Molecular Epidemiology of Feline Leukaemia Virus in Chilean domestic and non-domestic felids.

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Abstract

Feline Leukaemia Virus (FeLV) is a globally distributed gamma retrovirus infecting both domestic and non-domestic felids. The main consequences of FeLV infection are hematopoietic disorders and neoplasia. While non-domestic felids can be only infected by exogenous FeLV-A, domestic cats host both exogenous and endogenous viruses (ancestral copies of virus in the cat genome), allowing recombination events that generate FeLV-B and alter viral tropism.

This study employed various approaches to characterize the FeLV epidemiology and molecular diversity of FeLV in Chilean domestic and non-domestic felids. Epidemiological modelling from two regions in south-central Chile showed a high prevalence (53%) in domestic cats. Vaccination and being sampled in 2022 were both variables associated with a lower risk of infection, whereas geographic origin negatively influenced higher infection risk. On the other hand, a Kaplan-Meier survival analysis of retroviral-positive cats from a shelter in the United Kingdom was conducted to evaluate the effect of FeLV on lifespan compared with FIV-infected and uninfected cats. This analysis demonstrated a significantly reduced lifespan in FeLV infected cats (on average 4 years old), whereas FIV-infected cats did not differ significantly from uninfected cats until advanced age.

A comparison of Sanger (direct from amplicon and cloned in plasmids) and NGS methods (Illumina and Nanopore sequencing) was performed to characterize the *env* gene in eight FeLV infected cats. All methods successfully detected FeLV-A and FeLV-B variants. Phylogenetic and recombination (RDP4) analyses revealed distinct recombination breakpoints patterns driven by enFeLV insertion within FeLV-A sequences. Regarding FeLV-A, a Chilean cluster was identified with specific variations for Chilean cats, mostly in the Receptor Binding Domain (Surface Unit). This analysis also included synonymous site conservation and predicted RNA secondary structures identifying a complex structure for FeLV-A and another more stable one for the FeLV-B strain.

To explore broader retroviral diversity, Nanopore Adaptive Sampling was employed for real-time and bioinformatic enrichment of gamma retroviruses. This approach allowed the identification of FeLV-A, enFeLV, ERV-DC, Fc-gamma4, and RDRS (RD-114 virus-related sequences) elements. This analysis also mapped LTRs to the cat genome to determine the insertion sites of each retrovirus, comparing solo-LTR or viral-LTRs. These results were consistent with previous reports reflecting the endogenization ages, with Fc-gamma4 showing predominantly solo-LTRs and RDRS detected only in a minority of samples.

Finally, deep sequencing of the *env* gene from guigna samples (small Chilean non-domestic felids) confirmed infection with FeLV-A variants. Although domestic cats are the well-known primary source of FeLV infection for guignas, unique sequence variants and intra-host diversity within guignas suggest possible host-specific adaptation and ongoing intra-species transmission.

Together, these findings enhance the understanding of FeLV epidemiology and molecular evidence of the infection in Chilean felids, reinforcing the importance of introducing stronger measures to control the disease in domestic and non-domestic felids.

List of Abbreviations

Abbreviation	Term
Ag	Antigen
ALV	Avian Leukosis Virus
ASLV	Avian Sarcoma Leukosis Virus
BaEV	Baboon Endogenous Retrovirus
Ca	Capsid
CAEV	Caprine Arthritis Encephalitis Virus
cDNA	Complementary DNA
CIS	Common Integration Site
CITES	Convention on International Trade in Endangered Species
CPV	Canine Parvo Virus
CRAD	Capping Protein Inhibiting Regulator of Actin Dynamics
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
enFeLV	Endogenous Feline Leukaemia Virus
env	Envelope
ERV	Endogenous Retrovirus
ERV-DC	Endogenous Retrovirus Domestic Cat
exFeLV	Exogenous Feline Leukaemia Virus
FcERV	Feline catus Endogenous Retrovirus
FeLV	Feline Leukaemia Virus
FeMV	Feline Morbillivirus
FISH	Fluorescent *in situ* hybridization
FIV	Feline Immunodeficiency Virus
FPaV	Feline Paramyxovirus
gag	Group Antigen Glycoprotein
GALV	Gibbon Ape Leukaemia Virus
GPU	Graphic Processing Unit
HERV	Human Endogenous Retrovirus
HIV	Human Immunodeficiency Virus
HLTV	Human T-lymphotropic virus
iSNV	Intra-host Single Nucleotide Variation
IN	Integrase
INE	Instituto Nacional de Estadistica
IS	insertion sites
IUCN	International Union for Conservation Nature
JSRV	Jaagsiekte Sheep Retrovirus
KORV	Koala Retro Virus
LTR	Long Terminal Repeat
Mya	Millions of years ago
MA	Matrix
MHC	Major Histocompatibility Complex

miRNA	Micro Ribonucleic Acid
MLV	Murine Leukemia Virus
mRNA	Messenger Ribonucleic Acid
NAS	Nanopore Adaptive Sampling
Nc	Nucleocapsid
NCBI	National Centre for Biotechnology Information
NGS	Next Generation Sequencing
nPCR	Nested Polymerase Chain Reaction
nt	Nucleotide
ONT	Oxford Nanopore Technologies
ORF	Open Reading Frame
PBS	Primer Binding Site
PcEV	Papio Cynocephalus Endogenous Retrovirus
PCR	Polymerase Chain Reaction
PERV	Porcine Endogenous Retrovirus
PIC	Pre-integration Complex
pol	polymerase
PPT	PolyPurine Tract
pro	Protease
qPCR	Quantitative Polymerase Chain Reaction
RDRS	RD Virus-Related Sequence
RNA	Ribonucleic Acid
Rt	Reverse Transcriptase
RTC	Reverse Transcription Complex
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RBD	Receptor Binding Domain
SERV	Simian Endogenous Retrovirus
STR	Short Tandem Repeats
SU	Surface Unit
TB	*Mycobacterium tuberculosis*
TE	Transposable Elements
TM	Transmembrane Protein
tRNA	Transfer Ribonucleic Acid
XRV	Exogenous Retrovirus

Chapter 1: Literature Review

1.1 Felidae Conservation

According to the IUCN (International Union for the Conservation of Nature) Red List the Order Carnivora includes 16 families, among them, one of the biggest is the Felidae family, which includes 39 species. From these wild felids, 15 species (38.5%) are categorized as “vulnerable” and four (10.3%) as “endangered” (IUCN 2025). Direct anthropic effects like hunting, roadkill and farming conflicts are the main factors in the reduction of these wild populations, indeed, the felid range distribution has been diminished by up to 90% (Wolf and Ripple 2017). Among all felids, only the Iberian Lynx (*Lynx pardinus*) are currently increasing their population. This is the result of a strong conservation program, which involved a broad approach of areas such as: habitat management, traffic regulations, education programs, animal translocations, *ex-situ* breeding and control and prevention of infectious diseases (López et al. 2009, Rodriguez 2024, Palomares et al. 2010).

The Felidae phylogeny is subdivided into eight clades (**Figure 1**), with a common ancestor 35 million years ago (Mya) inhabiting all continents except Antarctica (Johnson et al. 2006). The first clade arose 10.8 Mya, the Panthera lineage, which includes genera *Neofelis* and *Panthera*. This lineage contains bigger felids such as the tiger (*Panthera tigris*) and lion *(Panthera leo)*. Around 9.4 Mya, the bay cat lineage (genus *Pardofelis)* arose. These species are associated with the *Panthera* lineage but are incompletely studied. The third is the *Caracal* lineage that branches off 8.5 Mya, which includes *Caracal* and *Leptailurus*, being mainly African cats. The fourth lineage includes most of the South American small cats belonging to the ocelot lineage, in the genus *Leopardus.* This lineage branched off at 8.0 Mya. The next clade arising 7.2 Mya, subdivided in the next lineage that includes *Lynx.* Afterwards, the sixth clade is the *Puma* lineage which includes the *Puma* and *Acinonyx* genera, differentiated at 6.7 Mya. The last two lineages were split approximately 6.2 Mya, these are the leopard cat (genera *Otocolobus* and *Prionailurus)* and genus *Felis,* including domestic cats (**Figure 1**) (Werdelin et al. 2010).

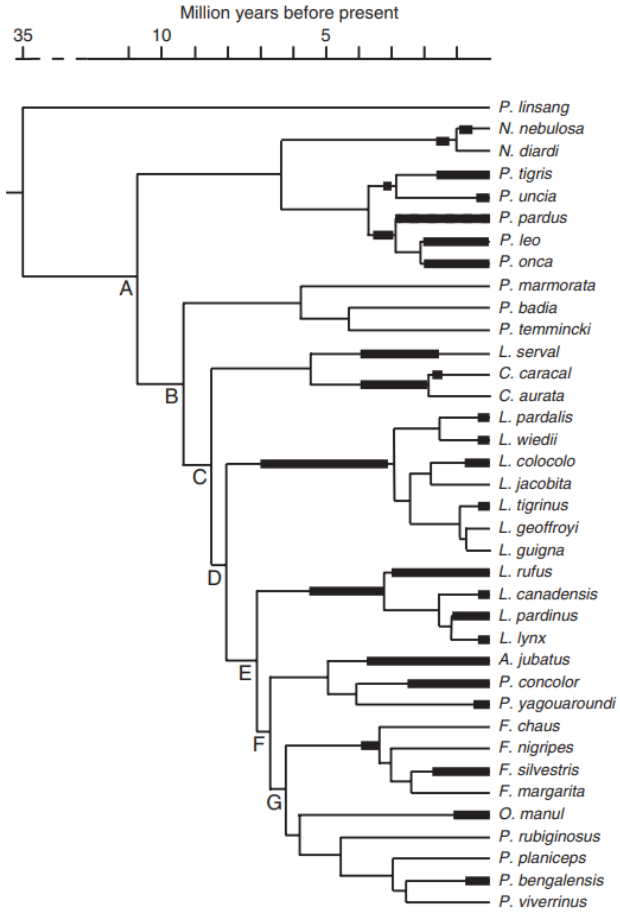

Figure 1 Phylogeny of extant felids based on autosomal DNA, Y-linked, X-linked and mitochondrial gene (22,789 bp). The letters show eight different clades that arose from a common ancestor 35 million years ago, being in order: A) Panthera lineage; B) Bay cat lineage; C) Caracal lineage; D) Ocelot lineage; E) Lynx lineage; F) Puma lineage; G) Small cats of the Old-World lineage. The broad lines show additional fossil records and thin lines the lack of fossil record. Extracted from (Werdelin et al, 2010).

Ecologically, Felidae has the top predator role being key for ecosystem conservation. Usually, they live in extensive habitats because they have higher prey requirements, with solitary organization and territorial behaviour in the majority of cases (MacDonald, Mosser, and Gittleman 2010). These characteristics make the felid family species highly prone to environmental change, specially aggravated in those populations with lower heterozygosity, namely, species with lower genetic variability and genetic bottle-neck events (O'Brien et al. 2006). The conservation difficulties associated with lower genetic diversity have been

exemplified in Florida panthers in United States, where there have been strong efforts to recover their populations, however, the reduced genetic diversity has caused continuous new challenges (van de Kerk et al. 2019). Although each felid population has different situations regarding their genetic diversity, overall, South America and Africa have higher levels of diversity than felids inhabiting Europe or North America (Azizan and Paradis 2021).

Due to diminished habitats and decreasing populations, many zoos have incorporated the concepts of the “modern zoo”, which means supporting knowledge development about wild species and supporting those highly endangered populations through reproduction plans (Deem 2015). In this scenario, zoological parks and breeding centres have developed successful *ex situ* programs to breed and release wild felids in their former habitats. They have achieved revitalization of endangered species such as the cases of the Amur leopard, Florida panther, Iberian lynx, and cheetah (Kelly, Stack, and Harley 2013). This has also included surveillance in captive species and work such as urban epidemiological monitoring stations for humans, wildlife, and domestic animals. This helps understanding of the relations between the host and pathogens and creates a zoo and public health network (McNamara 2007).

1.2 Chilean Situation

Chile is a long (4,300 km) and narrow strip of land (350 km in the widest zone) adjacent to three countries and limited by natural frontiers. In the north, Chile is limited by the Atacama Desert, having frontiers with Peru and Bolivia. In the east, it is limited by the Andes Mountains, sharing frontiers with Argentina. The west and south are limited by the Pacific Ocean, furthermore, the country has a terrestrial zone in the Antarctic circle. From west to east, the geography comprises four main landforms. It begins with the Andes Mountains, which reach an average height of 4,000 m and a maximum peak of 6,961 m. It is followed by the Intermediate Depression, where most of the settlements are located. This is naturally limited by the Coastal Range, a series of smaller mountain ranges and cliffs that reach up to 3,114 m at their highest point, influencing local ecosystems and microclimates. Finally, next to the Pacific Ocean, there are the coastal

plains, where many other major cities and ports, such as Antofagasta, Concepcion and Valparaiso are located (Pliscoff and Luebert 2018).

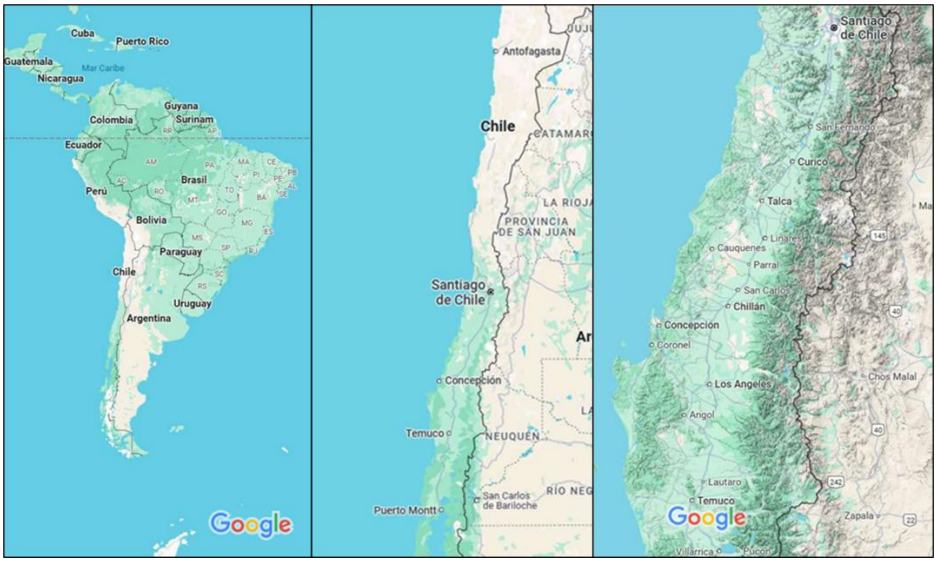

Figure 2 Map of South America, Central area of Chile, and terrain map from Google maps

From the North to South, Chile can be classified in four geopolitical zones: north, north-centre, south-centre and south. The first is characterized by extreme aridity and high thermic contrast, characterized by scarce vegetation and lower rain volumes. The North-centre is marked by the start of the Mediterranean climate zone and the appearance of sclerophyll forest. From the South-Centre dense forest areas are present, marked by *Nothofagus* forest and an increase in biodiversity (**Figure 2**). Finally, the southern area is represented by a mix of dense forest in the coast and in the intermediate depression, and Patagonia characterized by smaller flora adapted to cool and dry weather (Pliscoff and Luebert 2018).

The total Chilean population is 17,574,003 people, distributed in 15 regions, from north to south they are: Arica and Parinacota, Tarapaca, Antofagasta, Atacama, Coquimbo, Valparaiso, Metropolitana of Santiago, O'Higgins, Maule, Ñuble, Bio-Bio, Araucania, Los Rios, Los Lagos, Aysen Carlos Ibañez del Campo, and Magallanes and the Antartica Chilena. The major densities of people are in the Central area as is showed in **Figure 3**, this means 78.9% of the total population

is found in the Valparaiso region and the Araucania. From the total population, 87.9% live in urban areas, while only 12.2% live in rural areas (INE 2018).

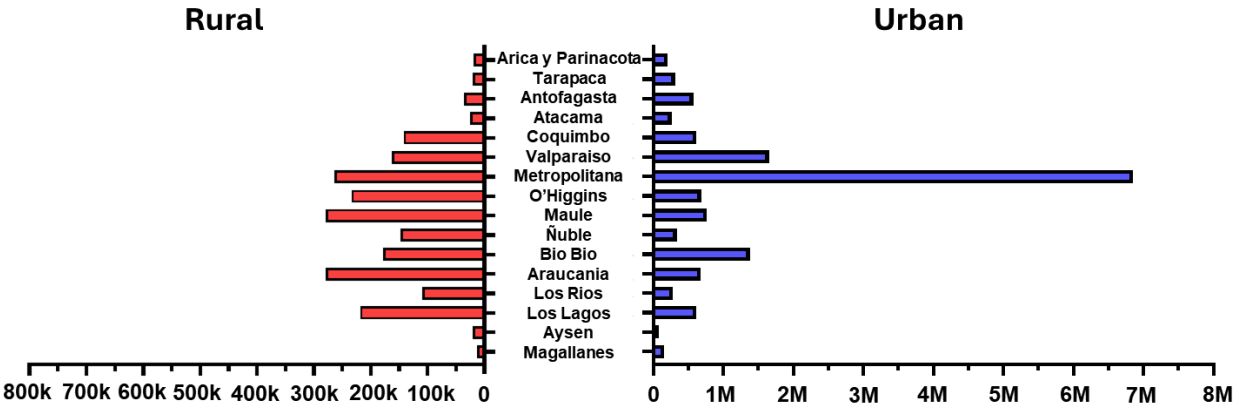

Figure 3 Chilean human population, sorted by region, urban and rural area. The biggest area is Metropolitana followed by Valparaiso and Bio Bio regions. The data were obtained from Instituto Nacional de Estadistica, Chile.

Similar to other countries, in Chile the development and growth of urban areas have led to increasing contact between wild and domestic species, producing detrimental effects for the wild fauna (Pauchard et al. 2006). The most common conflict with domestic animals is the predation by dogs on the wild fauna (Silva-Rodríguez et al. 2010), then (although with lower frequency) predation by wild animals on domestic animals has been recorded (Iriarte et al. 2013). Specifically in Chile, the domestic dog population density is amongst the largest in Latin America (Acosta-Jamett et al. 2015) and the cat population has not been assessed with respect to consequences on the wild fauna.

