelihood of FcaGHV1 DNA detection is congruent with previous reports (Beatty et al., 2014; Tateno et al., 2017; McLuckie et al., 2017; Kurissio et al., 2018) but contrasts with specific findings from Singapore where FeLV infection was significantly associated with FcaGHV1 detection (Beatty et al., 2014). On Tsushima Island, the prevalence rates of FeLV infection in Kamijima and Shimojima were 3.1% and 16.2%, respectively (Makundi et al., 2017). However, data from epidemiological survey of feline viruses in Singapore, and of particular interest FeLV, were inconsistent (McLuckie et al., 2017). Furthermore, it has been established that FeLV is primarily transmitted through non-aggressive interactions or vertical transmission, in contrast to the tendency of FcaGHV1 to be transmitted through fighting or territorial aggression (Beatty et al., 2014; Carver et al., 2015; Ertl et al., 2015; McLuckie et al., 2016a; 2017). Additionally, regional differences, different methodological approaches, and the characteristics of the study populations may account for the observed disparity between our results and those found in Singapore.

I found no significant association between increased FcaGHV1 detection and FIV infection. However, in most previous studies the probability of FcaGHV1 detection was significantly higher

in FIV-positive cats than in FIV-negative cats and FcaGHV1 viral loads were significantly higher in FIV-infected than non-infected cats (Beatty et al., 2014; Ertl et al., 2015; McLuckie et al., 2017; Kurissio et al., 2018). A possible explanation for this difference between my results and those of others is the different methodological approaches and the characteristics of the study population. For example, previous reports were based on well-defined age- and sex matched control populations (Beatty et al., 2014; Ertl et al., 2015; McLuckie et al., 2017) contrary to the current study where only data on individuals' sex were available. While the detection of FIV provirus DNA was conducted by qPCR (Ertl et al., 2015; Kurissio et al., 2018), I employed a serological FIV antibody test. In Singapore, where a large street cat population dominates and lives in free-roaming colonies, the possibility of FIV infection is likely to be high as the virus is spread through biting and fighting (Beatty et al., 2014). The characteristics of samples recruited for investigation also influence the results. For example, in Brazil, about 50% of FIV-positive cats were positive for FcaGHV1 due to the fact that all samples included in the study were collected from domestic cats with suspected infectious disease (Kurissio et al., 2018).

Comparison of the FcaGHV1 *gB* sequences in the 31 isolates obtained in this study with previously reported strains, detected nucleotide variations at positions 126, 323 and 420. Similarly, in Singapore, all FcaGHV1 *gB* isolates had thymidine in place of cytosine at nucleotide 126 of the KF840715 sequence (Beatty et al., 2014). In another study, one out of six Austrian isolates had guanine in place of adenine at nucleotide position 249 of the KF840715 sequence (Ertl et al., 2015). In the current study, 11 of 31 isolates were 100% identical to KF840715. Furthermore, previous studies have found that the FcaGHV1 partial *gB* sequences of 12 German, five Austrian, 10 Australian and 23 Japanese isolates were also identical to those found in the USA. This indicates that the FcaGHV1 *gB* gene is relatively conserved despite a few nucleotide polymorphisms.

The isolation of FcaGHV1 from TLCs is important not only to the welfare and health management of TLCs but also because it indicates that the virus may infect feline species other than its natural host. Close interactions between free-roaming domestic cats and TLCs have led to the interspecies transmission of several diseases including parasites and FIV (Tateno et al., 2013a; Nishimura et al., 1999a). These reports indicate that domestic cats play an important role in disease transmission to TLCs. Even though I found no TLCs positive for FeLV infections, I demonstrated that the virus can replicate in the cells of TLCs, which suggests that cross-species transmission is possible (Makundi et al., 2017). The higher prevalence of FcaGHV1 in Tsushima domestic cats than TLCs, and the location of the virus in both TLCs and domestic cats within the same genetic cluster, suggests that transmission from domestic cats to TLCs is likely.

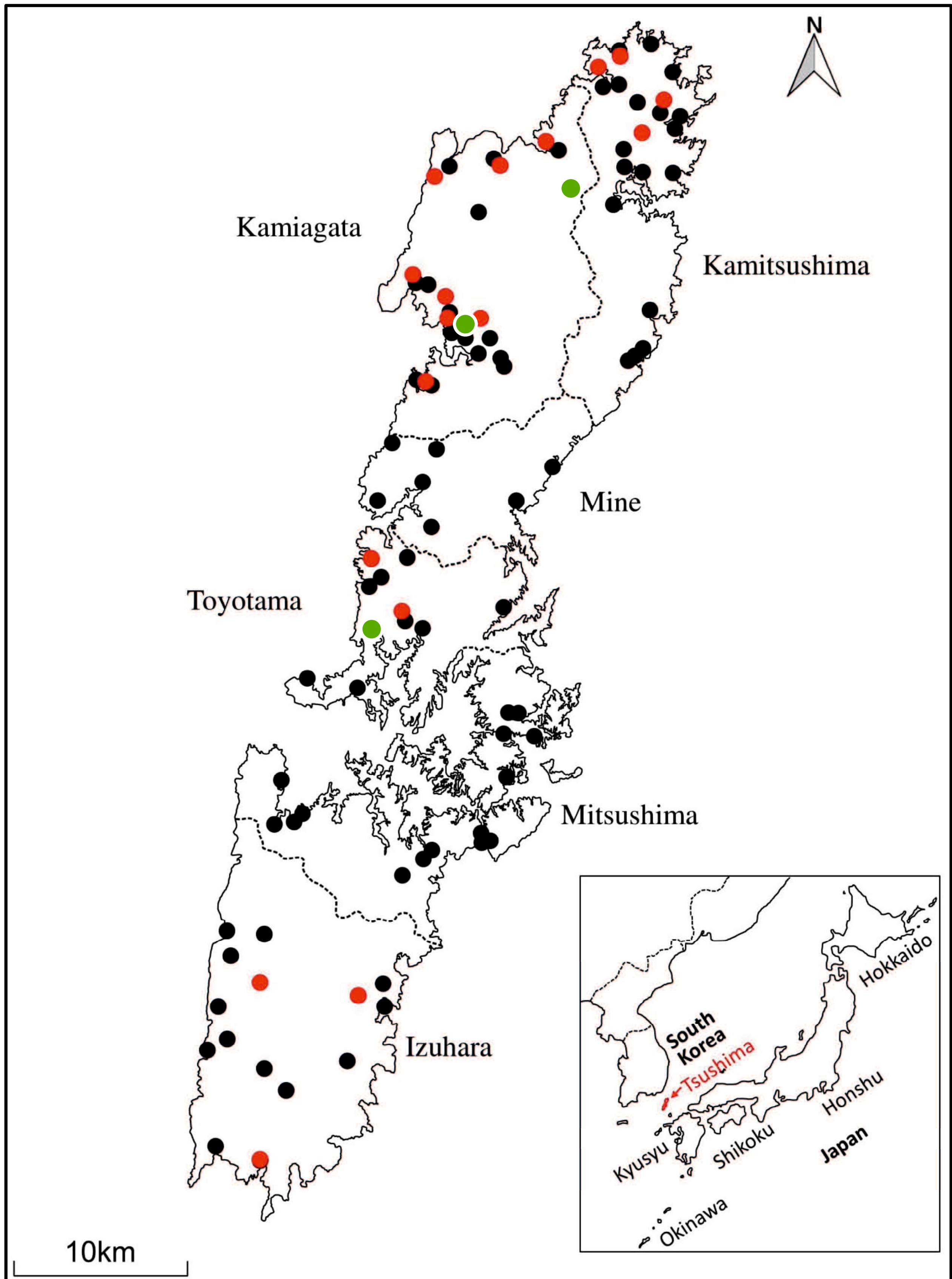

N
Kamiagata
Kamitsushima
Mine
Toyotama
Mitsushima
Izuhara
10km
South Korea
Tsushima
Hokkaido
Honshu
Kyusyu
Shikoku
Japan
Okinawa

Figure 13: Map of Tsushima Island showing sample collection sites, the locations of FcaGHV1-positive TLCs and domestic cats. Black dots indicate the sites where blood sampling of domestic cats was performed. The locations of FcaGHV1-positive TLCs are shown by green dots. Sites where FcaGHV1-positive domestic cats were detected are shown in red. The location of Tsushima Island is shown in the box.