1.3 Felidae Family in Chile

The family *Felidae* in Chile is represented by two genera. Firstly, genus *Leopardus* includes four species: Pampas cat (*Leopardus colocolo),* Geoffroy's cat (*Leopardus geoffroyi)*, Andean Cat (*Leopardus jacobita)* and Guiña (*Leopardus guigna).* The second is Genus *Puma* with only one species, the Andean puma (*Puma concolor*) (**Figure 4**).

Overall, these wild felids have a distribution that varies over several countries. The Puma has the widest distribution in the continent from North America to southern Chile. The Pampas cat is present in 8 countries in South America with a long distribution in Chile, mainly, associated with dry forest and scrub grasslands. In Chile, it has a long distribution associated with the Andes Mountains and a broad area in the Central zone. The Geoffroy's cat has a long distribution in Chile but is restricted to the mountain areas, using habitats similar to Pampas cats in open and dry eco-systems. Finally, Andean and Guiña cats have restricted areas. The first is only present in the Andes Mountain zone from Argentina, Bolivia, Chile and Peru. However, it inhabits rocky outcrops in the arid zones of the Andes Mountains, above 4,000 meters over sea level. Finally, Guiña are limited to the southern area in Chile and marginal zones in Argentina (Iriarte et al. 2013). In both countries these cats are associated with Valdivian and *Nothofagus* forest (MacDonald, Mosser, and Gittleman 2010).

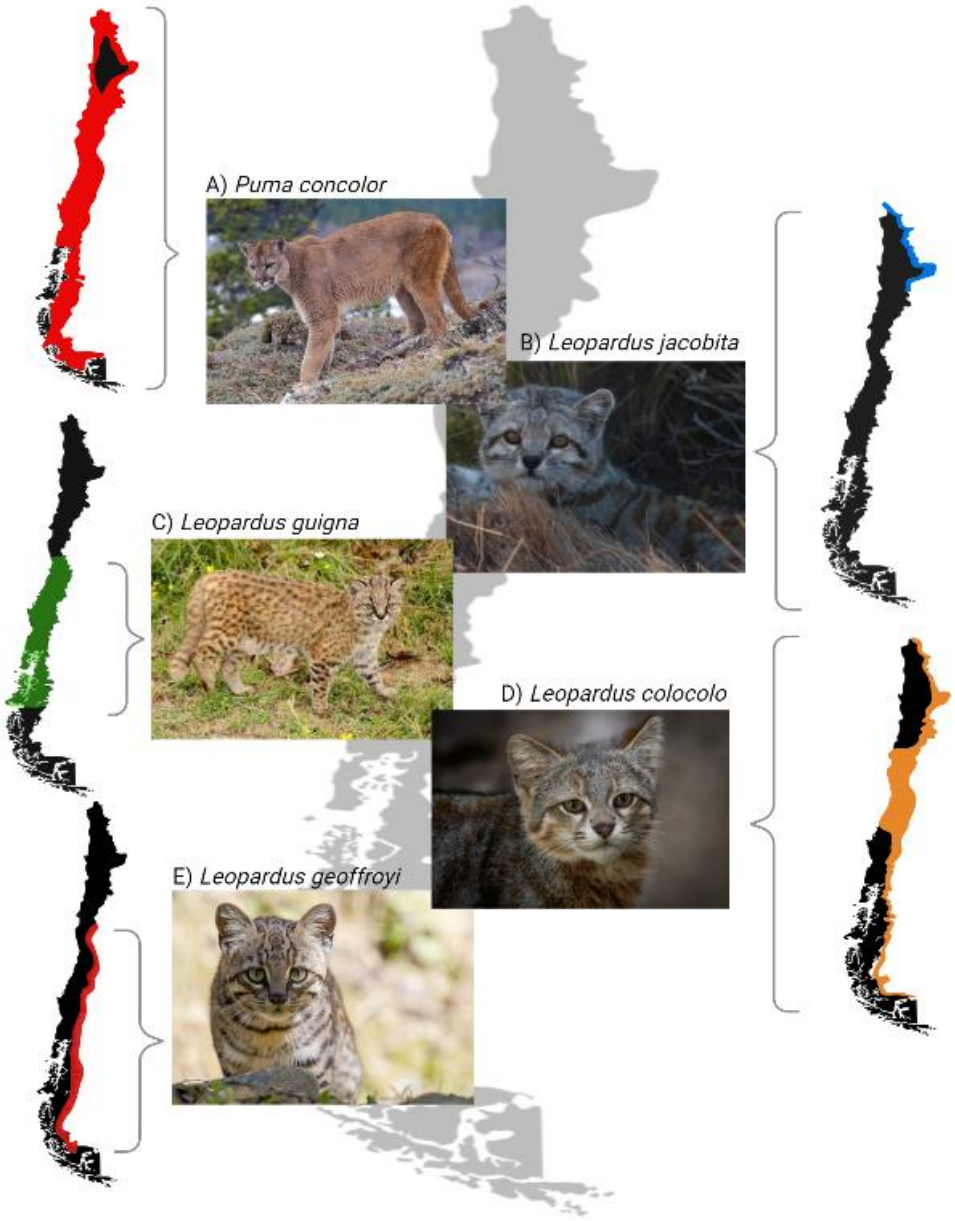

Figure 4 Distribution of the five wild felids inhabiting Chile marked in colour on a map of Chile. Map adapted from Iriarte *et* al. (2013), representing the distribution of the five wild felids presents in Chile. (A) In red Puma (*Puma concolor)* distribution; (B) in light blue Andean cat (*Leopardus jacobita)*; (C) in green guiña (*Leopardus guigna)*; (D) in amber pampas cat (*Leopardus colocolo);* (E) in darker red Geoffroy's cat (*Leopardus geoffroyi).*

1.4 Viral Diseases in Chilean wild Felids

The data on viral diseases in wild Felidae in Chile is scarce. Available studies are limited to the smallest wild felid, the guiña and limited to the central area of Chile (**Figure 4**). Two paramyxoviruses were described in 2020: feline morbillivirus (FeMV) related and feline paramyxovirus (FPaV) related virus. Thirty-five animals were analysed and eleven were positive for paramyxovirus by end-point PCR. Using the method of Sanger sequencing two clades were described, the FeMV-related and the FPaV-related. Both viruses were detected in the South-central zone of Chile. Similarly to other wild felid viral diseases, both viruses were closely related to other pathogens described in domestic cats from other countries (Sieg et al. 2020).

A different situation is apparent with canine parvovirus (CPV), where a young guiña tested positive by PCR. The virus detected was completely sequenced and showed a high nucleotide homology to CPV-2c circulating in the Chilean domestic dog population. The guiña was caught in the central zone of Chile close to an urban area (Ortega, R. et al. 2020).

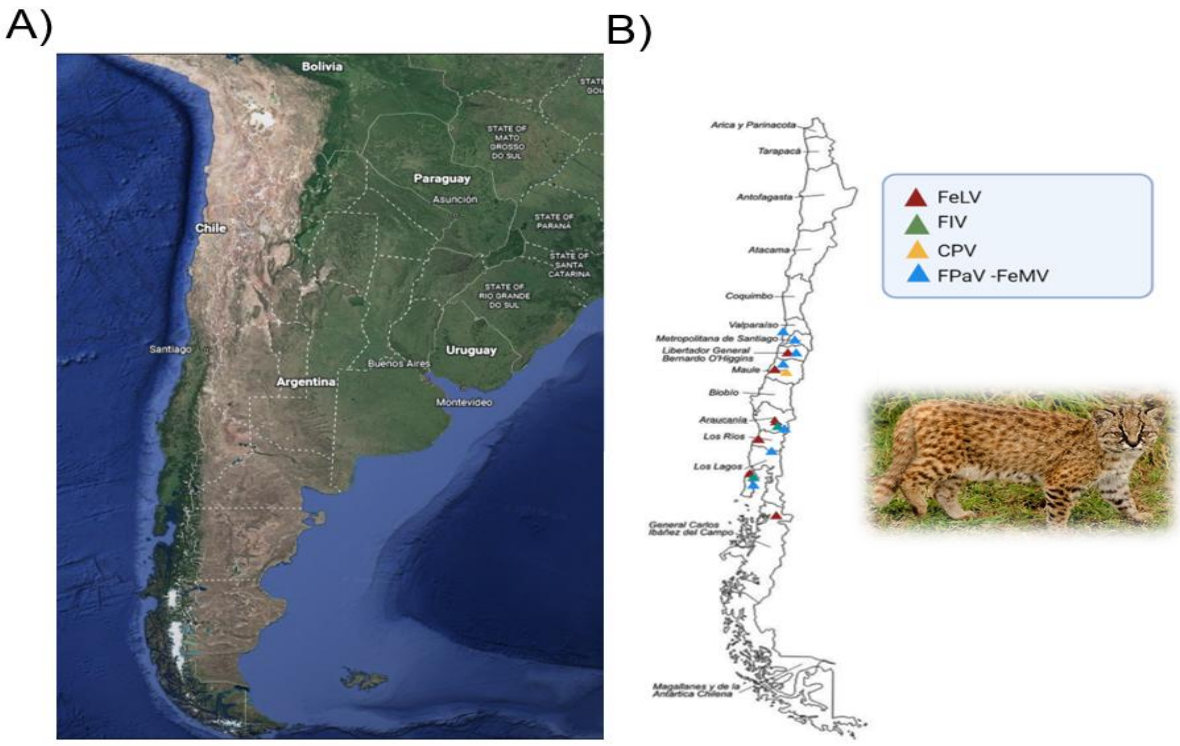

Figure 4. Summary of the viral agents described in wild felids in outlined map of Chile. A) Map from Google Earth showing the Chilean geography, an arid northern zone and forest in the southern area. The map is in the context of the South America map. The city of Santiago is the point of reference compared with Metropolitana de Santiago on the second map. B) Chilean Map showing 16 regions of the geopolitical sub-division. The red triangles display cases of FeLV (Feline Leukemia Virus), the green triangle display cases of FIV (Feline Immunodeficiency Virus), the yellow triangle shows a case of CPV (Canine Parvo Virus) and the light blue triangles display cases of FPaV (Feline Paramyxovirus) and FeMV (Feline Morbilivirus). All viral cases described were in guiñas and described by Mora et al. (2015), Sacristan et al. (2019), Ortega, R. et al. (2020), and Sieg et al. (2020).

Regarding retroviruses in Chilean wild felids, Feline Immunodeficiency Virus (FIV) has been detected in a few guiñas on Chiloé Island in the northern area. Nested PCR was used to amplify *gag* proviral genome region for FIV and U3 LTR provirus of FeLV. All positive cases were

phylogenetically related to FIV from Chilean domestic cats (Mora et al. 2015). Afterwards, a second study was conducted in a larger area, encompassing all South-central areas in Chile. FIV had a prevalence of 3.0% (like the previous study) and the FIV sequences from guiña, were closely related to domestic cat FIV (Sacristán et al. 2019).

Conversely, FeLV showed a high prevalence in both studies. Firstly, in Chiloé Island the prevalence reached 33% among the animals sampled (Mora et al. 2015). Secondly, FeLV in the larger analysis area, showed a prevalence of 20.6% of positivity (Sacristán et al. 2021a). All these sequences had a 98-99% nucleotide similarity to domestic cat viruses (Mora et al. 2015, Sacristán et al. 2021a).

1.5 Retroviruses

The *Retroviridae* family comprises two subfamilies: *Spumaretrovirinae* and *Orthoretrovirinae.* *Spumavirus* encompasses five genera: *Bovispumavirus*, *Equispumavirus*, *Felispumavirus*, *Prosimiispumavirus*, and *Simiispumavirus,* while the *Orthoretrovirinae* encompasses six genera: *Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus* and *Lentivirus* (Coffin et al. 2021). Specifically, feline leukemia virus is placed in the *Gammaretrovirus* genus and feline immunodeficiency virus in the *Lentivirus* genus (**Figure 5**) (Linial et al. 2005).

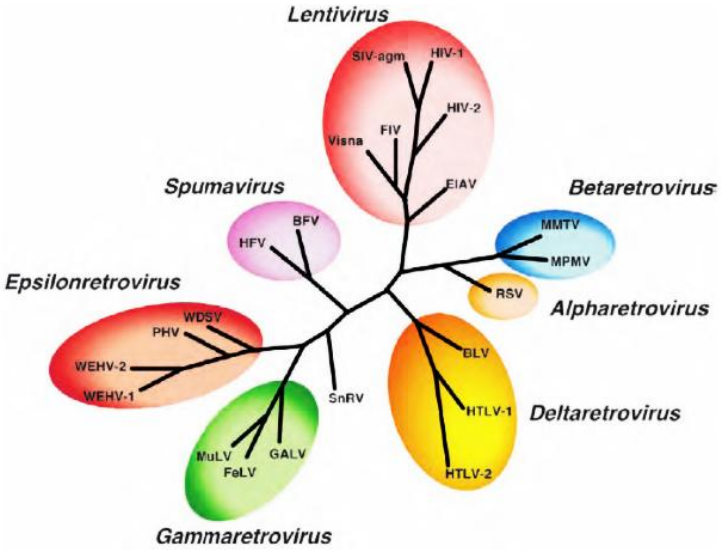

Figure 5 Unrooted neighbour-joining phylogenetic tree showing the six genera of *Ortho-retrovirinae* subfamily. Phylogenetic analysis of conserved regions of the polymerase genes of retroviruses source Linial et al. (2005)

The evolutionary history of retroviruses associates together with hepadna- and caulimo- viruses due to their similarity in the reverse transcription process. Additionally, the retroviruses are associated with retrotransposons and endogenous mobile elements through the similarities in *gag* and *pol* genes (Goff 2013).

Transposable elements (TE) have entered the germline during animal evolution. They are defined as a segment of nucleic acid that encodes the inherent ability to mobilize from one genomic location to another. They produce mutations and alter patterns of gene expression. This ability is conducted by two elements: reverse transcriptase (Class 1) or DNA transposase (Class 2). The main difference between both is the way that they can produce DNA movement. Class 1 or retrotransposons use an RNA intermediate to achieve movement. These are also categorized by whether they have a Long Terminal Repeat (LTR) or not. Those with LTR elements are known as endogenous retroviruses (ERVs). Class 2 are referred as DNA transposons, which only use DNA mechanisms to move fragments (Hancks and Kazazian 2016).

A second classification system for retroviruses splits them in two groups according to the presence of accessory genes. Theory suggests the simple retrovirus probably were the predecessors of the complex viruses, evolving with the independent acquisition of new genes (**Table 1**) (Goff 2013).

A further classification system using electron microscopy has also been used for retroviruses. This classification consists of: A-type which have a thick shell with a hollow, electron-lucent centre; B-type showing a round but eccentric inner core; C-type assembled at the plasma membrane and containing a central spherical inner core; D-type assembled in the cytoplasm and exhibiting a distinctive cylindrical core. However, nowadays genetic similarity is more commonly used as the classification system defining the different genus (**Table 1**) (Goff 2013).

Lentivirus are complex viruses having a common structure with *gag*, *pro*, *pol* and *env* genes. Furthermore, accessory genes such as *vif, vpr, tat* and *rev* are present. The most recognized lentiviruses are the human immunodeficiency virus (HIV-1), caprine arthritis encephalitis virus (CAEV), Maedi- Visna virus and feline immunodeficiency virus (FIV) (MacLachlan and Dubovi 2011).

On the other hand, Gamma-retroviruses have a C-type morphology. The most recognized members are murine leukemia viruses (MuLVs), feline leukemia virus (FeLV) and gibbon ape leukemia virus (GALV). This genus has a low complexity genome only containing *gag, pro, pol* and *env* (Goff 2013).

Table 1 Classification system of retroviruses following morphology, complexity type, and presence and type of accessory genes.

Genus	Type	Morphology	Accessory genes
Alpha retroviruses	Simple	C-type	
Beta retroviruses	Simple	B-type and D type	*sag* and *rem*
Gamma retroviruses	Simple	C-type	
Delta retroviruses	Complex	C-type	*tax* and *rex*
Epsilon retroviruses	Complex	C-type	*Orf-A, Orf-B* and *Orf-C*
Lentiviruses	Complex	Cylindrical or conical cores	*vif, vpr, vpu, upu, tat, rev and nef*
Spumaviruses	Complex	Immature	*tas/bel-1* and *bet*

1.5.1 Virus Morphology

Retroviruses are enveloped, with each particle between 80-100 nm in diameter with a heterogenous diameter. These virion characteristics make them sensitive to heat, detergent, and formaldehyde (Coffin et al. 2021).

The envelope protein subunits are the Surface (SU) and Transmembrane proteins (TM). The *env* gene encodes the protein involved in receptor binding and membrane fusion (Coffin et al. 2021). A second protein group is non-glycosylated structural proteins. These are Matrix (MA), Capsid (CA) and Nucleocapsid (NC) (**Figure 6**). The *gag* gene encodes all these proteins. The other three common proteins in all retroviruses are the Protease (PR) encoded by *pro* gene; Reverse transcriptase (RT) and Integrase (IN), the last two encoded by *pol*. PR, RT and IN are proteins directly involved in the process of genome replication (Linial et al. 2005).

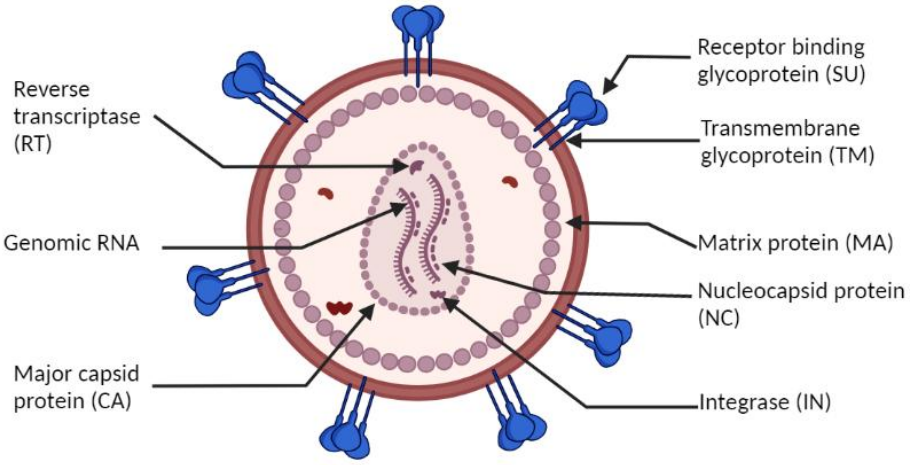

Figure 6 Generalized retro-virion morphology structure and components. In the figure are indicated the eight main components with the abbreviated nomenclature for each component. Adapted from (Linial et al. 2005).