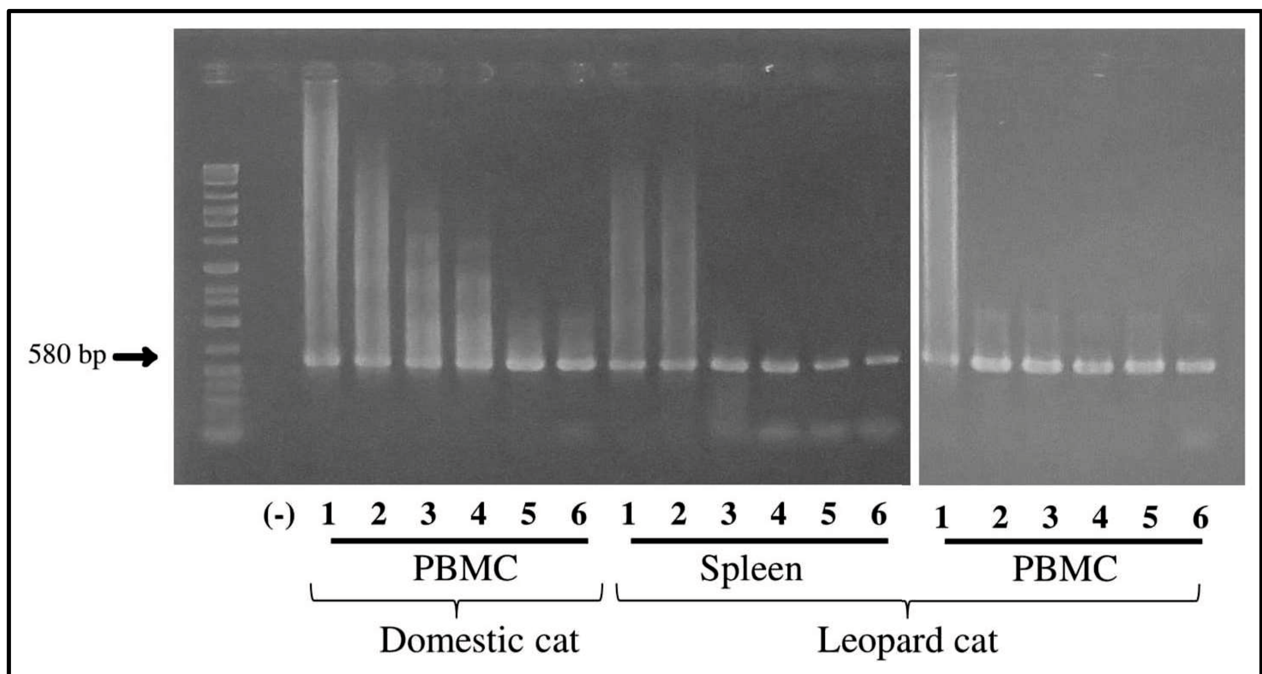


Figure 14: Determination of the sensitivity of FcaGHV1 *gB* nested PCR. Second-round FcaGHV1 PCR products from PBMCs of a domestic cat and the spleen and PBMCs of a Tsushima leopard cat. bp = base pairs; (-) = FcaGHV1 negative PBMC control; 1 = 1 μg DNA used in first round; 2 = 0.1 μg DNA; 3 = 1×10^{-2} μg DNA; 4 = 1×10^{-3} μg DNA; 5 = 1×10^{-4} μg DNA; 6 = 1×10^{-5} μg DNA.

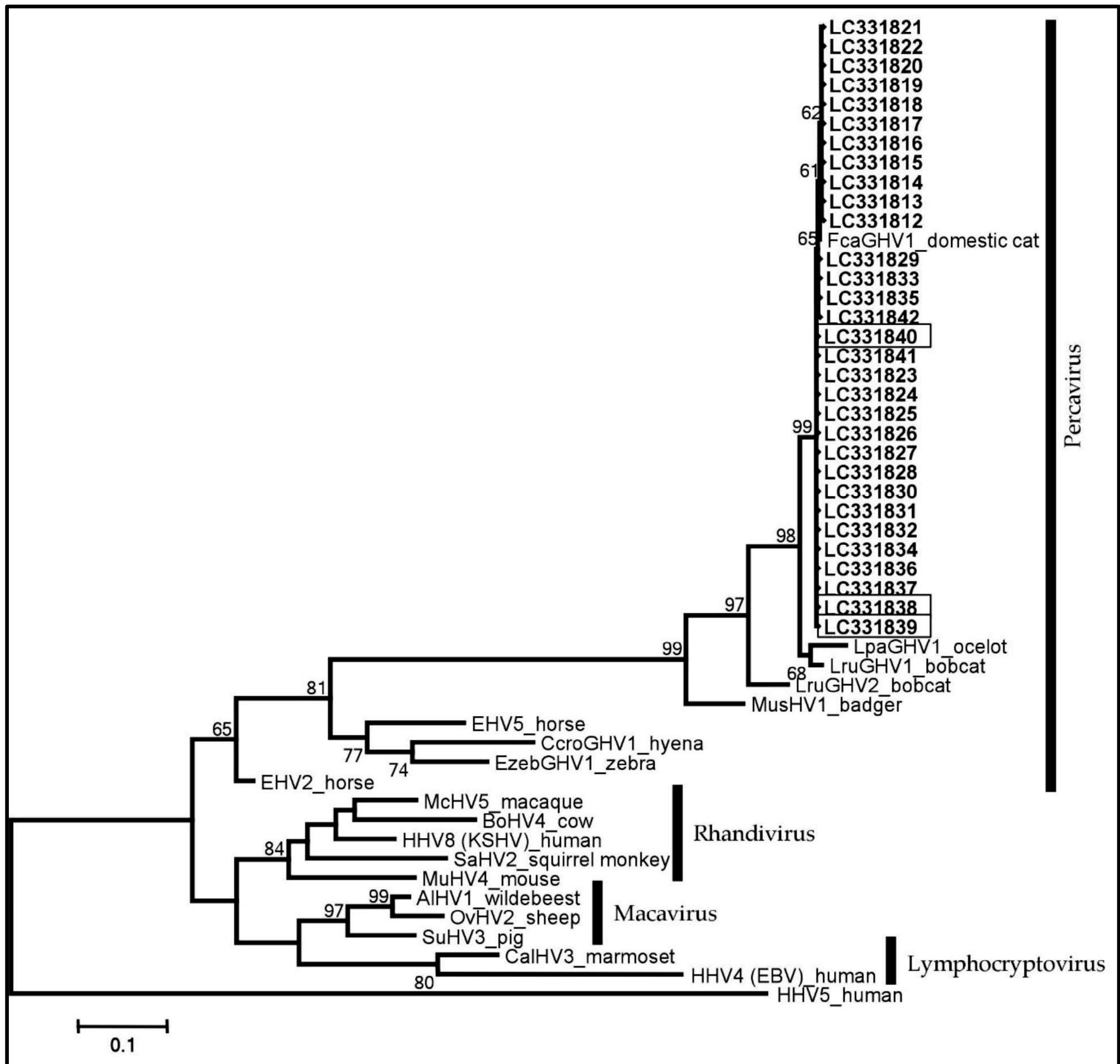


Figure 15a

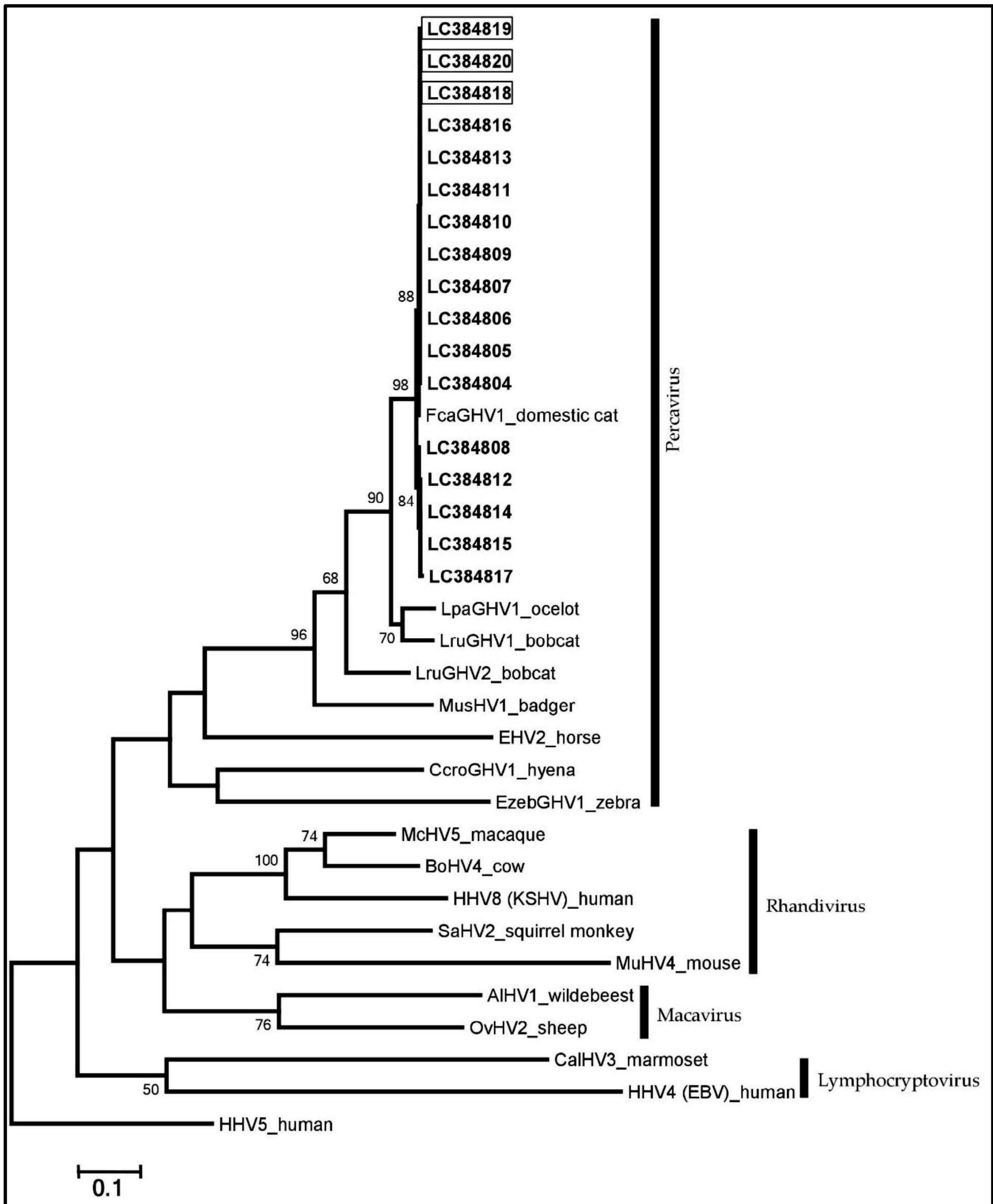


Figure 15b

Figure 15: Phylogenetic analysis of gammaherpesviruses using *glycoprotein B* and *DNA polymerase* nucleotide alignments. The trees display multiple GHV nucleotide sequences, and betaherpesvirus human cytomegalovirus (human herpesvirus 5; GenBank accession no. NC006273) represents the outgroup. (**a**) The 31 clones of FcaGHV1 gB detected in this study are shown in bold (LC331812–LC331842) including three clones isolated from TLCs (indicated with boxes); (**b**) The 17 clones of FcaGHV1 DNApol identified in this study are shown in bold (LC384804–LC384820) including three clones isolated from TLCs (indicated with boxes). Maximum-likelihood analysis was performed, and the Kimura 2-parameter model was used to calculate the