The genetic material is condensed by the NC. Its complex is wrapped by a protein core compounded by the CA. Thus, each genera have a different distinctive core shape. The MA surrounds the core, which is surrounded in turn by the lipid bilayer. The TM cross the envelope forming a trimeric structure to be a single-pass protein transmembrane. Finally, SU forms the spike structure and is located on the outside of the virion, allowing it to bind to the host cell receptor (Goff 2013).

1.5.1 Accessory proteins

Complex retroviruses also possess accessory proteins with different functions. Overall, these play a role in replication or allowing host immune system escape. Accessory genes include *tax, rex, vif, vpu, tat, bet*, among others and are shown in **Table 1**.

Lentiviruses have the most complex replication process due to the activity of seven accessory genes. Nonetheless, not all lentiviruses have all these genes, the most complex being human immunodeficiency virus (HIV) (Bannert, Fiebig, and Hohn 2010).

The Tat protein is an enhancer of the transcription from the viral LTR to enhance elongation (Ammosova et al. 2006). Rev protein mediates export of the unspliced and spliced viral RNAs from the nucleus and thus, Rev determines the proportions of mRNA left unprocessed (Lenasi, Contreras, and Peterlin 2010). Nef protein is a multifunctional protein with the main function to enhance virus replication. Its effects in target cells are a downmodulation of MHC class I antigens and CD4, facilitating virus release (Foster and Garcia 2007). Vpr protein has a function during replication being packaged at high levels into virion particles. This protein causes a strong cell cycle arrest in the G2 phase of the cell cycle providing the best conditions for LTR transcription. Additionally, it induces a T-lymphocyte apoptosis (Dembek et al. 2010). Vif is expressed at high levels in the cytoplasm and is packaged into virion particles. The main function is enhancing infectivity in lentiviruses by degrading or sequestering the APOBEC proteins from the host, which intervene in the reverse transcription and thus, Vif helps the virus to avoid the host immune system (Li et al. 2023). Vpu is associated with the inactivation of tetherins, which are involved in the morphogenesis, maturation and release of virions from infected cells (Mangeat *et al,* 2009). Vpx correspond to a specific protein in Simian Immunodeficiency Virus involved in the enhancement of virus replication in myeloid cells (Romani and Cohen 2012).

1.5.2 Viral Genome

These viruses are single-stranded RNA (ssRNA), positive sense and have a diploid genome. Each monomer comprises 7-12 kb copies of RNA and both sequences are identical (Goff 2013).

There are three regions in the genome, flanked by two fragments of Long Terminal Repeat (LTR) at both ends (MacLachlan and Dubovi 2011). All competent retroviruses comprise a minimum of these genes: *gag* (group antigen glycoprotein), *pol* (multifunctional protein, including reverse transcriptase) and *env* (antigenic glycoproteins and transmembrane protein), whereby all retroviruses have at least this genome structure 5'-*gag-pol-env*-3' that encode basic structural proteins and enzymes (MacLachlan and Dubovi 2011) (**Figure 7**).

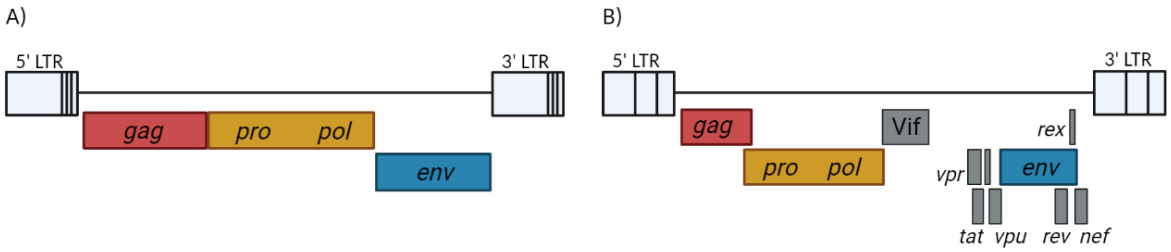

Figure 7 Comparison of genomics maps of Orthoretroviridae genera: (A) *Gammaretrovirus* genome, representing a simple retrovirus and (B) *Lentivirus* genome, representing complex retrovirus showing their accessory genes. Adapted from (Linial et al. 2005).

The basic genome structure includes identical LTR's at each end, 5' and 3'. The main function of the LTR is to drive transcription. According to each genus the segment size can vary, for instance, alpharetroviruses have the shortest LTR segments with 330 nt (avian leukosis virus), whereas spumavirus have the longest LTRs with 1758 nt (simian foamy virus 1) (Bannert, Fiebig, and Hohn 2010). The LTR can be divided into three fragments: U3 (untranslated 3'), R (repeated) and U5 (untranslated 5'). U3 is the most important segment in driving transcription with the core promoter. The R area shows a poly(A) sequence, which has the function of restricting transcription. The last segment is the U5, usually 200 nt long with actions principally in the integrase process (Uren et al. 2005). In the RNA genome, the 5' LTR is followed by the 5' untranslated region (UTR), which has the primer binding site (PBS) as a start point for reverse transcription (Bannert, Fiebig, and Hohn 2010). Additionally, the packaging signal (psi) sites can be found in the 5' LTR (**Figure 8**), which can increase the efficiency of RNA packaging into virions (Bannert, Fiebig, and Hohn 2010).

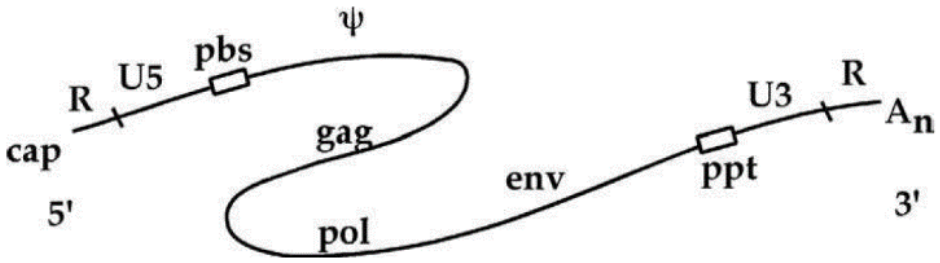

Figure 8 Retroviral RNA single-stranded genome structure represented by curved line. From 5' to 3' are shown 5' cap structure, R (for repeated), U5 (untranslated 5'), pbs (primer binding site), Ψ (packaging signal), *gag, pol, env,* ppt (polypurine tract), U3 (untranslated 3'), R in 3' and 3' poly(A) (source: Goff, 2013).

The *gag* gene encompasses between 1,200nt to 2,000nt depending on the genus. This fragment encodes the main structure of the virion, with three domains, Matrix (M), Capsid (C), and Nucleocapsid (NC). *Pro* has a length of roughly 700 bp and encodes the protease (PR), which has a function in the cleaving of viral proteins. The *pol* gene has a length from 2,500 to 3,500bp, this gene encodes, RT for the retro transcription process and the IN for the integration process (Bannert, Fiebig, and Hohn 2010).

Env has a length of approximately 1,500 to 3,000 bp, encoding surface (SU) and transmembrane (TM) glycoproteins, which form the viral envelope and determine most of the viral antigenic characteristics (Bannert, Fiebig, and Hohn 2010).

1.5.3 Life cycle

1.5.1.1 Entry and Uncoating

The virion first contacts the host cell. The infection can be achieved by fusion of the viral and cell surface membranes, or via receptor mediated endocytosis (Goff 2013). During the interaction between viral and cell receptors, SU and TM are disassociated. The TM protein is key to membrane fusion process, having contact with host cell membrane to induce changes to allow it to enter the target cell (Goff 2013).

The second step is the penetration and uncoating namely, the virion and host membrane fuse, and the core virion enters the cell. The process begins with Env protein activation, which has different pathways. In most cases, it is through cellular factors or by exposure to low pH (Mothes and Uchil 2010). The first is the case for the majority of retroviruses, especially gamma-retroviruses and involves interactions as the primary trigger for the Env protein activation (Mothes and Uchil 2010). Whereas other retrovirus groups use a pH dependent entry mechanism that often need endosomal interactions and additional co-factors (Goff 2013).

The uncoating process is still controversial. Previous studies showed an uncoating in the cytoplasm intimately linked with reverse transcription (Hulme, Perez, and Hope 2011, Mamede et al. 2017), while others show an uncoating close to the nuclear envelope during the nuclear import process (Fernandez et al. 2019). A new study demonstrated the viral core needs to enter in contact with the nuclear component to uncoat and develop the integration. This study revealed two different uncoating processes depending on whether the viral core goes across the nuclear membrane or not. Also, this process alters the place of the finalization of the reverse transcription process, possibly to evade the immune response (Burdick et al. 2020).

1.5.1.2 Reverse transcription

After entry and uncoating, reverse transcription must occur. The process starts with a tRNA annealed to the viral RNA genome, specifically to the PBS toward the 5’ end, synthesizing the minus-strand DNA. This product is named a minus-strand strong-stop DNA that consists of the R and U5 sequences. Afterwards, the RNase H activity transfers the minus-strand strong-stop DNA to the 3’ end continuing with the extension from the 3’ end to the 5’ end of the DNA minus strand. RNase H degrades the RNA genome except for the PPT (Poly purine tract) (Goff 2013). The PPT serves as the primer for the synthesis of plus strand DNA, using the minus strand DNA as template. A new strand is synthesized from the PPT to the 3’ end until a modified adenine residue terminates elongation. Again, the RNase H degrades the tRNA primer from the 5’ end in the minus-strand and the new plus-strand strong-stop DNA is then transferred to the 5’ end annealed to the PBS of the 3’ end minus strand DNA. The plus and minus are then extended and produce a double-stranded cDNA. As a result, the minus strand is a perfect polynucleotide,

as opposed to the plus strand which has gaps due to discontinuous DNA synthesis (Engelman 2010).

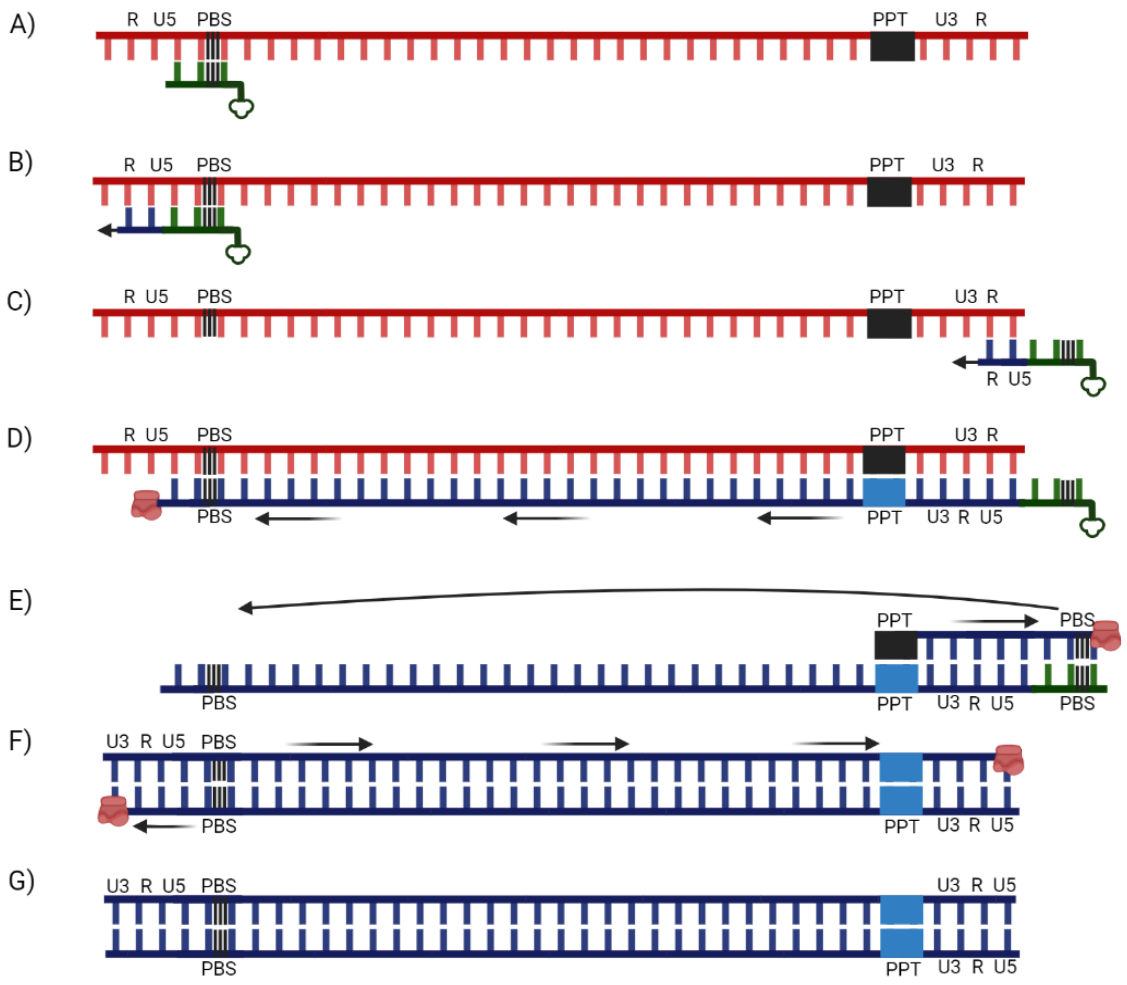

Figure 9 Viral genome reverse transcription process. The reverse transcription begins by (A) a cellular tRNA (in green) that anneals to primer binding site (PBS) in the 5' end viral RNA sequence (in red); (B) Reverse transcriptase synthetizes minus-strand strong-stop DNA (in blue), including R and U5 sequences; (C) First strand transfer to move tRNA to anneal to the 3' end of the viral genome; (D) Minus-strand is elongated from 3' end to 5' end (blue) and RNA plus-strand is degraded; (E) Using the PPT as a prime plus-strand DNA synthesis towards the 5' end forming plus-strand strong stop DNA, followed by second strand transfer to anneal in 3' PBS on the plus strand; (F) Plus strand is elongated from 3' to 5' and the minus strand is elongated to incorporate the plus strand strong stop DNA; (G) A complete double stranded DNA of the viral genome is produced to follow the replication process. Adapted from Engelman (2010).

Finally, the viral DNA fragments can be presented in three forms, one linear and two circular forms, which are differentiated by one or two LTR segments. These three types reach the cell core but only the linear form can be used to generate progeny (**Figure 9**) (Goff 2013).

1.5.1.3 Integration

The viral core, partially uncoated, starts the process of transport in the context of the reverse transcription complex (RTC). This RTC moves into the cell cytoplasm along microtubules. Afterwards, the viral genome goes to the pre-integration complex (PIC) being processed by the integrase. To pass through the nuclear membrane, different methods are used (Engelman 2010). For example, lentiviruses are efficient at infecting nondividing cells, because they use active transport through nuclear core complexes (Goff 2013). Meanwhile others like gamma-retroviruses are dependent on mitosis in the cell cycle when the membrane breaks down to reach the cellular DNA (Engelman 2010).

The provirus structure involves a 5' LTR, a viral sequence in the centre and a 3' LTR, inserted into the host genome. This process is driven by viral enzymes and can be separated into two steps: 3' end processing and strand transfer (**Figure 10**). The first step involves the integrase recessing in the 3' end and corresponding protruding 5' ends. The integrase protein uses water to hydrolyse the phosphodiester bond (Engelman 2010). Cleavage occurs in a CA/GT invariant zone in the 3' end of the unintegrated DNA and is usually a TT dinucleotide is released. Afterwards, in the strand transfer process a new 3'OH end is yielded as result of the processing step. The integrase then uses this molecule to attack the phosphodiester bonds in host DNA (Engelman 2010). The protruding 5' end in the viral genome is not joined by the IN but directly bounded by a transesterification. Finally, gaps produced in the previous processes are quickly repaired by the host repair enzymes and the final insertion is known as a provirus (Goff 2013).

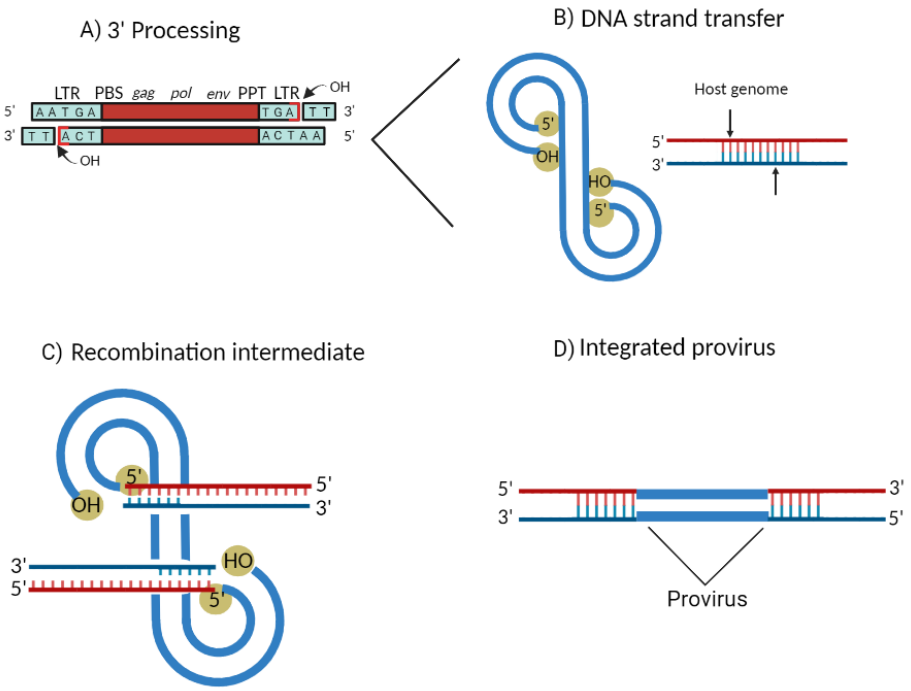

Figure 10 Viral genome Integration process. The integration process involves four steps. A) 3' Processing produces hydrolysis of each cDNA end to draw a conserved sequence, where the provirus sequence will be attached. B) Once a suitable chromatin acceptor site is found, the integrase uses the hydroxyl groups at the recessed 3' ends to cut target DNA to be joined to the viral ends to results in a 5' phosphates. C) The product from DNA strand transfer is a recombination intermediate harbouring gaps adjacent to the viral 5' ends. D) The integrated provirus is repaired by host DNA repair machinery, producing final provirus. (Adapted from Engelman, 2010).

This stage marks a threshold in viral replication, concluding early phase driven by viral enzymes, after which the subsequent steps are mediated by cellular enzymes (Goff 2013). Within the LTR region resides promoters, enhancers, and polyadenylation sequences to control transcription (Lenasi, Contreras, and Peterlin 2010). The transcription start site signal is located within the U3 and R regions, where the core promoters contain the TATA box recognized by the TATA-binding protein recruiting several factors to associate with the cellular RNA polymerase II system forming the pre-initiation complex (PIC) (Lenasi, Contreras, and Peterlin 2010). Afterwards, the PIC recruit splicing and polyadenylation factors to elongate the transcript. Transcription proceeds

through the genome and continues through the 3’ LTR yielding an RNA sequence, which is finished and cleaved at the R-U5 border of the 3’LTR (Goff 2013).

Conversely, the negative regulatory elements are LTR-mediated through the expression of negative regulatory proteins or mRNA to compete with binding sites in transcription (Goff 2013). Then the expression of down-regulation elements induces a decrease in viral replication some weeks after the initial infection to establish an environment for viral persistence (Lenasi, Contreras, and Peterlin 2010).

Polyadenylation of the transcript is the last step in the transcription, linked to a highly conserved polyadenylation signal. Due to the provirus having two identical LTRs, transcription can start at either end, 5’ LTR or 3’LTR. Usually, they start in the 5’LTR and finish in the 3’ LTR ends (Lenasi, Contreras, and Peterlin 2010). While several viruses can use the anti-sense transcription (from 3’ to 5’) some viruses like HIV and HLTV commonly use this mechanism, but it is not totally clear how this works in other viruses or in endogenous retroviruses (Manghera, Magnusson, and Douville 2017).

Once the transcript is finished it can be processed in several ways. It can be packaged into a progeny virion particle, exported, or used for translation to form the Gag and Pol polyproteins. Another portion can be spliced to generate sub-genomic mRNAs to produce Env glycoprotein or auxiliary proteins (Goff 2013). Furthermore, factors such as cell type, physiologic state and the integration site are key in determining the level of viral RNA produced (Goff 2013).

1.5.1.4 Translation and protein processing

All retroviruses contain *gag, pro, pol* and *env* as a minimum protein complement. Different strategies are used by each retrovirus. Often, protein expression results in large precursors that are subsequently cleaved. This strategy allows the yield of many proteins made from one ORF, which in turn has some advantages such as ensuring the protein production in correct ratios or working as a single entity during virion assembly (Goff 2013).

The first gene is *gag*, which is translated to form a Gag polyprotein precursor. The next gene translations vary between the families. The *pro* and *pol* gene, can be fused in the 5’end of *pol*

whereas others contain separate reading frames. *Pol* is required only in low levels for virus replication, usually this is not expressed as a separate ORF to improve viral efficiency (Goff 2013). Often it is produced as part of a Gag-Pro-Pol fusion, which is cleaved into different proteins afterwards. In this context, gamma-retrovirus produce the Gag-Pro-Pol as a fusion protein, whereas lentiviruses translate these proteins into separate frames. When Gag-Pro-Pol use the same ORF they are separated only by a UAG as stop codon, being ignored as a stop codon produces the other precursor proteins (Goff 2013).

Env is however always expressed from a spliced mRNA. *Env* uses an AUG initiator codon producing long proteins that are exported and cleaved in the Golgi into separate SU and TM subunits (Lenasi, Contreras, and Peterlin 2010).

1.5.1.5 Virion assembly, RNA packaging and virion maturation

The last steps in viral replication are virion assembly, RNA packaging and virion maturation. In this process, the Gag precursor protein is the main particle in the virion assembly, mediating in virion assembly and producing immature virus-like particles. The only exception is the spumaviruses, which need the Env glycoprotein for efficient budding (Goff 2013).

The assembly follows different pathways according to the viral morphology, with the CA domain critical to the virion morphology. The C-type morphology assembles in the plasma membrane. This occurs through a protein aggregation and forms a patch close to the plasma membrane, which releases a nascent virus to bud outwards. In contrast, the assembly of B and D-type viruses has two steps, these assemble in the cytoplasm and are afterwards transported to the plasma membrane, where they are enveloped and released (Goff 2013).

The RNA is condensed in a structure led by the dimer linkage sequences, which produces a tight binding with the NC and stimulates the packaging. The psi area, in the 5’ end, between the LTR and *gag* regions drives the RNA packaging interactions (Goff 2013). This process involves host and viral free tRNA being incorporated within the new virion and some of these tRNA function as the initiating primer annealed to the PBS area (Goff 2013).

Finally, maturation is driven by MA to the outer protein shell for the mature virion, which occurs either during or soon after viral budding. During this process, PR conducts the protein processing, where the cleavage process is directly linked to the assembly and budding through dimerization of the polyproteins. Thus, the polyproteins are cleaved producing small proteins (Goff 2013). The Gag-Pro-Pol polyprotein is cleaved to release mature PR proteins. Subsequently, Gag is divided into MA, CA, and NC, and MA, while SU and TM subunit cleavage is developed during transport through the cytoplasm activating virus to bind to the MA. NC is associated with viral RNA to protect it from degradation and CA forms a shell to envelope the inner core of the virus. Pol is cleaved into IN and RT subunits (Bush and Vogt 2014). Once the mature virus takes its form, this is a particle with condensed structure with various morphologies such as, cones, tubes or polyhedral structures (Bush and Vogt 2014) with a central core largely detached from the surrounding by the viral lipid envelope and is able to infect host cells (Goff 2013).

1.5.2 Endogenous Retroviruses

As a result of the reverse transcription process, the RNA viral genome is copied as cDNA, which is then integrated in the host genome. If the retrovirus infects germline cells, these fragments may be inherited in their offspring. ERVs have accumulated during millions of years in animal genomes during their own species evolution. Nonetheless, these ERVs have undergone various changes to subsist with the host. They have suffered recombination, deletion, or mutation events that may eliminate viral sections producing pathological, benign, or harmless proviruses (Denner 2010). In addition, robust restriction strategies to control the ERVs have arisen from the hosts. Thus, beneficial ERV insertions are frequently fixed in the host genome, whereas neutral or deleterious ERVs are more likely to be eliminated (Johnson 2019).

Based upon this evolutionary history, a large number of ERVs are present in animal genomes. For instance, in the human genome ~8% of it is ERV sequences, with a large portion of these proviruses being defective and incapable of replication (Hancks and Kazazian 2016). ERVs have been related to different effects in the hosts, some benign, and others exceptionally pathological for the host. Effects such as tumour development, immune regulation, protection

from exogenous virus or even physiological functions during pregnancy have been described (Denner 2010). When an endogenous retrovirus gains beneficial activity for the host, supporting their physiology, the process is called “ERV domestication” (Kawasaki and Nishigaki 2018).

While ERVs may encompass several viral regions, they are characterized by an LTR. Indeed, a lot of solo-LTRs have been described in animals and humans. This is a complete deletion of internal sequences owing to recombination between 5’ and 3’ LTR during cell division (Gifford et al. 2018). Solo-LTRs have a higher prevalence in contrast to full-length proviruses, a noteworthy case is the human endogenous retroviruses (HERVs), discovered in the late 1960s, where 30-50 copies of full-size ERV have been found, in contrast to their related LTRs with between 10,000 to 25,000 copies per haploid human genome (Sverdlov 1998). ERVs have the potential to be activated during cellular co-infection by other viruses. This happens in humans with the LTR directed transcription of HERV-W, which is activated by infection with herpes simplex virus type-1 (Denner 2010).

The co-existence of endogenous and exogenous retroviruses may create different outcomes. In some cases, ERVs play a role of protection against the exogenous retrovirus. For example, the endogenous Jaagsiekte sheep retrovirus (JSRV) provides protection against the exogenous JSRV through two mechanisms. The first is an early prevention through receptor interference, the second through a late prevention by a block produced by an excess of defective Gag protein that prevents the interaction of the exogenous virus with the cellular machinery (Spencer and Palmarini 2012).

A noteworthy case of deleterious endogenization is the endogenous Koala Retrovirus (KoRV). This is one of the youngest germline invasions being an ongoing endogenization which is under study (Johnson 2019). KoRV has been described as pathogenic for the koalas, with a relation between the presence of endogenous particles and a high probability of gene dysregulation with an increase in cancer rate (McEwen et al. 2021). Furthermore, KoRVs produce immune disorders which have been associated with increased susceptibility to chlamydia infections (Maher et al. 2019).

1.5.3 Endogenous Retroviruses in cats

Similar to humans and mice, 6 to 10% of the total sequence in domestic cat genomes are endogenous retroviruses (Pontius et al. 2007). Among them, several ERVs have been described including enFeLV (Soe et al. 1983), the ERV-DC groups (Anai et al. 2012), RD-114 (McAllister et al. 1972a), MAC-1 (Bonner and Todaro 1979), FERCmlu1 and mlu2 (Yuhki et al. 2008), ECE1, and FcEV (Van Der Kuyl, Dekker, and Goudsmit 1999a). Notably, cats exhibit an unusually high number of endogenous gammaretrovirus families with 47 families identified, in contrast to only 17 endogenous gamma retroviruses reported in dogs (Jo et al. 2012, Song et al. 2013). Although much remains to be understood, the ERVs listed here are among those for which much evidence is available.

1.5.3.1 Endogenous Retrovirus Domestic Cat (ERV-DC)

The ERV-DC are a group of endogenous gammaretroviruses, one of the youngest ERVs of domestic cats and European wildcats. Their standard structure follows the typical simple gammaretroviral structure, around 9kb in length, consisting of *gag*, *pro-pol* and *env* genes flanked by two LTRs. Integration into the cat genome likely occurred around 2.8 million years ago (Mya) (Anai et al. 2012). Thirteen different loci have been reported throughout the cat genome (Anai et al. 2012, Kuse et al. 2016, Pramono et al. 2024b), although 17 loci have been estimated to exist across cat populations (Kawasaki and Nishigaki 2018).

Based on phylogenetic relationships, ERV-DCs are classified into three genotypes (GI, GII, and GIII) (**Table 2**), reflecting different integration events, varying genome lengths (due to deletions), and differential impacts on the feline genome (Anai et al. 2012). Among these, ERV-DC8 and ERV-DC14 (from GI), as well as ERV-DC10 and ERV-DC18 (from GIII) have intact ORFs and are capable of producing infectious viral particles (Anai et al. 2012, Pramono et al. 2024b). Nonetheless, ERV-DC14 in European wildcats is defective due to a SNP in the *env* gene (Ngo et al. 2019). It is plausible that GI and GII infected the common ancestors of both wild and domestic cats, whereas GIII may have integrated more recently in the domestic cat lineage, as no evidence of GIII has been found in wildcats (Ngo et al. 2019).

Table 2. Classification and detail of genetic characteristics of endogenous retrovirus in domestic cats and present in wild felid species. (Adapted from Kawasaki and Nishigaki, 2018).

Genotype	Domestic Cat Provirus	Complete ORF	Replication competent	Functional gene	Wild Felid Present
Genotype I	ERV-DC1	-	-		
	ERV-DC2	*env*	Unknown		
	ERV-DC3	-	-		
	ERV-DC4	*gag-pol*	-		
	ERV-DC8	*gag-pol, env*	-		
	ERV-DC14	*gag-pol, env*	+		*F. Silvestris*
	ERV-DC17	*gag, env*	-		
	ERV-DC19	*env*	-		
Genotype II	ERV-DC7	SU	-	Refrex-1	*F. Silvestris*
	ERV-DC16	*gag,* SU	-	Refrex-1	*F. Silvestris*
Genotype III	ERV-DC6	*env*	Unknown		
	ERV-DC10	*gag-pol, env*	+		
	ERV-DC18	*gag-pol, env*	+		

ERV-DC elements can also be classified based on a *cis*-acting nucleotide variation within the LTR promoter region. The A-type subgroup (including ERV-DC10, DC18, DC7, DC16, and DC19) contains an adenine at a specific position, which is linked to higher promoter activity. In contrast, the T-type subgroup (ERV-DC1, DC4, DC8, DC14, and DC17) carries a thymine at the same position, resulting in attenuated promoter activity (Kuse et al. 2016, Kawasaki and Nishigaki 2018). This variation may have arisen during the endogenization processes, with the T-type likely derived from the A-type as an evolutionary strategy. This is based on the idea that the T-type LTRs attenuated promoter activity may help to evade the host immune system (Kuse et al. 2016).

Genotype I (GI) is the biggest group including 8 proviruses (DC1, 2, 3, 4, 8, 14, 17, and 19), encoding different complete or incomplete ORFs depending on each provirus (Kuse et al. 2016, Kawasaki and Nishigaki 2018). GI have been associated with recombination events with exogenous FeLV. FeLV-D is a recombinant form of FeLV that has acquired its *env* gene from GI ERV-DCs, and this recombination occurs *de novo* in each cat (Anai et al. 2012). The ERV-DC

env gene was likely acquired through interspecies retroviral transmission from primates to felids, resulting in a shift in receptor usage. The incorporation of ERV-DC sequences enables FeLV-D to utilize the receptor feCTR1 as a receptor for cellular entry, which is indeed the receptor used by ERV-DC14 and ERV-DC8 (Van Der Kuyl, Dekker, and Goudsmit 1999a, Anai et al. 2012, Tury et al. 2022, Pramono et al. 2024b). FeLV-D has been associated with hematopoietic malignancies, such as lymphomas and leukaemia (Anai et al. 2012).

Comparative studies of ERV-DCs in domestic cats from different regions of the world have revealed varying levels of endogenization. For example, the prevalence of ERV-DC8 in domestic cats is 77.5% in Japan and 65.7% in Spain (Ngo et al. 2019). In other countries, prevalence ranges from 52.6% to 85.8%, with the highest observed in Tanzania and the lowest in Vietnam and South Korea (Ngo et al. 2019). Notably, ERV-DC8 is absent in European wildcats (Ngo et al. 2019), suggesting that its integration happened later in the domestic cat lineage.

ERV-DC14 is another GI replication competent provirus, however its activity is reduced due to low promoter activity of its LTRs, resulting in a low frequency in the host population (Anai et al. 2012, Kuse et al. 2016). ERV-DC14 is inserted at chromosome C1q32 and has been detected in 2.5% of Japanese domestic cats evaluated (Kawasaki and Nishigaki 2018). Due to its low replication efficiency, ERV-DC14 is unable to persistently infect host cells by itself (Kuse et al. 2016). When compared with ERV-DC10 and ERV-DC18 (GIII), ERV-DC14 exhibits the lowest expression levels. This reduced replication efficiency is attributed to a single A-to-T mutation in the 5'LTR, which negatively impacts its promoter activity and viral replication (Kuse et al. 2016).

GII represents a domesticated group of ERVs, that have been disrupted by mutations and deletions in the *pol* and *env* genes. These modifications encode Refrex-1, which is an antiviral factor secreted extracellularly leading to receptor interference inhibiting ERV-DC GI and FeLV-D binding to the host receptor (Ito et al. 2013, Pramono et al. 2024b). Additionally, Refrex-1 lacks the immunosuppressive domain typically present in gamma retroviruses, which is often associated with immunosuppressive activities. Among the GII, ERV-DC7 is widely fixed in both domestic and wildcat populations, while ERV-DC16 is exclusively fixed in domestic cats. Furthermore, ERV-DC7 has an evolutionary divergence between domestic and wild cats. Both

ERV-DC7 and ERV-DC16 are truncated by early stop codons within the proline-rich region (Kawasaki and Nishigaki 2018).

GIII includes ERV-DC6, DC10 and DC18; however, only the last two are replication competent. ERV-DC18 is believed to have been recently endogenized through reintegration, reinfection, or transposition of ERV-DC10, as both differ by only single nucleotide variation in the primer-binding site of their full-length proviral genomes (Ngo et al. 2019). ERV-DC10 is located on chromosome C1q12-q21, while ERV-DC18 is inserted at chromosome D4q14 (Anai et al. 2012). ERV-DC10 has been described in 37.7% of Japanese cats and 24.5% of cats from Spain, whereas ERV-DC18 has been solely detected in Japanese cats, suggesting it is a recent and localized integration event (Ngo et al. 2019).

Host control over ERVs has been evaluated through analysis of CpG methylation patterns in 5' LTRs. Methylation happens downstream of the TATA-box, and this is inversely correlated with replication activity. The 5'LTRs of GIII ERVs are hypermethylated (ranging from 84 to 98% methylated), evidencing reduced replication fitness. In contrast, GII are hypomethylated (ERV-DC7 0% and ERV-DC10 17%) with higher expression levels. Intermediate levels of CpG methylation are associated with moderate viral expression levels (Kuse et al. 2016).

ERV-DC in wild felids has been detected only in cats from the *Felis* genus, specifically in European wildcats (*Felis silvestris silvestris*), however with some differences compared to domestic cats. In the European wildcats only the GI (ERV-DC14) and GII (ERV-DC7 and ERV-DC16) were detected, with no GIII detected, probably because GIII uses a different receptor to enter the cell than GI and GII. Another difference with domestic cats was that ERV-DC14 was found in high frequency but replication defective (Ngo et al. 2019).

1.5.3.2 RD-114 and RDRS clade

RD-114 is an ERV that is replication competent. This was initially isolated from a human rhabdomyosarcoma cell transplanted into foetal kittens (McAllister et al. 1972a), leading to initial confusion with an exogenous human retrovirus. RD-114 is now recognized as a chimeric virus composed of *gag-pol* genes derived from ERV-DC, and an *env* gene originating from a

betaretrovirus (**Figure 11**), baboon endogenous retrovirus (BaEV) (Van Der Kuyl, Dekker, and Goudsmit 1999). BaEV itself is also chimeric, encompassing a *gag-pol* ORF from *Papio cynocephalus* endogenous retrovirus (PcEV) and an *env* gene from simian endogenous retrovirus (SERV) (Van Der Kuyl et al. 1997).

Previously, RD-114 was considered as a xenotropic retrovirus (McAllister et al. 1972) but nowadays is considered a polytropic virus. This categorization depends on the ability to replicate in cells from different hosts and, thus a newer description infecting and replicating in cells from various species such as cat, pig, dog, mink, and rabbits, changed the categorization of RD-114 (Miyaho et al. 2015).