distance matrix of the aligned sequences, with all branch lengths measured as the number of substitutions per site. Bootstrap support out of 100 replicates is shown for each branch node (values of <50 are not displayed). Virus names, definitions and the GenBank accession numbers of the sequences used in the phylogenetic trees are as follows: Human herpesvirus 4 (HHV4, Epstein–Barr virus, NC007605); *Callitrichine herpesvirus* 3 (CalHV3, NC004367); *Alcelaphine herpesvirus* 1 (AlHV1, NC002531); *Ovine herpesvirus* 2 (OvHV2, NC007646); *Mustelid herpesvirus* 1 (MusHV1, AF376034); *Felis catus* gammaherpesvirus 1 (FcaGHV1, KF840715); *Lynx rufus* gammaherpesvirus 1 (LruGHV1, KF840716); *Leopardus pardalis* (LpaGHV1, KP721220); *Lynx rufus* gammaherpesvirus 2 (LruGHV2, KP721221); *Bovine herpesvirus* 4 (BoHV4, NC002665); *Saimiriine herpesvirus* 2 (SaHV2, NC001350); *Suid herpesvirus* 3 (AF478169); *Equid herpesvirus* 2 (EHV2, NC001650); *Equid herpesvirus* 5 (EHV5, AF050671); *Crocuta crocuta* gammaherpesvirus 1 (CcroGHV1, DQ789371); *Equus zebra* gammaherpesvirus 1 (EzebGHV1, AY495965); *Macacine herpesvirus* 5 (McHV5, NC003401); Human herpesvirus 8 (HHV8, Kaposi's sarcoma-associated herpesvirus, NC009333) and *Murid herpesvirus* 4 (MuHV4, NC001826).