The main characteristic of RD-114 comes from the receptors used to enter the cell, this is primarily the sodium-dependent neutral amino acid transporter type 2 (ASCT2/SLC1A5) and secondly ASCT1 (SLC1A4). ASCT2 is a highly conserved receptor between cattle, quail, dog, pig, horse, mink, ferret, cat, and sheep (Miyaho et al. 2015).

RD-114 contamination was detected in live attenuated vaccines for domestic dogs and cats manufactured in the United Kingdom and Japan (Miyazawa et al. 2010). The cross-contamination occurred due to the use of feline cells in vaccine production (CRFK cells, MCC cells, and FER cells). While the consequences of RD-114 transmission to dogs are not fully understood, it was speculated that, if the virus were to adapt efficiently in canines, it could potentially lead to immunosuppression or proliferative disease (Yoshikawa et al. 2014).

In addition, a group of sequences closely related to RD-114, known as RD-related sequences (RDRSs), have been described as non-replicative at different loci in the cat genome (Shimode, Nakagawa, and Miyazawa 2015). Reports have described between 8 to 13 distinct loci. These studies consistently observed highly conserved *gag-pol* genes, while the *env* gene showed high divergence compared to the to the original and replicative RD-114 from human RD cells (Spodick, Soe, and Roy-Burman 1984, Reeves and O'Brien 1984, Shimode, Nakagawa, and Miyazawa 2015).

Four of the six proviruses (RDRS A2, E3, D4, C2b) have identical 5' and 3' LTRs, suggesting they were integrated into the cat genome less than 0.2Mya, possibly through RDRV infection

(Shimode, Nakagawa, and Miyazawa 2015). The lack of divergence between 5' and 3' LTRs in A2, C2b, and E3, along with no evidence of LTR-LTR recombination, supports the idea of recent invasion events for these RDRS loci (Shimode, Nakagawa, and Miyazawa 2015).

The RDRS C2a is the oldest related provirus, showing approximately 95.7% sequence homology with the replication competent RD-114. C2a has been proposed as the ancestral virus that infected domestic cats around 1.6 Mya, after the divergence of the sand cat lineage. RDRS C2a is the most widely distributed among cat breeds, suggesting that it entered the feline genome with the domestic cat lineage. In contrast, other RDRSs may have been integrated into cats migrating through Europe. Additionally, most Asian cat breeds lack RDRSs, supporting the theory of recent invasion of RDRSs into the cat genome. The presence and distribution of RDRS vary between cat breeds, reflecting novel events of integration during the migration and geographic origin of domestic cats (Shimode, Nakagawa, and Miyazawa 2015).

RDRS are widely variable, for example RDRS_E3 is present only in certain cat breeds, indicating restricted distribution likely influenced by breeding and geographic history. Moreover, recombination events have contributed to the emergence of novel proviruses. Phylogenetic and recombination analyses based on the *gag-pol* region suggest that ERV-DC GIII shares homology with RD-114_SC3C, while GII shows genetic association with RDRS_C2a (Kawasaki and Nishigaki 2018). Additionally, the *pol* gene of RDRS_E3 appears to have been obtained from ERV-DC GI. Therefore, the complement of potentially active ERVS varies depending both on cat breed and geographical location (Shimode, Nakagawa, and Miyazawa 2015).

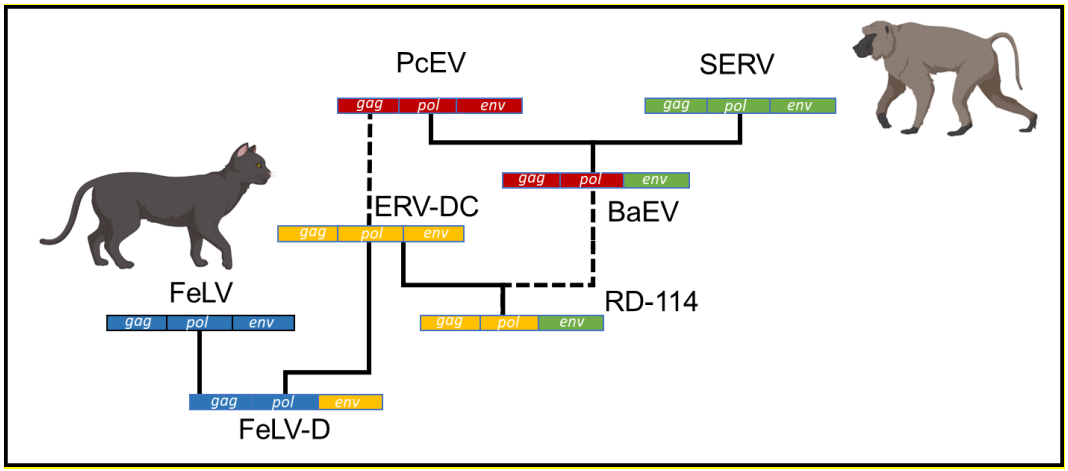

Figure 11 Origins of RD-114 and FeLV-D from recombination between retroviruses Simian endogenous retrovirus (SERV) arose. From Old world monkeys, *Papio cyanocephalus* endogenous retrovirus (PcEV) and Baboon endogenous retrovirus (BaEV) (from a recombination between *gag* and *pol* genes from PcEV and *env* gene from SERV). Secondly, in domestic cats the ERV-DC was acquired from interspecies transmission, similarly the *env* gene portion from BaEV and *gag* and *pol* from ERV-DC to generate RD-114. In parallel, the ERV-DC *env* gene recombined with *gag* and *pol* from FeLV to produce FeLV-D (source: Kawasaki and Nishigaki, 2018).

1.5.3.3 *Felis catus* endogenous gammaretrovirus 4

Another ERV reported in domestic cats is *Felis catus* endogenous gamma retrovirus 4 (FcERV-gamma4) described for the first time in 2013 (Song et al. 2013). FcERV-gamma4 is a non-coding viral fragment but can recombine with FeLV and produce defective RNA in feline tissues.

The FcERV-gamma4 are a group of ERVs estimated to have been inserted into the feline genome between one to 5.7 Mya (Kawasaki et al. 2017).

As is typical of gamma retroviruses, FcERV-gamma4 have a 5'-leader region located between the primer binding site and *gag* gene start codon. This region regulates viral replication by encoding signals for the primer binding site, the packaging signal, splicing donor sites and the dimerization initiation signal, it also carries an internal ribosome entry site, which enhances viral protein synthesis (Corbin and Darlix 1996, Miyazaki et al. 2011). FcERV-gamma4 has a ~1kb 5'-leader and nine loci have been identified with an extra locus predicted (gamm4-1, E2, 3, B3, A1, C1, X, B4, X2, and X2like). All these loci contain non-functional ORF due to deletions or insertions of a short interspersed nuclear element (Song et al. 2013, Kawasaki et al. 2017).

Gamma4-E2 is estimated to be the oldest insertion among the FcERV-gamma4, with an integration date of approximately 5.71Mya, whereas gamma4-X2 represents a more recent insertion in the cat genome. Furthermore, gamma4-X2 shows the lowest prevalence in the Japanese domestic cat population (Kawasaki et al. 2017).

More recently, XR-FeLV have been described, these are recombinants between FeLV and a portion of the 5'-leader sequence and the *gag* gene of FcERV-gamma4 (Kawasaki and Nishigaki 2018, Ngo et al. 2024). This region plays a critical role in regulating key processes essential for viral replication and this recombinant was detected in 6.4% of naturally FeLV infected cats and was statistically associated with hematopoietic tumours (Kawasaki et al. 2017) and recently was identified in 2 of 6 cats evaluated in another study detecting recombinants (Ngo et al. 2024).

1.5.3.4 Endogenous Feline Leukaemia virus (enFeLV)

Endogenous feline leukaemia virus (enFeLV) was integrated in the cat genome more than 2 Mya. Eight *Felis* species have been detected with enFeLV: *Felis catus, Felis lybica, Felis silvestris catus*, *Felis silvestris, Felis bieti, Felis nigripes*, *Felis margarita* and *Felis chaus* (Polani et al. 2010). In contrast, no other felid lineages have shown enFeLV, probably, due to the entrance of enFeLV happening in the final stages of Felidae divergence although before the *Felis* divergence. Additionally, FeLV have a high similarity to murine leukaemia virus (MLV) described in rodents, therefore it has been suggested that FeLV originated in the *Felis* lineage because of early rodent predation (Benveniste and Todaro 1975). Indeed, European wildcats have higher levels of enFeLV and the author suggested that possibly they have more contact with rodent retroviruses associated with a major dietary component (Tandon et al. 2007).

Analysis from *env*-LTR regions of enFeLV evidenced two major clades in the domestic cat lineage called Groups I and II. For domestic cat and the wildcat (the intermediate progenitor), both show a large diversity of enFeLVs, meanwhile other species like *Felis nigripes,* have a strong association solely with Group II. This also suggests that two different invasion events or successive proliferation events happened along the domestic cat lineage evolutionary history (Polani et al. 2010).

EnFeLV have similar behaviour to other endogenous retroviruses, for example, breed and age are not related to higher levels of enFeLV, nonetheless, the male gender has been demonstrated to have higher levels of enFeLV load. This happens due to enFeLV sites present on the Y chromosomes that have few zones of active transcription and fewer recombination events than X chromosomes, therefore male cats have less chance to lose enFeLV sequences (Roca et al. 2005, Tandon et al. 2007, Powers et al. 2018).

The major impacts of ERV in cat health are recombination events with exogenous virus. Similarly to FeLV-D, produced by ERV-DC and exFeLV, enFeLV can recombine with exFeLV to produce FeLV-B. This is the most common recombinant, producing a change in the receptor used to enter to the cell. FeLV-A uses thiamine transporter receptors (ThTR-1), while FeLV-B uses the phosphate transporter receptors (Pit-1/2) (Chiu, Hoover, and Vandewoude 2018a), which has been associated with progressive infections and more severe disease (Powers et al. 2018).

The enFeLV role in exFeLV infection is a complex situation not resolved yet. The first studies evidenced an association between higher loads of enFeLV, associated with exFeLV positive cats therefore enFeLV could be a predisposing factor to exFeLV disease (Tandon et al. 2007). Afterwards, other studies have demonstrated that higher loads of enFeLV, reduce the chances of developing a progressive infection, suggesting that the enFeLV may have a protective effect against exFeLV (Powers et al. 2018). Finally, the most recent studies have shown how higher loads of enFeLV in primary cells from cats are more resistant to FeLV infection and viral replication (Chiu, McDonald, and VandeWoude 2021). Furthermore, several factors may be involved in regressive or progressive infection. Some tissues produce more gene segments than others, for instance there is higher load of lymphoid enFeLV-LTR that could act as a miRNA inhibitor of exFeLV helping to control the infection. In contrast, an infection of nonlymphoid tissues with lower levels of LTR may result in possible recombination with enFeLV-*env* and higher chances to overwhelm RNA restriction and develop a progressive infection (Chiu, McDonald, and VandeWoude 2021). The relevance of enFeLV-*env* is because in those worse clinical outcomes, recombination between enFeLV and exFeLV occurs in the 3’ half of the

genome, probably associated with a change of viral receptor (Chiu, Hoover, and Vandewoude 2018a).

1.5.4 Feline leukemia virus

FeLV belongs to the family Retroviridae, genus gamma-retrovirus, described for the first time in 1964 in cats with lymphoma (Jarrett et al. 1964). The pathogen has a wide distribution among the domestic cat populations, being one of the most important infectious diseases for cat health (Hartmann 2012). The prevalence has a wide range of levels, depending on several factors, among them: cat density, cat behaviour, vaccination, housing status, and cat origins (Gleich, Krieger, and Hartmann 2009, Burling et al. 2017, Studer et al. 2019, Capozza et al. 2021). The main clinical signs are lymphadenopathy, anaemia, bone marrow suppression, immune suppression, lymphoma, leukaemia, and ultimately death (Willett and Hosie 2013, Hartmann and Hofmann-Lehmann 2020, Diesel et al. 2024)

1.5.4.1 FeLV Subgroups

The FeLVs have been classified using interference assays and analysis focussed on the SU protein (Miyake et al. 2016, Chiu, Hoover, and Vandewoude 2018, Biezus et al. 2023). Six subtypes have been described, being FeLV-A, B, C, D, E and T. These arose from recombination with endogenous retrovirus or mutations and recombination with FeLV-A itself. Thus, FeLV-B and FeLV-D arise from FeLV-A and an endogenous retrovirus, in contrast to FeLV-C, FeLV-E and FeLV-T, which have arisen from deletion, insertions or substitutions from FeLV-A (Miyake et al. 2019, Anai et al. 2012, Erbeck et al. 2021). This was confirmed by a recent study using SU protein sequences available in GenBank. They described two main clades, FeLV-A clade, including FeLV-A, FeLV-C, FeLV-E and FeLV-T; and another clade, the enFeLV clade including only enFeLV and FeLV-B. The FeLV-D was not considered because it has too great a variability compared with the other sub-types (Cano-Ortiz et al. 2022).

FeLV-A is the most widespread subtype and was until recently thought to be the only subtype horizontally transmitted between cats. FeLV-A uses uniquely the thiamine rransporter protein (ThTR-1) to enter the cell (**Table 2**), in contrast to other sub-variants using different receptors

(Mendoza, Anderson, and Overbaugh 2006). FeLV-A produces overall mildly pathogenic signs such as immunosuppression and hematologic disorders, but also in the worse cases can produce macrocytic anaemia and lymphomas (**Table 2**) (Willett and Hosie 2013). Other variants like FeLV-D and FeLV-T are barely detected in cat populations and are thought to arise within individual cats without spread between animals (Little et al. 2020). The second most widespread subtype is FeLV-B (Ahmad and Levy 2010, Erbeck et al. 2021), historically this was considered not to transmit between cats, but recent studies have postulated that FeLV-B may be transmitted horizontally by itself, without requiring FeLV-A (Chiu and Vandewoude 2021, Erbeck et al. 2021).

FeLV- B is more virulent than FeLV-A in terms of disease pathogenesis but is usually replication-defective and requires a FeLV-A helper virus for successful replication (Boomer, Sarah et al. 1997, Willett and Hosie 2013). This subgroup is the second most distributed, found in up to 68% of FeLV positive cats (Powers et al. 2018). The high diversity is produced because the infection arises *de novo* in the host by recombination between the exogenous and endogenous viruses (Watanabe et al. 2013). Nonetheless, newer studies have suggested that FeLV-B may have a horizontal transmission. Erbeck et al (2021) evaluated 189 shared variants inside a cat population, confirming a high identity between the circulating viruses suggesting a horizontal transmission of FeLV-B. An additional study showed FeLV-B cross-species transmission in Florida panthers, a species without enFeLV, which must have arisen by a FeLV-B horizontal transmission (Chiu et al. 2019).

The increased oncogenic potential of FeLV-B has been linked with mediastinal and multicentric lymphoma. Experimentally, kittens inoculated with FeLV-B developed lymphoma within one year and died in less than a year post inoculation (Ahmad and Levy 2010). FeLV-C, formerly referred as C-Sarma, is also highly pathogenic and causes fatal aplastic anaemia in cats (Riedel et al. 1986). This pathogenicity is attributed to variation in the FeLV-C *env* gene that alters receptor usage to FLVCR1 for cell entry. This protein is the major heme exporter, its blockage interferes with signal transduction pathways involved in erythropoiesis, leading to cytotoxic accumulation of heme in erythroid precursors. Affected cats develop severe non-regenerative anaemia with macrocystis without reticulocytosis (Hofmann-Lehmann and Hartmann 2020).

There are other FeLVs, but these have been less well described (**Table 2**). FeLV-D has been detected in blood or tumour tissues of 1.1% of FeLV infected cats and is a recombinant between exFeLV and RD-114 (Anai et al. 2012). FeLV-T or FeLV-61C, is a T cell-tropic retrovirus that was detected in experimental infections (Overbaugh et al. 1988). Like FeLV-B, FeLV-T uses the phosphate transporter 1 to enter to the cell but also needs to interact with FeLIX (FeLV infectivity X-essory protein) as a co-receptor. FeLIX is encoded by a defective endogenous provirus secreted from feline T lymphocytes (Overbaugh 2000). Another experimental subgroup was the FeLV-FAIDS (feline acquired immunodeficiency syndrome), this was highly immunopathogenic infecting T lymphocytes and B lymphocytes, causing immunosuppression induced with substantial losses of helper cells and cytotoxic suppressor cells (Quackenbush et al.1996).

Table 2 The six main subgroups of FeLV described in domestic cats. The table describes main clinical signs, frequency and receptor used to access cells.

Subgroups	Frequency	Main clinical signs	Entry receptor
FeLV-A	~99%	Mildly pathogenic, highly contagious. Associated with immunosuppression and other FeLV associated diseases.	Thiamine transport protein 1 (THTR1)
FeLV-B	40%-60%	Hematopoietic neoplasia, nonreplicating and noncontagious, highly virulent	Phosphate transporter 1 (Pit-1)
FeLV-C	Rare	Nonregenerative anaemia, nonreplicating and noncontagious, highly virulent	FLVCR1
FeLV-D	Rare	Hematopoietic tumors (lymphoma and leukemia)	Not completely described
FeLV-E (TG35-2)	One case	Stomatitis and loss of appetite. Incompletely described	Feline Reduced Folate Carrier (RFC)
FeLV-T	Experimental	Highly cytolytic on immune cells leading to a severe immune suppression	Pit-1 and co-receptor FeLIX

The last variant described is FeLV-TG35-2, also referred to as FeLV-E. It was discovered in a 1-year-old cat with stomatitis and loss of appetite, but pathogenicity, prevalence and mode of action is incompletely understood (Miyake et al. 2016, Miyake et al. 2019), although a noteworthy characteristic of this variant is its use of the reduced folate carrier as a receptor to enter the cell (Miyake et al. 2019).

1.5.5 FeLV Transmission and Prevalence

The virus is excreted in bodily fluids, among them, saliva, nasal secretions, milk, urine and faeces (Torres, Mathiason, and Hoover 2005). The main transmission route is via saliva, therefore social behaviour such as grooming, sharing food and water dishes are the main risk behaviours for spread of the virus. Secondly and less probably, it can be transmitted via blood contact, milk, faeces, urine (sharing of litter trays) and during fighting by bites (Hartmann and Hofmann-Lehmann 2020). FeLV is quickly inactivated in the environment by disinfectants, heat, soap and drying, therefore it can be effectively controlled in veterinary hospitals by routine cage disinfection and hand washing between procedures on different patients (Möstl et al. 2015).

FeLV antibodies rise progressively as a consequence of viral exposure during the cat's life, reducing the risk of developing infection. Formerly, age had been described as a factor in resistance, independent of antibody presence (Hartmann 2012). However, in newer studies, adult cats have been shown to be more likely to develop progressive infection, though this has not been detected in all studies (Hofmann-Lehmann and Hartmann 2020).

Vertical transmission via the trans-placental route can occur during high viral load infections, also transmission from the queen to kittens is highly common during the nursing care of kittens. Additionally, regressively infected cats even if they have a negative p27 antigen test can spread the virus to their offspring. Focal atypical FeLV infection in utero can produce fetal resorption, abortion, and neonatal death (Hofmann-Lehmann and Hartmann 2020.

The prevalence can vary wildly in different geographical zones (**Table 3** and **Table 4**), usually, a lower prevalence is found in developed countries (1-8% prevalence) (Levy et al. 2006, Burling et al. 2017, Studer et al. 2019), in contrast to developing countries where the prevalence can be up to 60% in some cities (Ortega, C. et al. 2020, Muz et al. 2021, Capozza et al. 2021). Overall, in countries with strong awareness of the disease and preventive measures, including vaccination and early testing, the FeLV prevalence has been diminished in the domestic cat population. For example, in Germany, the prevalence reduced from 6% to 1% between 1993-2002 (Gleich, Krieger, and Hartmann 2009). However, over recent years, the prevalence in many countries has stagnated (Hofmann-Lehmann and Hartmann 2020, Giselbrecht et al. 2023).

Table 3 FeLV antigen prevalence in seven main areas around the world.

Geographic Area	Prevalence
North America	4%
Caribbean	9%
Latin America	13%
Northern Europe	7%
Southern Europe	12%
Middle East/Africa	14%
Asian/Pacific	6%

Extracted from Little et al. 2020

Various factors may increase the prevalence of FeLV detected including the health status of the cats. In sampling focused on non-specific clinical signs, the prevalence can beup to 38%, in contrast to healthy cat surveys where the prevalence of FeLV is around 8% of cats positive (Levy et al. 2006, Hofmann-Lehmann and Hartmann 2020). Cat behaviour is also linked to FeLV prevalence, whether they are an outdoor or strictly indoor cat will have a big effect on prevalence. Cat sex is predisposition factor, with male cats having higher predisposition due to aggressive and sexual behaviour interactions (Cristo et al., 2019; Garigliany et al., 2016; Spada et al., 2012). Older cats have also a higher predisposition to be positive (Garigliany et al. 2016). Cats that live in shelters under high control and management or areas with high cat density will have a higher prevalence rate of FeLV (Stavisky, Dean, and Molloy 2017). Furthermore, legislation and cultural issues affect FeLV prevalence. For example, designated stray cat protection areas in Italy increasing prevalence risk (Studer et al. 2019), while regions with lower income levels and reduced purchasing power often have weaker preventive measures and less effective animal control, contributing to higher FeLV prevalence (Ludwick and Clymer 2019).

Table 4 FeLV prevalence in domestic cats in different cities from Latin American countries.

Country	Prevalence	Type of diagnostic	Number of cats	Source
Brazil	22.26%	Ag p27	274	Biezus et al. 2019
	3%	Ag p27 / nPCR	200	Lacerda et al. 2017
	47.2%	nPCR	608	Coelho et al. 2008
Argentina	7.69% / 11.82%	Ag p27 / nPCR	255	Galdo Novo et al. 2016
Colombia	59.44%	Ag p27	96	Ortega, C. et al. 2020
	23.3%	Ag p27	60	Tique et al. 2009
Chile	33%	nPCR	78	Mora et al. 2015
	20.2%	nPCR	262	Sacristán et al. 2021
	20%	qPCR	50	Tabilo et al. 2018
	3.36%	PCR	50	Bilbao et al 2008.

*Ag= Antigen; nPCR= nested PCR; qPCR= real-time PCR; FeLV Pathogenesis

Once the virus infects the host, the virus goes to the local lymphoid tissue and then spreads using monocytes and lymphocytes (Rojko et al. 1979). The outcome of this infection is dependent on various factors linked to viral and host conditions. These factors include the age at the time of infection; the presence of humoral and cell-mediated immunity, mainly affected by vaccination status; the infection pressure, driven by the FeLV viral load in the environment and chronic viral exposure; and the virus virulence, dependent on which subgroup has developed and possible mutations near to proto-oncogenes (Hofmann-Lehmann et al. 2007, Flynn et al. 2002, Hofmann-Lehmann and Hartmann 2020). All these factors may have three possible outcomes:

Progressive infection: If the host does not develop an efficient and early immune response, the virus will have a strong and extensive replication in the cat, disseminating through lymphoid tissue, bone marrow, mucosal and glandular epithelial tissue. Viremia will persist for more than 16 weeks, and the cat will be persistently infected, continuously excreting virus. Thus, the virus can be detected by antigen tests after this period. The virus is excreted in body fluids, and the host starts to spread it to other animals (Hartmann 2012). These infected cats will have progressively lower levels or undetectable levels of neutralizing antibodies and a persistent viremia (over 16 weeks), resulting in the worst prognosis compared with other infection outcomes. The virus is readily transmitted, and the cat is highly likely to develop an FeLV related disease syndrome. Finally, the majority of these cats will die within three years (Torres, Mathiason, and Hoover 2005, Hartmann 2012).

Regressive infection: The regressor cat has an efficient immune response. The viral replication is restricted only to the first stage or an early bone marrow infection. In so doing, antibodies can be detected, the cat may have some mild clinical signs such as fever, lymphadenopathy, and immune deficiency, but the infection will be contained. The virus will be integrated into the cat genome, then the cat will be negative to antigen test but provirus positive (Hartmann 2012). The virus may be reactivated during immune-suppressive processes (following an infective stage, shedding viruses and clinical symptomatology). During pregnancy it is possible for the virus to be reactivated, linked to the immune suppressive actions of progesterone. Then, the regressive cats may test negative to p27 antigen between 1 to 6 weeks later if the virus is successfully controlled (Hartmann and Hofmann-Lehmann 2020). Viremia may also be reactivated when the antibody levels decline during cases of immunosuppression, such as stressful situation or pregnancy in queens. However, with elapsed time the future reactivation decreases, in fact, after one or two years, the likelihood of reactivation is drastically reduced. During reactivation events, p27 antigen may also be detected (Hartmann 2012).

Abortive infection: The viral replication is controlled by the immune system, led by effective humoral and cell-mediated immune responses. The unique sign of this infection is the presence of antibodies with no DNA or RNA detectable in blood or tissues, nor able to be cultured (Little et al. 2020). This type of infection has been largely demonstrated through experimental inoculation and negative viral diagnosis results. Because there is no viremia, direct viral detection methods are negative, and no evident clinical sign are observed. Abortive infection typically occurs following low-dose exposure and in cats that already possess antibodies from previous viral exposure (Hartmann and Hofmann-Lehmann 2020).

1.5.5.1 Tumoral activity

Among the most characteristic, clinical signs of FeLV are the development of malignant lymphomas and leukaemia as a consequence of viral provirus insertions close to cellular oncogenes, this dysregulation of these cellular oncogenes will produce a cell proliferation with different tumoral outcomes depending on which oncogene was affected (Hartmann 2012). The most common gene affected is c-*myc*, a proto-oncogene, furthermore alterations of *c-myc*

collaborators *bmi*-1 and *pim*-1 occur (Fujino 2003, Fujino, Ohno, and Tsujimoto 2008). This gene can be activated either by insertional mutagenesis near its locus or via transduction of the viral v-*myc* contained in some recombinant FeLV strains (Tsatsanis et al. 1994). This over-expression was observed in 32% of cats evaluated. Additionally, another five loci have been identified in lymphoma development, these are *flvi*-1, *flvi*-2, *fit*-1, *pim*-1 and flit-1, which are also implicated in tumorigenesis (Fujino et al. 2009, Tsatsanis et al. 1994, Levy et al. 1993).

1.5.6 FeLV diagnostics

The diagnosis of FeLV is complicated due to the variable course of infection. Commonly the routine diagnostic used in veterinary hospitals are tests based on detection of the p27 antigen, this is done through ELISA or immunochromatographic devices which detect a part of the capsid protein (Gag) that is highly abundant during viral replication (Little et al. 2020). These devices have a lot of advantages in that they are cheap, easy to manipulate, quick and have good sensitivity and specificity (98-99% for both parameters in some cases) (Levy, Crawford, and Tucker 2017). But this diagnostic is limited to the presence of viral antigen, namely, an active viremia. This will not occur in some infections like a regressor cat (Beall et al. 2019, Diesel et al. 2024). In general, these diagnostics have great levels of sensitivity and specificity (>95%), but different studies have been conducted using different reference standards, therefore the test quality could be different among the different manufacturers (Little et al. 2020, Levy, Crawford, and Tucker 2017).

In general, the detection of antigen in the indicates antigenemia and viremia. However, exceptions exist; for example, in regressive infections, low antigen levels may be undetectable or antigenemia may take longer to develop in some cats. Under natural conditions, only viraemic cats can excrete virus that is infectious for other cats. Thus, a cat with progressive infection and regressive infection in the early phase or after reactivation can be detected easily by an antigen test (**Table 5**) (Little et al. 2020).

Viral genome detection can be performed during an active replication to detect the RNA circulating in the blood or saliva by RT-PCR (Gomes-Keller et al. 2006). Alternatively, detection

can be through PCR detecting the proviral DNA in blood or bone marrow (Torres, Mathiason, and Hoover 2005). DNA detection allows detection of the virus during wider periods and kinds of infection. As shown in **Table 6** for a successful diagnostic in cats the use of a correct assay is critical (Hofmann-Lehmann and Hartmann 2020).

The detection of RNA from saliva only during the FeLV shedding phase has been used. This happens between 1-3 weeks post-exposure. This method can be useful to evaluate a group of cats sharing the same space, for instance, in shelter cats where resources can be scarce (Little et al. 2020, Gomes-Keller et al. 2006).

Table 5 Possible diagnostic results according to the target assay and kind of infection.

Diagnostic	Progressive Infection	Regressive Infection	Abortive Infection	Focal Infection
FeLV p27 antigen	Positive	Negative (positive during transient viremia of post-reactivation)	Negative	Alternating of low positive
Proviral FeLV (PCR)	Positive	Positive	Negative	Negative (low positive)
Anti-FeLV antibodies	Negative	Positive	Positive (variable titers)	Positive
Viral RNA (RT-PCR)	Positive	Negative (Some exceptions)	Negative	Negative (Some exceptions)
Virus Shedding	Positive	Negative	Negative	Variable

Due to the characteristics of surveys in wild species, there has been much discussion about which diagnostic should be used. For several years, antigen assays were used (Daniels et al. 1999, Marker et al. 2003, Ramsauer et al. 2007) but this has only limited information and can detect during only specific infection conditions (**Table 5**). Afterwards, with the increase of access to viral molecular methods, most surveys have been designed to detect viral genomes, especially detecting the provirus in regressor felids. Overall, the detection of the U3-LTR has been widely used to diagnose wild felids as it is a highly conserved gene (**Table 7**) encompassing a wide range of FeLV variants, and distinguishing between enFeLV and exFeLV (Torres, Mathiason, and Hoover 2005, Cattori et al. 2006). The main disadvantage of LTR

analysis is that is not specifically able to differentiate between the FeLV variants in the majority of cases (Chiu et al. 2019)

Subsequently, studies have been conducted using alternatives to PCR, like nested PCR (nPCR), to improve the specificity and sensitivity. Although these techniques are limited by drawbacks such as high possibilities of contamination and the long process to achieve the diagnostic or analyses (Franklin et al. 2007). Nowadays, access to new technology in molecular analysis, like the use of next-generation sequencing (NGS) has allowed changes to the traditional approach to research in FeLV, assessing the whole viral genome or at least the *env* gene (specifically SU region) to describe different variants of FeLV and possible recombination variants circulating in wild and domestic cats (Geret et al. 2011, Chiu, Hoover, and Vandewoude 2018).

1.5.7 FeLV in wild felids

Several non-domestic species have been described with FeLV infection. Using p27 antigen tests, FeLV has been diagnosed in cheetahs (*Acynonyx jubatus*) (Marker et al. 2003, Krengel et al. 2015); african lions (Ramsauer et al. 2007); wildcats (*Felis silvestris)* (Daniels et al. 1999, Heddergott et al. 2018); iberian lynx (Luaces et al. 2008, Meli et al. 2010a, Nájera et al. 2024), pampas cat (Filoni et al. 2012); puma (Cunningham et al. 2008), and tigers (Tangsudjai et al. 2010), among others. Regarding molecular diagnostics, FeLV has been detected in fewer species, usually, using diagnostics detecting the LTR segment. Additionally, more studies have been conducted in free-range animals than captive animals (**Table 6**). Two wild species have suffered outbreaks of FeLV: the Iberian Lynx (*Lynx pardinus*) and Florida Panthers (*Puma concolor*) (Nájera et al. 2024, Chiu et al. 2019).

Another factor involved in the outcome of an FeLV infection in wild populations is the reduced genetic variability in the host, being more susceptible in those bottle-necked populations with lower heterozygosity (Chiu et al. 2019, O'Brien et al. 2006). Linked to these factors, the loss and fragmentation of habitat provoke an increase of risk factors facilitating the disease spreading,

because this can cause some effects such as reduction of animal numbers, isolation of populations and close contact with domestic animals (Millán et al. 2009).

Table 6 Wild felid species FeLV positive diagnosed by molecular assays, origin of the animals, type of molecular assay, genes analysed, and fragment amplified (if fragment was amplified).

Species	Origin	Type of diagnostic	Gene analysed	Source
Acinonyx jubatus	Wild	PCR	*Env* (SU*)* 155-bp	Marker et al. 2003
Felis bengalensis	Captive	nPCR	LTR (166bp)	Tangsudjai et al. 2010
Felis silvestris	Wild	PCR	enFeLV	Ngo et al. 2019
	Wild	PCR	U3-LTR	León et al. 2017
Herpailurus yagouaroundi	Wild	qPCR	U3-LTR	Filoni et al. 2012
Leopardus guigna	Wild	nPCR	U3-LTR	Mora et al. 2015
Leopardus pardalis	Captive	nPCR	LTR-Gag (601bp)	Guimaraes et al. 2009
Leopardus tigrinus	Captive	nPCR	LTR-Gag (601bp)	Guimaraes et al. 2009
Lynx pardinus	Wild	PCR	U3-LTR	Luaces et al. 2008
	Wild	PCR	U3-LTR	Meli et al. 2009
	Wild	PCR	U3-LTR	Nájera et al. 2021
	Wild	PCR and NGS	*env* (794 bp)	Geret et al. 2011
Neofelis nebulosa	Captive	nPCR	LTR (166bp)	Tangsudjai et al. 2010
Panthera pardus	Captive	nPCR	LTR (166bp)	Tangsudjai et al. 2010
Panthera tigris	Captive	nPCR	LTR (166bp)	Tangsudjai et al. 2010
Prionailurus viverrinus	Captive	nPCR	LTR (166bp)	Tangsudjai et al. 2010
Puma concolor	Wild	PCR	*env*-LTR (437bp-1,700bp)	Brown et al. 2008
	Wild	PCR	Whole genome (8,448bp)	Chiu et al. 2019
	Wild	qPCR	LTR and *env*	Chiu and VandeWoude 2020

The Iberian lynx was one of the most endangered felids with small populations inhabiting Spain (Meli et al. 2009). The main threat has been the decline of its basic prey, fragmentation and loss of habitat and direct anthropogenic factors such as poaching and road kills (Palomares et al.

2010). The strong population reduction up to early 2000s , contributed to a reduced genetic variability in the remaining populations (Johnson et al. 2004). Thus, an FeLV outbreak happened in 2006 where out of 12 lynxes, 11 were found positive for p27 antigen and seven of them were found dead (Meli et al. 2010a). While the first interventions to support the Iberian lynx population were to add supplementary feeding stations and drinking points, the presence of positive animals prompted an FeLV control program. This program considered: removing viraemic lynxes, vaccinating susceptible animals, eliminating domestic cats in the habitat, and changing or directly eliminating structures to reduce contact between the animals (López et al. 2009).

Molecular analysis was carried out targeting the SU of the *env* gene, amplifying 1302bp. The phylogenetic analysis showed 95-100% identity with a previous outbreak in the lynx's population and 97.4% identity to FeLV-A/Rickard. The evidence suggested that at least two virus introductions to the population had occurred, being highly probably spill-over from the domestic cat (Meli et al. 2010a).

The continuous surveillance of the Iberian lynx involves detection of the pathogen in the domestic cat population. Indeed, sympatric (animals sharing geographical areas) cats living in reintroduction areas have shown a frequency of infection between 30% to 46.4% for FeLV (León et al. 2017). This situation is further aggravated by predation of domestic cat by Iberian lynx, highlighting the importance of preventive measures (Nájera et al. 2019).

The second outbreak occurred in Florida panthers, a sub-species of the puma (*Puma concolor coryi)*. These are a population threatened by hunting and habitat destruction, causing an increase of in-breeding and reduction of genetic variability (Roelke, Martenson, and O'Brien 1993). Thus, the FeLV outbreak started in 2001, where p27 antigen was detected in some animals and dead animals were soon found (Cunningham et al. 2008). Using primers to amplify *env* (437bp and 1,689bp) and *env*/LTR (725bp) molecular characteristics showed high similarities among the puma strains and indicated a possible origin from a domestic cat (Brown et al. 2008).

In the following years, various interventions have been carried out, like surveillance leading directly to FeLV detection, vaccination campaigns and elimination of feral cats from protected

areas to eradicate the FeLV risk. Nonetheless, a newer study conducted during 2010-2016, to describe the whole genome of FeLV found at least two introductions of domestic cat FeLV in the puma population (Chiu et al. 2019).

A noteworthy event described by Chiu et al (2019) is the record of a puma infected by a FeLV-B variant. As was described previously, FeLV-B arises from recombination between enFeLV and FeLV-A but the enFeLV is lacking in felidae outside the *Felis* genus. Therefore, the only spread possible to a puma is through horizontal transmission. This has been described in jaguar and guiña but in both cases only small fragments of the LTR gene (230bp roughly) were analysed (Silva et al. 2016, Sacristán et al. 2021). The presence of FeLV-B (a highly oncogenic variant) in wild felids lacking enFeLV, could represent a great threat because these animals do not have the protection conferred by enFeLV against exFeLV (Chiu and VandeWoude 2020).