```
USA     1  ccaggtccagacaacatatgctactccagacccatcgtcacattcagattcaaaaacggt  60
TLC     1  CCAGGTCCAGACAACATATGCTACTCCAGACCCATCGTCACATTCAGATTCAAAAACGGT  60
TDC1    1  CCAGGTCCAGACAACATATGCTACTCCAGACCCATCGTCACATTCAGATTCAAAAACGGT  60
TDC2    1  CCAGGTCCAGATAACATATGCTACTCCAGACCCATCGTCACATTCAGATTCAAAAACGGT  60
TDC3    1  CCAGGTCCAGACAACATATGCTACTCCAGACCCATCGTCACATTCAGATTCAAAAACGGT  60

USA    61  actgacatcttcactggacaactgggccctagaaatgaaattctgttatcaacaaactta  120
TLC    61  ACTGACATCTTCACTGGACAACTGGGCCCTAGAAATGAAATTCTGTTATCAACAAACTTA  120
TDC1   61  ACTGACATCTTCACTGGACAACTGGGCCCTAGAAATGAAATTCTGTTATCAACAAACTTA  120
TDC2   61  ACTGACATCTTCACTGGACAACTGGGCCCTAGAAATGAAATTCTGTTATCAACAAACTTA  120
TDC3   61  ACTGACATCTTCACTGGACAACTGGGCCCTAGAAATGAAATTCTGTTATCAACAAACTTA  120

USA   121  gtggagacttgtagagactcagctgttcactactttcagtcaggacatcaaatgcacaag  180
TLC   121  GTGGAGACTTGTAGAGACTCAGCTGTTCACTACTTTCAGTCAGGACATCAAATGCACAAG  180
TDC1  121  GTGGAGACTTGTAGAGACTCAGCTGTTCACTACTTTCAGTCAGGACATCAAATGCACAAG  180
TDC2  121  GTGGAGACTTGTAGAGACTCAGCTGTTCACTACTTTCAGTCAGGACATCAAATGCACAAG  180
TDC3  121  GTGGAGACTTGTAGAGACTCAGCTGTTCACTACTTTCAGTCAGGACATCAAATGCACAAG  180

USA   181  tatgtaaactatcaacataaaagcacaacagatattcagaatttttcaactctcaacacc  240
TLC   181  TATGTAAACTATCAACATAAAAGCACAATAGATATTCAGAATTTTTCAACTCTCAACACC  240
TDC1  181  TATGTAAACTATCAACATAAAAGCACAATAGATATTCAGAATTTTTCAACTCTCAACACC  240
TDC2  181  TATGTAAACTATCAACATAAAAGCACAACAGATATTCAGAATTTTTCAACTCTCAACACC  240
TDC3  181  TATGTAAACTATCAACATAAAAGCACAACAGATATTCAGAATTTTTCAACTCTCAACACC  240

USA   241  ttcataactttaaacctaacattcattgaaaacattgactttgaggtagtcgaattgtat  300
TLC   241  TTCATAACTTTAAACCTAACATTCATTGAAAACATTGACTTTGAGGTAGTCGAATTGTAT  300
TDC1  241  TTCATAACTTTAAACCTAACATTCATTGAAAACATTGACTTTGAGGTAGTCGAATTGTAT  300
TDC2  241  TTCATAACTTTAAACCTAACATTCATTGAAAACATTGACTTTGAGGTAGTCGAATTGTAT  300
TDC3  241  TTCATAACTTTAAACCTAACATTCATTGAAAACATTGACTTTGAGGTAGTCGAATTGTAT  300

USA   301  tccaaggaagaaaaaagattggctaacgtactggacatagaaagcatgtttagagaatat  360
TLC   301  TCCAAAGAAGAAAAAAGATTGGCTAACGTACTGGACATAGAAAGCATGTTTAGAGAATAT  360
TDC1  301  TCCAAAGAAGAAAAAAGATTGGCTAACGTACTGGACATAGAAAGCATGTTTAGAGAATAT  360
TDC2  301  TCCAAGGAAGAAAAAAGATTGGCTAACGTACTGGACATAGAAAGCATGTTTAGAGAATAT  360
TDC3  301  TCCAAAGAAGAAAAAAGATTGGCTAACGTACTGGACATAGAAAGCATGTTTAGAGAATAT  360

USA   361  aactactacacacaaaggttgtcaggtattagcaaggagttacacaacaccgttgaaaac  420
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TDC1  361  AACTACTACACACAAAGGTTGTCAGGTATTAGCAAGGAGTTACACAACACCGTTGAAAAC  420
TDC2  361  AACTACTACACACAAAGGTTGTCAGGTATTAGCAAGGAGTTACACAACACCGTTGAAAAC  420
TDC3  361  AACTACTACACACAAAGGTTGTCAGGTATTAGCAAGGAGTTACACAACACCGTTGAAAAC  420

USA   421  aacagggatgcaattattaggcagtttggaaacatccttcaggatcttggaaatattggt  480
TLC   421  AACAGGGATGCAATTATTAGGCAGTTTGGAAACATCCTTCAGGATCTTGGAAATATTGGT  480
TDC1  421  AACAGGGATGCAATTATTAGGCAGTTTGGAAACATCCTTCAGGATCTTGGAAATATTGGT  480
TDC2  421  AACAGGGATGCAATTATTAGGCAGTTTGGAAACATCCTTCAGGATCTTGGAAATATTGGT  480
TDC3  421  AACAGGGATGCAATTATTAGGCAGTTTGGAAACATCCTTCAGGATCTTGGAAATATTGGT  480

USA   481  tctgtggttgtgaatgtagccagtggggtatttaccctatttggttctgttgttacaggc  540
TLC   481  TCTGTGGTTGTGAATGTAGCCAGTGGGGTATTTACCCTATTTGGTTCTGTTGTTACAGGC  540
TDC1  481  TCTGTGGTTGTGAATGTAGCCAGTGGGGTATTTACCCTATTTGGTTCTGTTGTTACAGGC  540
TDC2  481  TCTGTGGTTGTGAATGTAGCCAGTGGGGTATTTACCCTATTTGGTTCTGTTGTTACAGGC  540
TDC3  481  TCTGTGGTTGTGAATGTAGCCAGTGGGGTATTTACCCTATTTGGTTCTGTTGTTACAGGC  540

USA   541  cttatcaatttta  553
TLC   541  CTTATCAATTTTA  553
TDC1  541  CTTATCAATTTTA  553
TDC2  541  CTTATCAATTTTA  553
TDC3  541  CTTATCAATTTTA  553
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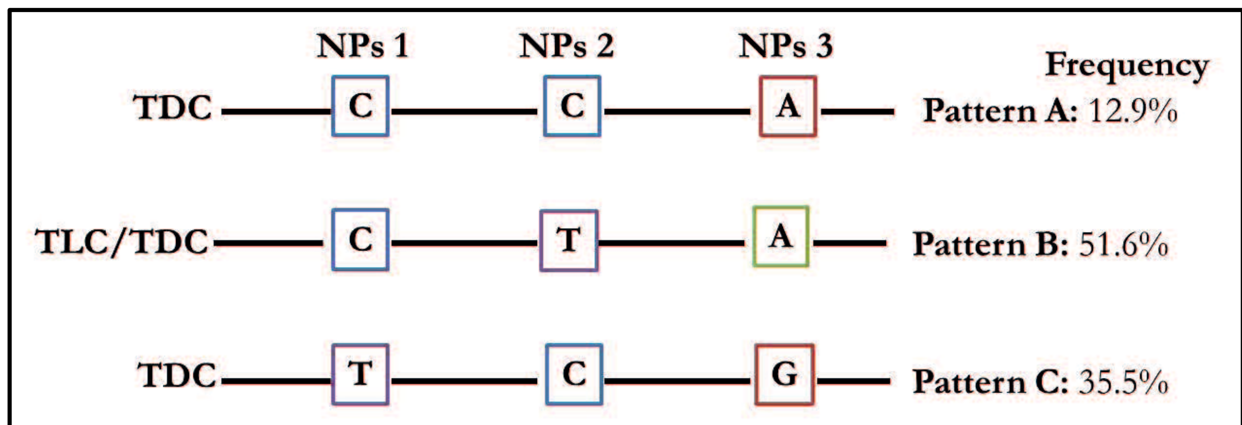


Figure 16: Nucleotide sequences alignment of partial FcaGHV1 gB gene in TLCs and domestic cats. Three positions of nucleotide polymorphisms (NPs) were detected, SNP1, SNP2 and SNP3 (black arrows). Three patterns of FcaGHV1 strains based on NPs were identified, pattern A, B, and C. The frequency of each pattern is indicated in percentage. USA represent the reference FcaGHV1 strain, GenBank KF840715 isolated from United States of America. TLC represent FcaGHV1 isolates from Tsushima leopard cats. TDC represent FcaGHV1 isolates from domestic cats on Tsushima Island.