1.5.8 FeLV Vaccine

To achieve effective disease control, it is necessary to implement general testing of infected cats and vaccination of susceptible animals, contrasting to other retroviruses, FeLV vaccination has been highly successful to control the disease (Hofmann-Lehmann et al. 2007). Indeed, unvaccinated cats with bite wounds were 7.5 times more likely to be infected by FeLV than vaccinated cats (Goldkamp et al. 2008).

Among the available vaccines there are adjuvanted, inactivated whole virus, recombinant subunit vaccines and genetically engineered subunit recombinant canarypox vector vaccines (Little et al. 2020). These were all developed using FeLV-A sub-type (Rickard strain or Glasgow strain) either whole virus or specific elements from the envelope glycoproteins and capsid proteins (Patel et al. 2015, Sparkes et al. 2015).

Different vaccines may have different levels of protection (Westman, Malik, and Norris 2019a), and some vaccines have failed to prevent provirus integration (Torres et al. 2010). However, all vaccines are efficacious at preventing progressive infection, therefore avoiding the worst clinical outcomes (Little et al. 2020).

Several studies showed better levels of protection from whole virus adjuvanted vaccines compared with recombinant canarypox-vectored vaccines (Stuke et al. 2014, Patel et al. 2015). Although, a newer study found no statistical differences between both vaccine groups and suggested that the previous discrepancy in the results could be influenced by the different methods of viral challenge or experimental conditions in the animals evaluated (Grosenbaugh et al. 2017). Better performance of whole virus adjuvanted vaccine in immune-compromised animals has also been reported (Patel et al. 2015).

The vaccination guidelines suggest establishing negative FeLV test prior to vaccination, since the FeLV vaccine has no therapeutic value and is an unnecessary vaccination in infected cats (Little et al. 2020). The focus of control programs is the vaccination of all kittens and susceptible negative cats tested. The immunization of younger cats is key because they are more susceptible to developing a progressive infection and more likely to engage in high-risk behaviours, such as sexual activity, outdoor roaming, and physical aggressions (Scherk et al. 2013).

Overall, the vaccination scheme includes two doses, as early as eight weeks old, followed by the second dose 3-4 weeks later. Then, a booster at 1-year should be administered. The next booster is dependent on whether the cat has a low risk of infection, in which case the vaccine would not be necessary. The cats at lower risk should be vaccinated every two years and cats at higher risk should be carried out yearly. According to the Feline Vaccination Advisory Panel, the recommended site to administer is subcutaneously in the left hindlimb distal to the stifle joint, to facilitate complete tumour removal in the event that feline injection-site sarcoma develops (Little et al. 2020). According to the WSAVA (World Small Animal Veterinary Association) FeLV vaccines should be considered as core vaccines for young cats and adult cats with outdoor lifestyle in high-risk areas and cats should be annually revaccinated in high-risk areas (Squires et al. 2024). Mean-while, in areas of low FeLV prevalence, vaccines have been taken out of market due to low sale volumes, increasing the risk of new outbreaks (Luckman and Gates 2017).

FeLV vaccination in wild species has been conducted in Iberian lynxes and Florida panthers. In the first case, the animals have routinely had a recombinant vectored vaccine applied from 2009 (Meli et al. 2009) until now (Nájera et al. 2021). While the authors suggest that the immunization program has contributed to protection against FeLV infection (Nájera et al. 2021), this has not been confirmed by objective methods.

In the case of Florida panther, the immunization program started in 2003 (Cunningham et al. 2008) and was continuously carried out until 2007. In the following years, the Florida panthers were only opportunistically vaccinated. In 2010, cases associated with a new introduction of FeLV from the domestic cat were detected (Chiu et al. 2019). Therefore, the success of the immunization program has not been evaluated.

Aims of the Thesis

Describe epidemiology and phylogenetic relationships of FeLV circulating among free-range and captive wild felid populations inhabitant in Chile to determine viral factors (e.g., strain, risk factors, recombination events) associated with spillover from domestic cats or establishment of endemic circulation in Chilean wild felids. The thesis will lay essential groundwork for the development of FeLV control programmes in endangered Chilean felids.

1.5.9 Objectives

1. Determine spill over from domestic cats by sequencing *env* gene PCR from both, domestic and wild felids, examining phylogenetic trees of the output.
2. Determine if FeLV-A has established within host transmission in guiña in Chile and if FeLV B transmission is occurring.
3. Determine the optimal sequencing method for exFeLV variants and quasispecies (Sanger sequencing, Illumina or Nanopore sequencing).
4. Modelling risk factors to assess the impact of FeLV in Chilean cats. Modelling survival analysis to investigate the long-term effects of FeLV in a UK shelter.

Chapter 2: Modelling Survival Analysis of Domestic Cats in the UK and FeLV prevalence and Risk Factors in Chile

2.1 Lifetime Analysis: 20-year lifetime histories of FIV and FeLV infected Domestic Cats in a Shelter in the UK indicates an impact of retroviral infection on longevity.

This section includes contributions from:
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Statement of contribution: This section of the work has been prepared for manuscript submission. Cristobal Castillo-Aliaga performed the statistical analysis, wrote and edited the text, Stephen Dunham (SVMS) collected data, managed stakeholder interaction, conceptualised the study and edited the manuscript, Helen McCallum-Chanter collated the original data into a database and performed initial univariate analysis as part of her undergraduate degree requirements, Rachael Tarlinton conceptualised the study, and edited the manuscript.

2.1.1 Abstract

This study presents retrospective analysis of life expectancy in domestic cats housed at a feline sanctuary in the United Kingdom. The sanctuary primarily houses cats infected with feline immunodeficiency virus (FIV), feline leukaemia virus (FeLV) and cats with behavioural problems. Lifetime records included 22 retrovirus negative cats, 82 FIV, 24 FeLV, and 4 co-infected (FeLV/FIV^{+}) cats. Kaplan-Meier survival analysis was used to assess life expectancy based on their retroviral status. Clinical conditions commonly associated with retroviral infections, such as respiratory, ophthalmic, gastrointestinal signs, weight loss, neoplasm, oral disease, and anaemia were also analysed.

The median life expectancy was 160.3 months for uninfected cats, 149.1 months for FIV^+ cats, 41.5 months for $FeLV^+$, and 74.2 months for FeLV/FIV co-infected cats. A highly significant reduction in survival time was observed for $FeLV^+$ cats ($p<0.000$), while a smaller difference was observed between FIV^+ and uninfected cats ($p=0.037$). Neoplasia was significantly associated with FeLV infection ($p<0.000$), with lymphoma being the most common tumour type (76.9%). Oral disease was significantly associated with FIV infection ($p=0.004$). Male cats were overrepresented in the FIV positive group (87.8%), and to a lesser extent in the $FeLV^+$ group (62.5%). The uninfected group had a balanced sex ratio.

In conclusion, FIV infection did not significantly impact life expectancy except in extremely aged cats, with FIV positive individuals reaching lifespans similar to the general UK cat population. In contrast, FeLV infection was associated with a markedly reduced lifespan and a higher incidence of lymphoma.

2.1.2 Introduction

Three feline retroviruses have been described: feline foamy virus, feline immunodeficiency virus (FIV), and feline leukaemia virus (FeLV). However, only the latter two are considered a threat to cat health. FIV has attracted interest in the past due to its similarity with Human Immunodeficiency Virus (HIV), and it was used as model for retrovirus research (Dunham 2006, Bęczkowski and Beatty 2022).

FIV belongs to the genus *Lentivirus* and is primarily transmitted horizontally through saliva via bite wounds. Iatrogenic transmission through parenteral routes has also been demonstrated (Burkhard and Dean 2005). Although, the risk of transmission is low in cats sharing a household with minimal fighting (Litster 2014), the possibility of infection always exists (Addie et al. 2000).

FIV infected cats exhibit general clinical signs such as fever, skin and ocular infections, lymphadenopathy, mild and severe oral problems, weight loss, lethargy, urinary issues, and upper respiratory tract infections (Little et al. 2020). Progression of disease in some cats can lead to an immune deficiency syndrome associated with opportunistic infections, hematopoietic disorders, or neoplasia (Hartmann 2012). However, FIV is now regarded as relatively well

adapted to cats with the majority of infected cats living a close to normal life span and retroviral induced clinical signs tending to become a problem only later in the disease course (Möstl et al. 2015).

On the other hand, FeLV, a gamma-retrovirus with a high virulence in infected cats has been associated with a significant reduction in cat's lifespan (Spada et al. 2018, Beall et al. 2019). The main clinical syndromes associated with FeLV include tumours, immunosuppression, neurological disorders, and bone marrow suppression (Lutz, Hunsmann, and Schüpbach 2010). The clinical outcome and prognosis will be led by viral factors and host immune response, leading to four clinical courses: regressive, progressive, abortive, or focal (Torres, Mathiason, and Hoover 2005, Hofmann-Lehmann and Hartmann 2020, Beall et al. 2019). In abortive and focal infections, the viral replication is controlled and does not produce viremia, therefore the infection status is difficult to determine in natural conditions (Major et al. 2010). In regressive cats, the immune system controls the infection and minimize the effects on cat health (Helfer-Hungerbuehler et al. 2015), whereas progressive infections lead to severe clinical conditions, with cats having shorter survival times than regressive cats (Hofmann-Lehmann et al. 2007, Helfer-Hungerbuehler et al. 2015, Beall et al. 2021).

Survival analyses comparing the effects of FeLV and FIV have been conducted, showing strong evidence of the differing outcomes for these retroviral infections (Spada et al. 2018, Kent et al. 2022, Luckman and Gates 2017). In general, FIV infected cats have shown close to normal life expectancy whereas FeLV infection has been strongly correlated with a shorter life expectancy, with most infected cats dying within four years after initial infection (Addie et al. 2000, Ravi et al. 2010, Liem et al. 2013, Kent et al. 2022). Regarding FeLV infection, cats maintained under good conditions may have longer life expectancy (Spada et al. 2018). For example, progressively infected cats kept under experimentally conditions have lived up to 6.5 years, whereas cats regressively infected and clinically healthy have lived up to 12.7 years with the virus (Helfer-Hungerbuehler et al. 2015).

Survival analyses have been conducted in various scenarios, including shelters or multi-cat environments under natural infection conditions (Addie et al. 2000, Beczkowski et al. 2015, Beall

et al. 2019), as well as under experimental conditions (Kohmoto et al. 1998, Helfer-Hungerbuehler et al. 2015). Other studies have used clinical records from owned cats, having the disadvantage of high numbers of individuals lost to follow-up for reasons such as voluntary euthanasia (used as population control for FIV^+ or $FeLV^+$ cats), traffic accidents, or unexplained disappearances (Gleich, Krieger, and Hartmann 2009, Ravi et al. 2010, Liem et al. 2013, Spada et al. 2018). Studies conducted in closed environments like shelters have the advantage of keeping strict supervision over cats and similar feeding conditions, veterinary access and general care.

In the UK, the widespread use of highly effective vaccines against FeLV has led to a significant decrease in disease prevalence (Studer et al. 2019), however no effective vaccines are available in the UK for FIV (Dunham 2006, Bęczkowski and Beatty 2022). In one study in 2002 conducted in a RSPCA (Royal Society for the Prevention of Cruelty to Animals) shelter for stray cats in Birmingham, they found infection rates of 10.4% for FIV, and 3.5% for FeLV (Muirden 2002). Later, another study conducted between 2011 and 2012 at two RSPCA rehoming centres in the Midlands reported an FIV prevalence of 11.4% and FeLV prevalence of 3% at one site, while the other site showed 3% for FIV with no FeLV-positive cases. These differences may be explained by different strategies for disease control implemented at each institution (Stavisky, Dean, and Molloy 2017). FIV and FeLV have been common reasons for euthanasia in shelters (Murray, Skillings, and Gruffydd-Jones 2008), though this has been slowly changing during the last decades.

In this study, we present a survival analysis of cats housed in a shelter with a high number of individuals infected with feline retroviruses. This shelter aims to provide long term care for FIV and FeLV positive cats. Most of these cats were admitted after being displaced from other homes or shelters due to their retroviral status. Each cat was monitored throughout its lifetime, until it either died of natural causes or was humanely euthanized to prevent unnecessary suffering in cases of severe illness.

2.1.3 Methods

2.1.3.1 Data collection

The dataset was retrospectively collected from paper and electronic records, from Quantock Veterinary Hospital, of a cohort of cats that have lived at Catwork Sanctuary in Somerset (UK). Data were collected until a cat's death was recorded. Catwork Sanctuary is a private sanctuary for cats difficult to rehome, most of them stay at Catwork until their death, rarely being rehomed. The centre focus on FIV positive, FeLV positive and cats with severe behaviour problems. The cat origin was variable, including previously stray cats (mainly kittens), or sourced from owner's houses, veterinary practices, or other shelters where they were going to be euthanized. The cats at Catwork shared areas according to their social requirements and retroviral status, FeLV and FeLV/FIV^{+} cats were co-housed separately to other animals, FIV and non-infected cats were however free to mix. Datasets included estimated date of birth (assigned as January 1st of the respective year if the exact date was not available), date of death, retroviral status, and clinical records. Data from 132 cats were collected and entered manually into an Excel spreadsheet (McCallum 2015).

The diagnostics were conducted using two methods according to availability and some records included confirmatory test results. The methods were mostly commercial rapid enzyme-linked immunosorbent assay (ELISA) kit (SNAP® Combo Plus FeLV Ag/FIV Ab, IDEXX Laboratories, Europe) which are very sensitive and specific methods. For FeLV this test has a 100% sensitivity and specificity. For FIV this is 97.9% sensitivity for antibody presence, and 99% specificity (Levy, Crawford, and Tucker 2017). Some cats were retested at the centre using a commercial laboratory PCR test conducted at the University of Glasgow.

The cat dataset was categorized by retroviral infection status: FIV positive (FIV^{+}), FeLV positive (FeLV^{+}), co-infected (FeLV/FIV^{+}), and non-infected. Age at infection or arrival was grouped into four categories: Kittens (between 1 month to ≤6 months), young adult (>6 months to 2 years), adult (>2 years to 6 years) and old (>6 years old).

From clinical records, data related to retroviral pathogenesis were retrieved. The data were grouped in binary categories for statistical analysis. The categories selected were sex (female or male), reproductive status at arrival (neutered or intact), and clinical conditions were collected in binary mode (yes or no) and these were: neoplasm diagnosis, ophthalmic signs, chronic diarrhoea (persistent for at least 3 months or until death), respiratory signs, anaemia, at least 20% of weight loss during last year before death, and oral diseases.

2.1.3.2 Statistical Analysis

The dataset was exported, and descriptive analysis were calculated in StataCorp (2023) with Fisher's exact test, and these are presented in **Table 7**.

Kaplan-Meier survival analysis was conducted in Rstudio Team (2020), using the package "survival" and pairwise comparison was done using the Mantel-Cox log-rank test. Plots were drawn in the "survminer" package. Survival times were classified in four groups based on the viral test result ($FeLV^+$, FIV^+, $FeLV/FIV^+$, or non-infected) and were calculated from the date of birth to the date of death (natural or euthanasia). Kaplan-Meier survival analysis was performed to estimate median survival of cats infected by retroviruses, and cats were censored (excluded) if date of birth was missing. Age comparisons were performed using the non-parametric Mann-Whitney *U* test for un-paired samples to calculate statistical differences between the median ages of the groups evaluated.

Since identifying the exact moment of infection is not possible for FIV or FeLV under natural conditions due to their subtle initial clinical signs, calculating survival time post-diagnosis was not the primary aim of this study. However, median survival time from diagnosis or arrival at the shelter (as all cats were positive at arrival) was used to categorize and compare survival times with previously published studies.

The chosen variables were selected according to biological significance and are presented with 95% confidence intervals, these were: sex, reproductive status before diagnosis, neoplasia cases, ophthalmic signs, diarrhoea during the last year of life, respiratory signs, anaemia, oral

diseases and weight variation during the last year of life. Univariable Cox regression analysis was performed, and variables with a *p*-value <0.2.

2.1.3.3 Results

Out of 132 cats that lived in the Catwork Sanctuary, death was recorded for 127 cats. With regards to their infection status: 22 (16.7%) were negative for both viruses, 82 (62.1%) were positive for FIV, 24 (18.2%) were positive for FeLV, and 4 (3.0%) were FeLV/FIV^{+}. Regarding to the cat's sex, males were overrepresented, with 102 (77.3%) males compared to 30 (22.7%) females.

Kaplan-Meier survival analysis was performed for 127 cats and observations from 6 cats were censored as no date of birth records were available. The Kaplan-Meier curve (**Figure 12**) shows a significant influence on life expectancy (*p*-value<0.0001) based on the type of viral infection. The median survival for all cats was 136.8 months (CI 95%: 128.4 to 148.8 months). FeLV^{+} cats had a median survival of 41.5 months (CI 95%: 32.7-71.0 months), while FIV^{+} cats showed a median survival time of 149.1 months (CI 95%: 137.9-158.1 months). Non-infected cats had a median survival of 160.3 months (CI 95%: 138.1-206.2 months), and FeLV/FIV dual positive (FeLV/FIV^{+}) cats had a median survival of 74.2 months (CI 95%: 36-NA).

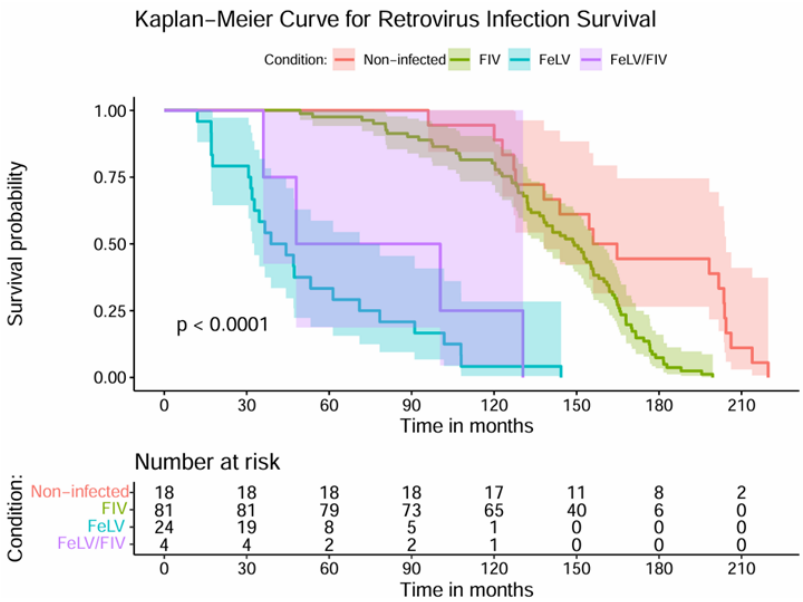

Figure 12 Kaplan-Meier analysis curve showing comparison life expectancy of cases of FeLV (light blue), FIV (green), FeLV/FIV⁺ (purple), and uninfected (red) cats. Time zero indicates birth of cats.