CHAPTER THREE

4.0. *Felis catus* Gammaherpesvirus 1 (FcaGHV1) detection in Feline lymphoma/leukemia samples investigated for B-or T- lymphocyte Clonality

This study hasn't yet published.

4.1. Abstract

Felis catus Gammaherpesvirus 1 (FcaGHV1) is a panlymphotropic gammaherpesvirus that infects domestic cats and can be transmitted to other closely related feline species. FcaGHV1 DNA have been detected in B- and T- lymphocytes in peripheral blood of infected, asymptomatic cats. Studies to examine broad range of tissues and involving large group of cats including Feline immunodeficiency virus (FIV) -uninfected cats are essential to understand the FcaGHV1 pathobiology. I investigated FcaGHV1 DNA in feline lymphoma/leukemia samples that were submitted for B-lymphocyte and or T-lymphocyte clonal analysis by molecular techniques. In an assay designed to detect FcaGHV1 glycoprotein B (*gB*) gene by nested PCR, 9 out of 175 samples examined 5.1% (95% confidence interval [CI], 2.38%–9.54%) were positive. FIV positive tumors were significantly associated with FcaGHV1 detection (P= 0.0049). Feline leukemia virus infection were not associated with FcaGHV1 detection (P= 0.2745). Sequence analysis of partial FcaGHV1 *gB* gene revealed 99% identity with previously reported studies. These results demonstrate that FcaGHV1 DNA can be detected in various feline lymphoma/leukemia tissues including blood, lymph node, effusions, biopsies, spleen, intestine and peritoneal masses.

4.2. Introduction

Lymphoma is the most common hematopoietic malignancies in domestic cats. Feline immunodeficiency virus (FIV) infected cats have a 5–6 fold increased risk of developing lymphoid malignancies compared to uninfected cats (Shelton et al., 1990). On the other hand, it has been reported that *Felis catus* gammaherpesvirus 1 (FcaGHV1) infection and the viral load is significantly higher in FIV-infected cats than uninfected cats (Beatty et al., 2014; Ertl et al., 2015). While the pathogenicity of FcaGHV1 and its involvement in lymphomagenesis is under active investigation, the association between FcaGHV1 infection and other retroviruses such as Feline leukemia virus and FIV have been reported (Ertl et al., 2015; McLuckie et al., 2018). In addition to that detection of FcaGHV1 DNA in B and T lymphocytes may raise the possibility of an association between viral latency and feline lymphoproliferative disorders (McLuckie et al., 2016b). To begin the search for the potential roles of FcaGHV1 in tumorigenesis, identifying the FcaGHV1 latency-associated transcripts in cancer cells is an essential step. In their study, Aghazadeh et al. (2018) identified limited repertoire of FcaGHV1 transcripts in five tumors, including homologs of oncogenic latency-associated transcripts, latency-associated nuclear antigen, and lytic genes.

Investigation of tissue tropism for FcaGHV1 DNA loads was conducted in three adult FIV-infected domestic cats. FcaGHV1 exhibits the ability to infect a broad range of tissues, however displays putative tropism for the small intestine prompting its significance in viral shedding or pathogenesis (Beatty et al., 2014). Studies to examine broad range of tissues and involving large group of cats including FIV-uninfected cats are essential to understand the FcaGHV1 pathobiology. I investigated several feline tissues submitted for clinical tests of clonal lymphocyte growth from private veterinary hospitals in Japan to identify FcaGHV1 DNA and evaluated the association between FcaGHV1 detection and FeLV and FIV co-infections.

4.3. Materials and Methods

4.3.1. Ethical Approval

This study was approved by the University of Yamaguchi Animal Ethics and Research Committee and conducted in accordance with the guidelines of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

4.3.2. Samples and DNA Preparation

Feline tissues such as blood, lymph node, body effusions, mass, and organs were submitted to Laboratory of Molecular Immunology and Infectious Disease, Yamaguchi University for PCR testing for FcaGHV1 detection. These samples had been submitted for tests of clonal lymphocyte growth from private veterinary hospitals in Japan. Chromosomal DNA were extracted by using a QIAmp DNA mini kit (Qiagen, Hilden, Germany) or PureLink Genomic DNA mini kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions. A total of 175 samples were available for this study. Age, sex, and clonality status data were available for all samples.

4.3.3. FeLV and FIV detection

FeLV provirus was detected by PCR as previously described (Watanabe et al., 2013; Kawamura et al., 2015). Nested PCRs were carried out for detection of *env* (V3–V5) and *gag* regions of FIV proviral DNA as previously described (Nishimura et al., 1999b; Cammarota et al., 1996).

4.3.4. FcaGHV1 DNA detection and Sequencing of FcaGHV1 *gB*

Nested PCR for FcaGHV1 DNA detection and sequencing of the viral *gB* gene have been described (Makundi et al., 2018).

4.3.5. Phylogenetic analyses

The GHV partial *gB* and *DNApol* nucleotide sequences were aligned using ClustalW 1.6 and phylogenetic analysis was performed using MEGA6 (Tamura et al., 2013). Maximum likelihood (ML) phylogenetic analyses were conducted based on the Kimura 2-parameter model with all areas containing gaps being ignored. The betaherpesvirus human cytomegalovirus (HCMV; human herpesvirus 5{HHV5}) was used as an outgroup to root the tree. Bootstrap analysis was performed with 100 iterations to evaluate the stability of the tree.

4.3.6. Prevalence and association between FcaGHV1 detection and FIV and FeLV co-infections

Prevalence of FcaGHV1 detection was estimated using maximum likelihood, with 95% confidence intervals profiled. Fisher's exact test was performed to analyze the association between FcaGHV1 detection and co-infections (FIV and FeLV). All statistical analyses were conducted using Minitab Statistical program (Minitab version 18, Minitab Inc., Shanghai, China, 2018).

4.4. Results

4.4.1. Prevalence of FcaGHV1

Samples from 175 feline tumors were available for FcaGHV1 testing (Table 9). FcaGHV1 DNA was detected in blood, lymph node, body effusions, mass, and organs from domestic cats. Overall prevalence of FcaGHV1 was 5.1% (95% confidence interval [CI], 2.38%–9.54%). FcaGHV1 DNA was detected across various specimens (Table 10).

Table 9: Contingency tables of categorical variables with outcome, FcaGHV1 detection

Variables	Categories	FcaGHV1 Status		Total	% Positive
		Positive	Negative		
Sex	Male	4	91	95	4.21
	Female	5	71	76	7.04
FIV infection	Negative	4	144	148	2.70
	Positive	5	22	27	18.52
FeLV infection	Negative	8	110	118	6.78
	Positive	1	56	57	1.75

Table 10: Summary of positive FcaGHV1 in lymphoma/leukemia specimens

Accession/ sample Id	Clonal growth	Sex	Age (years)	FIV	FeLV	Specimen
LC437912	T	M	13	+	–	Blood
LC437917	T	F	18	+	–	Intestine
LC437922	T	F	17	+	–	Lymph node
LC437923	T	F	13	–	–	Blood
LC437924	T	M	10	–	–	Blood
4956	B	F	5	–	+	Lymph node
LC437929	B	F	6	–	–	Lymph node
LC437930	T	M	7	+	–	Pleural effusion
LC437931	B	M	8	+	–	Mesenteric lymph node

4.4.2. Association between FcaGHV1 detection and co-infections

FIV positive tumors were significantly associated with FcaGHV1 detection (Fisher's Exact, p=0.0049) whereas FeLV infection were not associated with FcaGHV1 detection (Fisher's Exact test, p= 0.2745).

4.4.3. FcaGHV1 *gB* sequence variation

The partial FcaGHV1 *gB* gene was detected in 8 of the 175 lymphoma/leukemia cases. DNA sequencing and BLAST analysis revealed that all the sequences were highly similar (99% identity) to FcaGHV1 accessions KT595939 and KF840715 (from USA) and isolates recently reported in Japan (Makundi et al., 2018). Partial FcaGHV1 *gB* sequences of 553 nucleotides were used for analyzing nucleotide polymorphisms. Comparison of the nucleotide sequences from the 8 FcaGHV1 *gB* sequences obtained in this study revealed one nucleotide substitution at position 126 of KF840715, in which thymidine replaced cytosine. One sequence (accession number, LC437925) had additional two substitutions at positions 160 and 379 of the KF840715. These two substitutions in this sequence might have arisen due to PCR or sequencing error and therefore considered not significance.

4.4.4. Phylogenetic relationship of gammaherpesviruses (GHVs)

I aligned the FcaGHV1 partial *gB* sequences to sequences of previously reported viruses for phylogenetic analysis. All FcaGHV1 sequence data detected in this study formed one cluster with other GHVs within the Percavirus genus (Figure 17).

4.4.5. Nucleotide sequence accession numbers

The nucleotide sequences of the partial FcaGHV1 *gB* reported in this study have been deposited in the DDBJ and GenBank databases under accession numbers LC437912 – LC437931.

4.5. Discussion

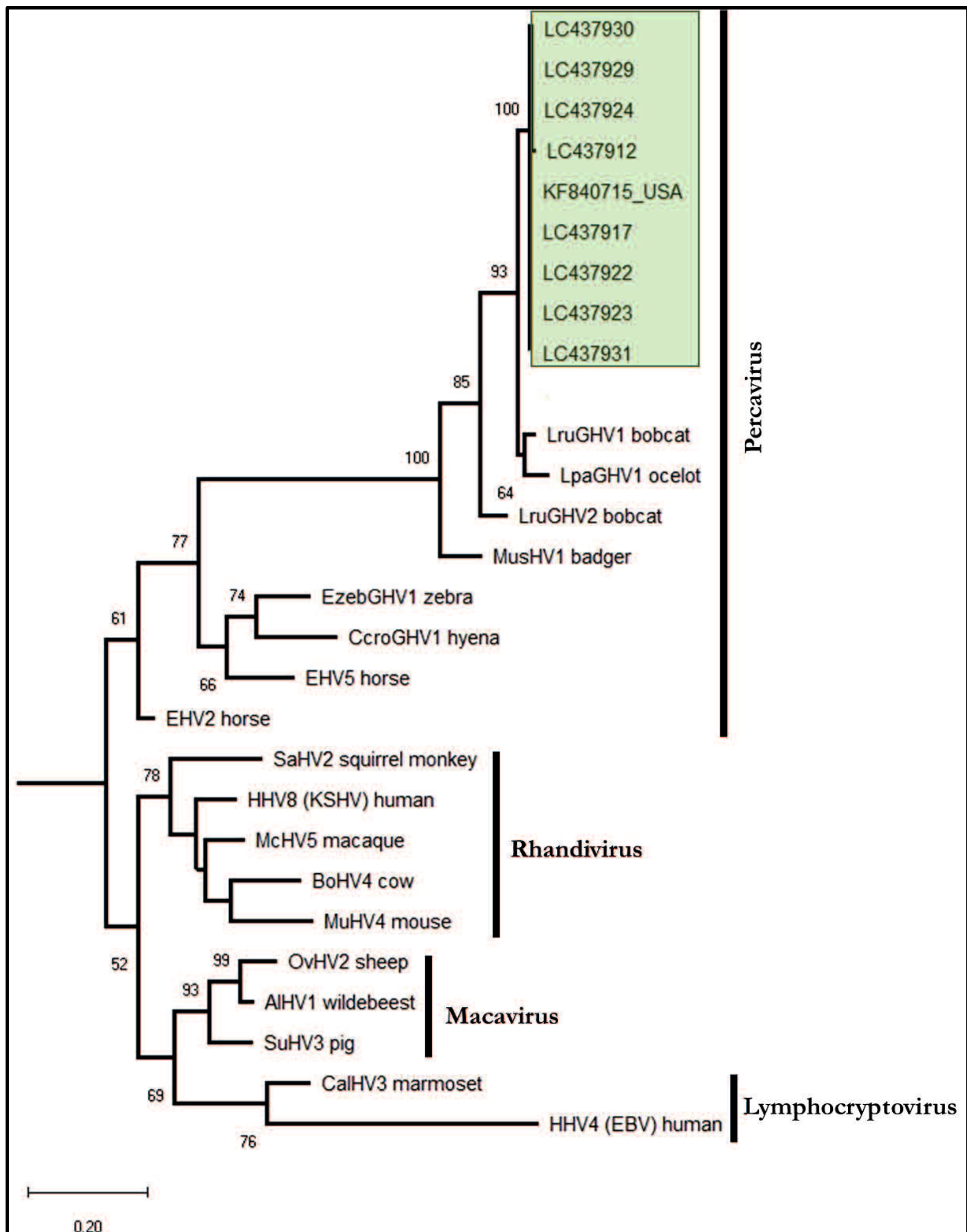
In this study I have detected FcaGHV1 DNA in various feline lymphoma/leukemia samples that were submitted for B-lymphocyte and or T-lymphocyte clonal analysis. Identification of FcaGHV1 DNA in various tissues and organs extend our understanding on the pathogenic potential and viral tissue tropism for FcaGHV1 infection. Clarifying the pathogenicity of FcaGHV1 will rely on accumulating evidence from multiple lines of investigation (Aghazadeh et al., 2018). Due to fluctuation of viral load among tissues and limit of quantitative real-time PCR (qPCR) assay detection, the use of specimen for FcaGHV1 DNA detection from only one source of tissue may hamper FcaGHV1 investigation. McLuckie et al. (2016b) emphasized that qPCR of whole blood taken on a single occasion cannot be relied upon to detect all FcaGHV1 infected cats.

The prevalence of FcaGHV1 detection reported here is consistent to previously reported frequencies of between 9.6% and 23.6% on whole blood qPCR (Troyer et al., 2014; Beatty et al., 2014; McLuckie et al., 2016a; Ertl et al. 2015; Kurissio et al., 2018). The development of new virus specific nested PCR for investigation of FcaGHV1 infection efficiently detected FcaGHV1 DNA in whole blood and spleen of Tsushima wild cats and domestic cats at the frequencies of 3.37% and 13.02% respectively (Makundi et al., 2018).

FIV-infected cats showed a significantly higher probability for being FcaGHV1 positive than uninfected cats. Previous studies reported that FIV infection increases the risk of FcaGHV1 detection

by five times compared to FIV uninfected cats matched for sex and age (Beatty et al., 2014; Ertl et al., 2015). These reports support my similar findings reported here.

FcaGHV1 DNA were detected in various feline lymphoma/leukemia of B- and T- origin. This finding is consistent with the previous report where by FcaGHV1 DNA was detected in B- and T-lymphocytes in peripheral blood of chronically infected, asymptomatic cats (McLuckie et al., 2016b). Another study conducted to explore the association between FcaGHV1 detection and feline lymphoma found no evidence of FcaGHV1 DNA being distributed preferentially in lymphoma tissue (McLuckie et al., 2018). Therefore, detection of FcaGHV1 DNA in various tissues shows that FcaGHV1 exhibits the ability to infect a broad range of tissues as exemplified by Beatty et al. (2014).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LC437924
LC437912
KF840715_USA
LC437917
LC437922
LC437923
LC437931
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LpaGHV1 ocelot
LruGHV2 bobcat
MusHV1 badger
EzebGHV1 zebra
CcroGHV1 hyena
EHV5 horse
EHV2 horse
SaHV2 squirrel monkey
HHV8 (KSHV) human
McHV5 macaque
BoHV4 cow
MuHV4 mouse
OvHV2 sheep
AlHV1 wildebeest
SuHV3 pig
CalHV3 marmoset
HHV4 (EBV) human
Percavirus
Rhandivirus
Macavirus
Lymphocryptovirus
100
93
85
100
64
77
74
61
66
78
52
99
93
69
76
0.20

Figure 17: Phylogenetic analysis of FcaGHV1 isolates and other gammaherpesviruses using glycoprotein B gene nucleotide alignments. The tree display multiple GHV nucleotide sequences, and betaherpesvirus human cytomegalovirus (human herpesvirus 5; GenBank accession no. NC006273) was used to root the tree but is not displayed due to space constraints. The 8 clones of FcaGHV1 *gB* detected in this study are shown in accession numbers. Maximum-likelihood analysis was performed, and the Kimura 2-parameter model was used to calculate the distance matrix of the aligned sequences, with all branch lengths measured as the number of substitutions per site. Bootstrap support out of 100 replicates is shown for each branch node (values of <50 are not displayed). Virus names, definitions and the GenBank accession numbers of the sequences used in the phylogenetic trees are as follows: Human herpesvirus 4 (HHV4, Epstein–Barr virus, NC007605); Callitrichine herpesvirus 3 (CalHV3, NC004367); Alcelaphine herpesvirus 1 (AlHV1, NC002531); Ovine herpesvirus 2 (OvHV2, NC007646); Mustelid herpesvirus 1 (MusHV1, AF376034); *Felis catus* gammaherpesvirus 1 (FcaGHV1, KF840715); *Lynx rufus* gammaherpesvirus 1 (LruGHV1, KF840716); *Leopardus pardalis* (LpaGHV1, KP721220); *Lynx rufus* gammaherpesvirus 2 (LruGHV2, KP721221); Bovine herpesvirus 4 (BoHV4, NC002665); Saimiriine herpesvirus 2 (SaHV2, NC001350); Suid herpesvirus 3 (AF478169); Equid herpesvirus 2 (EHV2, NC001650); Equid herpesvirus 5 (EHV5, AF050671); *Crocuta crocuta* gammaherpesvirus 1 (CcroGHV1, DQ789371); *Equus zebra* gammaherpesvirus 1 (EzebGHV1, AY495965); Macacine herpesvirus 5 (McHV5, NC003401); Human herpesvirus 8 (HHV8, Kaposi's sarcoma-associated herpesvirus, NC009333) and Murid herpesvirus 4 (MuHV4, NC001826).

5.0. GENERAL DISCUSSION AND CONCLUSION

5.1. Discussion

Infectious diseases negatively affect the health and production of livestock and wild animals. On the other hand, animal pathogens (zoonoses) that are transmitted to humans may not only cause health problems but also interferes economic and social growth of mankind. Both livestock and wildlife can be infected by pathogens that are capable to be transmitted among similar or different animal species leading to devastating effects on the livelihood of livestock owners and animals themselves. Infectious diseases spill-over from domestic animals to wildlife or vice versa significantly impair health and production of non-adapted host. For the small population of endangered animal species the risk of infectious diseases may accelerate its extinction. Increased human activities such as agriculture, deforestation, development projects including roads construction, housing, etc. have caused not only loss of habitat to wildlife but also enhances the contact between the wildlife and domestic animals. This interaction between wildlife and domestic animals has been the main pathway of disease transmission.

Now, identifying and characterizing wildlife and domestic animal diseases, defining the diseases transmission dynamics and proposing the management strategies on Tsushima Island, Japan is the main focus of this Dissertation.

Tsushima leopard cat (TLC) is a small wild cat inhabiting Tsushima Island, Nagasaki, Japan. TLC is classified as a critically endangered species due to drastic decrease of its population caused by several factors including habitat loss (deforestation), road kills, and infectious diseases (Izawa et al., 2009). Management strategies including breeding have been implemented to maintain the existence of the species (Mitani et al., 2009). Several diseases and pathogens have been reported to infect TLC including feline immunodeficiency virus (FIV), protozoans, hemoplasmas, and several species of

helminthes. There are cumulative evidence that most of these infections were originated from domestic cats and they were horizontally transmitted to TLC.

The first chapter of this dissertation reported about Feline leukemia virus (FeLV) infections in domestic cats and TLCs on Tsushima Island. The prevalence of FeLV infections in domestic cats was 6.4% based on FeLV antigen p27. FeLV infections were not detected in TLC by either FeLV antigen p27 or PCR. There could be several reasons as to why I didn't find positive TLC. First, TLCs were true negative and were not infected with FeLV. Secondly, TLCs could have been infected but were able to clear the infection. In this regard, FeLV p27 antigen test would provide negative results indicating absence of antigenemia (viraemia). Third, TLCs could have significantly lower FeLV provirus loads to be detected by PCR (Hofmann-Lehmann et al., 2001).

After I identified no FeLV infections in TLCs I hypothesized that FeLV could potentially infects TLCs. Now, to test this hypothesis I conducted infection assay in vitro. I infected primary skin fibroblasts from TLCs with two different strains of FeLVs, FeLV-A and FeLV-B. The TLC fibroblast cells were susceptible to both viral strains indicating that FeLV could replicate in TLC cells. These results suggest that there is high probability of cross-species transmission of FeLV infections between the two cats population. The primary route by which FeLV is transmitted is thought to be via oronasal exposure to virus-containing secretions. High levels of FeLV are present in the saliva of viraemic cats. However, since the virus is relatively labile in the environment, it is thought that intimate contact between animals during grooming, sharing feeding bowls or through fighting are the most likely routes of transmission (Willett and Hosie, 2013). Previous studies reported that TLCs were infected by FIV that were transmitted from domestic cats. Therefore, the possibility of FeLV transmission from domestic cats to TLCs remain to be high.

Sequence analysis and phylogenetic analyses of FeLV infections in domestic cats on Tsushima Island revealed that all FeLV isolates belong to Genotype I clade 3 which is prevalent and widespread on Kyushu, Japan indicating that FeLV strains on Tsushima may have originated in Kyushu. Furthermore, FeLVs strains on Tsushima were clearly separated into two areas according to geographical regions. FeLV Genotype I clade 3–1 was found in Kamijima while Genotype I clade 3–2 was circulating in Shimojima. These results suggest that FeLV on Tsushima could have been transmitted at least twice in the past however, the exact time of arrival of these FeLV strains are unknown. The source of FeLV infections on Tsushima Island could be explained by how does the Tsushima is connected with other regions. Tsushima is connected by ship and air transportation. Sea routes link the ports of Hitakatsu and Izuhara from the side of Tsushima and port of Hakata in Fukuoka. Similarly, air transport connect Tsushima airport and Fukuoka and Nagasaki airports. This connection method between Tsushima and Fukuoka and or Nagasaki may probably be the main source of FeLV on Tsushima since FeLV strains from Tsushima were of the same genotype and clade as those circulating in Kyushu region. On the other hand, Tsushima Island is also connected with South Korea mainly through ship/ferries transportation. Therefore, FeLV transmission from South Korea is also likely to occur. However, this method of connection may face tough check and control such as restrictions on animal movement between the two countries.

The second chapter of this dissertation explains about identification of *Felis catus* gammaherpesvirus 1 (FcaGHV1) in TLCs on Tsushima Island. The main reason why I decided to conduct gammaherpesviruses (GHVs) survey in TLCs is the presence of relationship between FcaGHV1 and FIV infections. FIV infection carries significant risk of FcaGHV1 DNAemia with median loads up to 5 times higher than in retrovirus uninfected controls (McLuckie et al., 2017). Based on the previous reports, FIV were detected in TLCs and its prevalence was significantly higher in domestic

cats on Tsushima Island compared to other regions of Japan (Hayama et al., 2010). In this regard, I hypothesized that TLCs were at similar high risk of being infected with GHVs. To test this hypothesis, I developed new FcaGHV1 virus-specific nested PCR system to detect GHVs in TLCs. FcaGHV1 DNA was detected in 3 out of 89 TLCs investigated. For the purpose of TLCs management and determining where could be the source of FcaGHV1 infection in TLCs, I tested domestic cats on Tsushima Island and I found that 28 out of 215 were positive for FcaGHV1 DNA.

Sequence analysis and phylogenetic analyses revealed that FcaGHV1 strains in TLCs and domestic cats were of the same identity. On nucleotide sequence alignments, all three positive TLCs had similar nucleotide sequences forming one FcaGHV1 pattern which they also shared with domestic cats (Figure 16). Additionally, two different patterns of FcaGHV1 strains were found only in domestic cats (Figure 16). These results demonstrate that domestic cats harbor all three patterns of FcaGHV1 strains probably due to the fact that domestic cats is the natural host of this virus. One pattern of FcaGHV1 strain was transmitted from domestic cats to TLCs. The likelihood of FcaGHV1 transmission from domestic cats to TLCs is supported by the following findings; first, FcaGHV1 was identified in domestic cats which is the natural host of this infection, the high frequency of FcaGHV1 DNA detection in domestic cats than in TLCs suggest that the infections is endemic in domestic cats, and lastly, TLCs and domestic cats FcaGHV1 strains formed one genetic cluster on phylogenetic analyses.

The third chapter of this dissertation was the study about FcaGHV1 DNA detection in feline lymphoma/leukemia samples that were submitted for investigation of B- or T-lymphocyte clonal growth. FcaGHV1 is a panlymphotropic GHVs and have been detected in B- and T- lymphocytes in peripheral blood of FcaGHV1 chronically infected cats (McLuckie et al., 2016b). Feline lymphoma remains to be the most common malignancy of domestic cats. Studies engaged to explore the

association between lymphoma and various etiologies and of specific interest, FcaGHV1 are of significant importance for the welfare of domestic cats. FcaGHV1 DNA was detected in feline blood, lymph node, effusions, biopsies, spleen, intestine and peritoneal masses. These results suggests that FcaGHV1 DNA is exclusively distributed in lymphoma/leukemia tissues of B- and T-lymphocyte origin. Co-infection with FIV was found to be the risk factors for FcaGHV1 detection. Previous studies also reported higher viral loads in co-infected cats suggesting the pathogenic interactions between FcaGHV1 and FIV (Ertl et al., 2015).

5.2. Conclusion and Recommendation

The majority of Tsushima Island is private land, therefore the cooperation of landowners is essential in preventing the spread of FeLV infections from domestic cats to TLCs. In particular, I recommend cat owners to keep their cats indoors so as to reduce the possibility of contact between infected domestic cats and TLCs. In addition, cat owners are advised to adhere to vaccination programs and should consider routine FeLV tests. Monitoring the TLCs population for FeLV infections is highly recommended as part of the surveillance and management strategies for TLCs. This report extends awareness of factors involved in the transmission dynamics and spread of FeLV infections which need to be considered and evaluated careful because the measures required to control diseases in wild populations are difficult and faces a lot of challenges.

The isolation of FcaGHV1 from TLCs is important not only to the welfare and health management of TLCs but also because it indicates that the virus may infect feline species other than its natural host–domestic cats. Identification of GHVs on Tsushima Island provides additional epidemiological data about the status of feline diseases as the baseline information necessary for setting up effective control measures. Furthermore, detection of FcaGHV1 DNA in TLC confer

additional health risk factor for TLCs survival. GHVs are host-specific. Detection of FcaGHV1 DNA in TLCs indicates that the virus is circulating in TLCs. Further studies of FcaGHV1, including the genetic diversity of this virus and its pathogenic potential in other feline species, are needed. In addition, as it has been previously reported that felids can be infected with more than one GHV, future studies to identify other GHVs in TLC populations are warranted.

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SUPPLEMENTARY MATERIALS

Table 11: Supplementary Table 1 showing FeLV *env* sequences used in the phylogenetic analysis

FeLV *env* gene	FeLV genotype/clade	Gene accession
AC6-3	GI/C7	AB635535
AT37-4	GII	AB635491
EH20-1	GI/C5	AB635529
FO2-1	GI/C3	AB635851
FO8-1	GI/C3	AB635854
FO10-1	GI/C3	AB635856
FO11-1	GI/C3	AB635858
FO19-3	GI/C3	AB635862
FO20-1	GI/C3	AB635863
FO26-1	GI/C3	AB635761
FO32-1	GI/C3	AB635758
FO36-1	GI/C3	AB635755
GF37-1	GI	AB635849
IK40-1	GI/C2	AB635658
KC2-1	GI/C4	AB635538
KG20-1	GI/C6	AB635640
KM1-1	GI/C3	AB635750
KM4-2	GI/C3	AB635748
KM5-2	GI/C3	AB635744
KM6-1	GI/C3	AB635742
KM7-1	GI/C3	AB635707
KM8-1	GI/C3	AB635741
KM11-3	GI/C3	AB635740
KM13-1NF	GI/C3	AB635739
KM16-2	GI/C3	AB635736
KM17-1	GI/C3	AB635705
KM18-1	GI/C3	AB635733
KM20-1	GI/C3	AB635731
KM22-1	GI/C1	AB635764
KM23-3	GI/C3	AB635727
KM25-1	GI/C3	AB635726
KM26-1	GI/C3	AB635724
KM29-1NF	GI/C3	AB635722
KM30-1	GI/C3	AB635719
KM32-1	GI/C3	AB635718
KM33-1	GI/C3	AB635715
KM34-1	GI/C3	AB635713
KM35-2	GI/C3	AB635709

KM36-1	GI/C3	AB635708
KM38-2	GI/C3	AB635704
KS2-1	GI/C2	AB635656
ME12-1	GI/C2	AB635553
MZ17-1	GI/C3	AB635829
MZ29-6	GI/C3	AB635831
MZ38-1	GI/C3	AB635842
NS1-2	GI/C3	AB635803
NS6-3	GI/C3	AB635806
NS10-4	GI/C3	AB635814
NS11-3	GI/C3	AB635816
NS17-1	GI/C3	AB635819
NG30-1	GI/C3	AB635752
NS33-2	GI/C3	AB635821
NS36-2	GI/C3	AB635825
OI10-1	GI/C3	AB635827
OY18-5	GI/C2	AB635672
ON33-1	GIII-2	AB635483
SA5-2	GI/C3	AB635693
SA11-1	GI/C3	AB635692
SA12-1	GI/C3	AB635691
SA29-3	GI/C3	AB635690
SA39-1	GI/C3	AB635689
TG22-3	GII	AB635503
TK4-1	GI/C1	AB635765
TY26-2	GI/C5	AB635514
WY15-1	GI/C1	AB635636
YG13-1	GI/C4	AB635545
FeLV-A clone33	GI/C1	AB060732
FeLV-A Glasgow1	GIII/C1	M12500
FeLV-A Rickard	GIII/C1	AF052723
FeLV-A 61E	GIII/C1	M18247
FeLV-A 945	GIII/C2	AY662447
enFeLV AGTT	None	AY364318