The exact diagnosis date was available for 33 (40%) FIV cats and 20 (83%) FeLV cats, for other cats the arrival date to death was considered for median survival time calculation. The median age at FIV diagnosis was 75 months (6.25 years), and 27 months (2.25 years) for the FeLV cohort. The median survival time after diagnosis or arrival was 48 months for FIV⁺ cats, 16 months for FeLV⁺ cats. The Mann-Whitney *U* test showed that FeLV⁺ cats had a significantly lower survival time ($p<0.000$) compared to FIV⁺ cats. Due to the low number of dual FeLV/FIV⁺ positive cases were not considered further in the survival analysis.

The most common pathologies associated with retroviral infections identified in this cat population are listed in **Table 7**. The occurrence of neoplasia, anaemia, respiratory signs, and oral diseases were identified as statistically significant using Chi-square or Fischer's exact test. Thirteen (54.2%) neoplasia cases were observed in FeLV⁺ cats, 9 (10.9%) were in FIV⁺ cats, 4 (18.2%) were in FeLV/FIV⁺ cats. Ophthalmic signs were observed in 17 cats: 2 (8.3%) were FeLV⁺, 12 (14.6%) were FIV⁺, 3 (13.6%) cases of uninfected. Chronic diarrhoea (duration >3 months) was observed in 17 cats: 1 (4.2%) was FeLV⁺, 15 (18.3%) were FIV⁺, and 1 (4.5%) was uninfected. Twenty-six cats were diagnosed with respiratory signs, of these 4 (16.7%) were

FeLV$^+$, 16 (19.5%) were FIV$^+$, 6 (27.3%) were uninfected. Seventeen cats were diagnosed with anaemia: 4 (16.7%) were FeLV$^+$, 11 (13.4%) were FIV$^+$, 2 (50%) were FeLV/FIV$^+$, and uninfected cat had anaemia. Cats that had weight loss during the year before death were 61, out these 8 (33.3%) were FeLV$^+$, 44 (53.7%) were FIV$^+$, 1 (25%) were FeLV/FIV$^+$, and 8 (36.6%) were non-infected. Finally, 60 cats had oral diseases, 6 (25%) were FeLV$^+$, 47 (57.3%) were FIV$^+$, 1 (1.7 %) was FeLV/FIV$^+$, and 6 (27.3%) were uninfected. Conditions that demonstrated a statistical association with retroviral status in univariate analysis (chi squared or fishers exact test) were neoplasia and anaemia for FeLV$^+$, and oral diseases for FIV$^+$. Due to the low numbers of animals in many categories further multivariate analysis of clinical conditions was not attempted.

With regards to tumours found among the cats evaluated, 14 (50%) were confirmed as lymphomas, 5 (17.9%) were identified as thoracic tumours (including mediastinal mass, lung tumours, and undetermined thoracic tumour), 3 (10.7%) were liver tumours, 2 (7.1%) were soft tissue sarcomas, and 1 (3.6%) jaw tumour, 1 (3.6%) stomach tumour, 1 (3.6%) oral tumour, 1 (3.6%) case was only recorded as “cancer”. For FeLV$^+$ cats the median survival age of cats without tumours was 3.9 years, and cats with tumours was 3 years. Of total FeLV$^+$ tumours, 10 (76.9%) were lymphomas, two (15.4%) were chest tumours, and the remaining case was a jaw tumour. In the FIV$^+$ cats, the median age of cats without tumours was 12.7 years and cats with tumours was 10 years old. FIV$^+$ cats showed 2 cases each of lymphoma (18.2%), sarcoma (18.2%), liver tumour (18.2%), and thoracic tumour (18.2%), as well as 1 case each of oral tumour (9.1%), stomach tumour (9.1%), and undetermined tumour (9.1%). Finally, two cases of lymphoma (50%), and 1 case each of liver (25%), and thoracic neoplasia (25%) were identified in uninfected cats.

Table 7 Descriptive statistics of clinical records grouped by retrovirus condition.

Variable	Modalities	Group				
		Negative (n =25)	FIV^+ (n = 83)	$FeLV^+$ (n = 24)	$FeLV/FIV^+$ (n = 4)	*p*-value
Sex	Female	11 (50)	10 (12.2)	9 (37.50)	0 (0)	0.000
	Male	11 (50)	72 (87.8)	15 (62.50)	4 (100)	-
Reproductive Status	Neutered	-	55 (67.07)	12 (50)	1 (25)	0.000
	Intact	1 (4.55)	9 (10.98)	-	-	-
	n/a	21 (91.45)	18 (21.95)	12 (50)	3 (75)	-
Age at infection or arrival	Kitten	-	1 (1.27)	4 (17.39)	0 (0)	0.000
	Young Adult	-	5 (6.33)	11 (47.83)	2 (50)	-
	Adult	-	37 (46.84)	8 (34.78)	2 (50)	-
	Old	-	36 (45.57)	-	-	-
Tumour	No	18 (81.82)	71 (86.59)	11 (45.83)	4 (100)	0.000
	Yes	4 (18.18)	11 (13.41)	13 (54.17)	-	-
Ophthalmic	No	19 (86.36)	70 (85.37)	22 (91.67)	4 (100)	0.736
	Yes	3 (13.64)	12 (14.63)	2 (8.33)	-	-
Diarrhoea	No	20 (90.91)	63 (76.83)	23 (95.83)	4 (100)	0.07
	Yes	2 (9.09)	19 (23.17)	1 (4.17)	-	-
Respiratory	No	16 (72.73)	66 (80.49)	20 (83.33)	4 (100)	0.58
	Yes	6 (27.27)	16 (19.51)	4 (16.67)	-	-
Anaemia	No	22 (100)	71 (86.59)	20 (83.33)	2 (50)	0.03
	Yes	0 (0)	11 (13.41)	4 (16.67)	2 (50)	-
Weight Loss	No	14 (63.64)	38 (46.34)	16 (66.67)	3 (75)	0.17
	Yes	8 (36. 63)	44 (53.66)	8 (33.33)	1 (25)	-
Oral Disease	No	16 (72.73)	35 (42.68)	18 (75)	3 (75)	0.004
	Yes	6 (27.27)	47 (57.32)	6 (25)	1 (25)	-

2.1.4 Discussion

This study followed 132 cats, including non-infected, FIV^+, $FeLV^+$, and $FeLV/FIV^+$ individuals, over a 20-year period. All cats resided at Catwork Sanctuary, a centre providing care for retrovirus infected cats to avoid euthanasia. This explains the exceptionally high frequency of retroviral infection observed in this study compared to other survival analyses. However, the low impact of FIV^+ and high impact of $FeLV^+$ on longevity observed here are consistent with previous studies (Addie et al. 2000, Gleich, Krieger, and Hartmann 2009a, Ravi et al. 2010, Liem et al. 2013, Spada et al. 2018, Kent et al. 2022, Luckman and Gates 2017). While low transmission rates and low viral loads for FIV^+ have been suggested as factors contributing to its limited impact on cat lifespan (Courchamp et al. 1995, Gleich, Krieger, and Hartmann 2009), this study provides further robust evidence supporting the low impact of FIV^+ in a population with high infection frequency. In contrast, $FeLV^+$ cats experienced a severe reduction in lifespan compared to the other groups, with two-thirds of them dying before 5-of age.

Interestingly, a small difference was identified when comparing FIV^+ and uninfected cats, with uninfected cats showing a slightly longer survival time. The Kaplan-Meier analysis demonstrated

a similar curve between FIV⁺ and uninfected cats until approximately month 165 (13 years), at which point the survival of FIV⁺ cats continued declining, while that of uninfected cats showed an extension due to the extreme longevity of eight cats evaluated. These cats were over 17 years old, an unusually long-life expectancy for domestic cats. In the UK, the general life expectancy for domestic cats is 11.74 years (Teng et al. 2024), which is similar to the lifespan observed in our FIV⁺ cats (12.4 years). This unique lifetime cohort study provides evidence that under consistent husbandry conditions, while FIV⁺ cats can be expected to attain an average life span, they are less likely to live to extreme old age. The factors that determine whether a cat progresses to the terminal-stage or remains asymptomatic are still unknown though the virus likely has a subtle effect on maximum possible lifespan (Kohmoto et al. 1998, Bęczkowski and Beatty 2022)

The survival analysis was not conducted from the time of diagnosis to death, as FIV infection typically presents with subtle clinical signs, making the exact timing of natural infection difficult to determine (Bęczkowski and Beatty 2022). Therefore, the median age at diagnosis and survival times were calculated solely for comparison with previously published studies.

In our study, the median age at FIV diagnosis was slightly higher than that reported in earlier studies (Levy et al. 2008, Gleich, Krieger, and Hartmann 2009, Ravi et al. 2010). However, when considering only cats with the exact diagnosis date (excluding arrival date), the age aligned more closely with previous reports.

Regarding survival times, while published studies report FIV⁺ survival ranging from 2.0 to 5.6 years (Gleich, Krieger, and Hartmann 2009, Levy et al. 2006, Ravi et al. 2010, Spada et al. 2018), our findings indicated a median survival time of 4.0 years post-diagnosis. It should be noted that this likely represents an underestimate of total survival time with infection, as the actual survival time may be substantially longer, considering that cats were not always immediately moved to Catwork after their initial diagnosis.

Our study included a more complete dataset for FeLV⁺ diagnosis compared to FIV⁺ cases. The median age at diagnosis (approximately 2 years) was consistent with previous reports (Gleich, Krieger, and Hartmann 2009, Spada et al. 2018). However, we observed a shorter median

survival time (1.3 years) for FeLV$^+$ cats. Other reports have described survival times between 2.4 to 4 years (Levy et al. 2006, Spada et al. 2012, Kent et al. 2022). FeLV$^+$ cats lifespan varies significantly depending on infection type (i.e. regressive, progressive, abortive). In experimentally infected cats with a progressive infection, the median survival time was 3.1 years (0.6-6.5 years) (Helfer-Hungerbuehler et al. 2015). However, cats with higher viral loads had a median survival of 1.37 years (Beall et al. 2021).

With regards to neoplasia diagnosis, the uninfected cohort showed that 18.8% of cats had a neoplasia diagnosis, while the FIV$^+$ cats had a lower incidence, with 10.9% cases. Base rates of neoplasia in the UK are likely around 10.8% based on a report evaluating over 3,000 cats (O'Neill et al. 2015). This is very similar to our FIV$^+$ cohort and is within normal range of the general cat population in the UK (O'Neill et al. 2015). Whereas the extreme age of many animals in the retroviral negative cohort in this study may have inflated the neoplasia prevalence, which increases with age in cats as with other species (Manuali et al. 2020).

In contrast, around half of FeLV$^+$ cats died due to neoplasia . Although domestic cats already have an increased risk of developing lymphomas and leukaemia (Stützer et al. 2010), FeLV exposure significatively increases this risk compared with FIV and uninfected cats. For example, in multi-cat households, the risk of developing lymphoma can be up to 40 times higher (Shelton et al. 1990, Hartmann and Hofmann-Lehmann 2020), with the most common neoplasms being mediastinal and multicentric lymphoma (Hofmann-Lehmann and Hartmann 2020). Our results indicated a strong association between FeLV infection and neoplasia, with lymphoma accounting for three-quarters of all neoplasias. This may help explain the shorter survival time, as affected cats lived nearly one year less than FeLV$^+$ cats without neoplasia.

Secondary infection as a consequence of retroviral infections is commonly detected in FIV$^+$ and FeLV$^+$ domestic cats (Burling et al. 2017, Addie et al. 2000, Little et al. 2020). Our results indicated that only oral diseases were closely associated with FIV$^+$ cases, where more than half of FIV$^+$ cats presented with some form of oral disease (gingivitis, feline odontoclastic resorptive lesion, ulcer, or tooth loss). However, over 80% of oral diseases occurred in cats older than 7 years. The cases of oral diseases in FeLV$^+$ cats (25%) were similar to that in uninfected cats

(27.5%), likely because FeLV$^+$ cats tend to die at a young age and are less likely to develop such conditions.

Additionally, anaemia was found to be statistically more likely in both FIV$^+$ and FeLV$^+$ infected cats compared with uninfected cats. Anaemia was not identified in non-infected cats, whereas approximately a quarter each of FIV$^+$ and FeLV$^+$ cats presented with anaemia. Anaemia has been previously associated with decreased survival times in cats infected by retroviruses (Spada et al. 2018). In FeLV$^+$ cases, the anaemia is typically a direct consequence of viral infection, which causes bone marrow aplasia (Hartmann 2012). In contrast, in FIV$^+$ cases, anaemia is usually caused by secondary infections such as Mycoplasma (Little et al. 2020).

In the general UK population, all cats (FIV positive or not) are more likely to die from stochastic events such as being hit by a car, unexpected disappearance, or metabolic diseases such as renal or cardiovascular diseases (O'Neill et al. 2015, Kent et al. 2022).

As an additional factor to consider in this analysis, the cats that lived in Catwork were mostly males, excepting the uninfected group which was balanced between male and female. It is well known that there is a predisposition to FIV infection in male cats due to their aggressive behaviour (Levy et al. 2008, Little et al. 2020). This imbalance between male and females may influence overall lifespan. According to general lifespan analysis, female cats have a 1.33 year longer life expectancy compared with male cats (Teng et al. 2024).

This study has confirmed the significantly reduced life span of FeLV$^+$ and FeLV/FIV$^+$ cats and has provided further evidence that FIV positive cats largely reach an average life expectancy. The extended life expectancy observed in the retrovirus negative cats in this study suggest that FIV does influence the chances of a cat surviving into extreme old age. Some authors have suggested that multi-cat households may affect survival time (Beczkowski et al. 2015, Spada et al. 2018). However, this was not a factor in this study as all cats lived in multi- cat groups, though these were stable social groups with hiding spots and opportunities to avoid conflicts. Overall, in this study, under controlled health, diet, and husbandry conditions, such as those at the Catwork sanctuary, we observe an effect of FIV on longevity with FIV cats less likely to survive into extreme old age.

2.2 Prevalence and Risk Factors in Chilean Domestic Cats: High Prevalence and Risk Factors of Feline Leukaemia Virus Infection in Chilean Urban Cats (*Felis catus*).

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Statement of contribution: This section of the work has been prepared for manuscript submission. Cristobal Castillo-Aliaga performed the statistical analysis, collected data, wrote and edited the text. Susana Castro-Seriche and Alonso Jerez-Morales (Haiken Lab) collected samples and data, performed end-point PCR diagnosis, and edited the manuscript. Rachael Tarlinton conceptualised the study and edited the manuscript.

2.2.1 Abstract

Feline Leukemia Virus is a retrovirus causing fatal disease in domestic cats. While FeLV has been controlled in many countries, it remains a major concern in Latin American countries. This study conducted an epidemiological survey of FeLV in 182 Chilean domestic cats using PCR to detect presence of proviral DNA. The results were analysed using Multivariate Logistic Regression to examine risk factors associated with FeLV detection. The FeLV prevalence was 54.95%, and statistically significant associations (p <0.05) were found for two protective factors and one risk factor. Cats from Concepcion city (95%CI 0.08-0.56%) and cats sampled in 2022 (95%CI 0.1-0.06%) had lower odds ratios for provirus positivity, whereas non-vaccinated cats (95%CI 2.3-15.8%) had an increased odds ratio. No other factors were statistically significant. The high FeLV prevalence is similar to other Latin American countries and the geographical differences highlighted in this study likely correspond to the socioeconomic status of the owners. This study highlights the need for improved FeLV control measures such as promoting FeLV

vaccination, implementing health screening prior to adoption of new cats, and educating owners about FeLV to control its circulation.

2.2.2 Introduction

Feline Leukemia Virus (FeLV) is a gamma-retrovirus with a worldwide distribution among domestic cat populations. The main clinical signs are related to neoplasia, aplastic anaemia, bone marrow suppression, lymphoma, leukaemia and ultimately death (Little et al. 2020). The virus is excreted in bodily fluids and can be horizontally transmitted during close contact (grooming, food and water dishes, blood contact, milk, during fights) (Tandon et al. 2005). It also can be transmitted trans-placentally, but less frequently (Hardy et al. 1976a). The wide distribution and the high mortality rate mean that FeLV is one of the most common and important infectious threats to domestic and non-domestic felids (Hartmann 2012).

Cats are routinely tested for FeLV. This is carried out using point-of-care tests to detect free viral p27 antigen in blood, determining antigenemia in viraemic cats (Levy et al. 2006). The test has the advantage that is easy and quick to use in daily clinical practice (Little et al. 2020). Although this test has shown high sensitivity and specificity, under certain circumstances these can produce false-negative results, therefore PCR is suggested to confirm results (Westman et al. 2019, Beall et al. 2021).

There are three main courses of infection outcomes produced by FeLV, led by host immune and viral factors, and these outcomes can cause discrepancies between the tests (Hofmann-Lehmann and Hartmann 2020). The most severe outcome is progressive infection. The cat does not develop an efficient and early immune response, being persistently infected leading to positive results for DNA (provirus) and antigen tests (p27) (Torres, Mathiason, and Hoover 2005). In a regressive infection the cat's immune response is efficient enough to control the viral replication. In consequence, the antigen test will be negative 1 to 6 weeks later. However, the provirus is integrated into the host genome, and it will remain PCR positive and the infection can be reactivated under stressful situations (Tandon et al. 2005, Hartmann and Hofmann-Lehmann 2020). The most benign infection course is abortive infection. A competent immune system

eliminates the virus before proviral insertion, in consequence, there are no antibodies detectable, nor DNA or RNA in peripheral blood. The cat will be negative to all types of tests (Hofmann-Lehmann and Hartmann 2020).

FeLV prevalence has a wide range of levels, based on factors such as country, cultural issues, veterinary care availability, characteristics of the cat population, and geographic area. In general, developed countries have lower prevalence rates than developing countries (Gleich, Krieger, and Hartmann 2009, Burling et al. 2017, Studer et al. 2019, Ortega, C. et al. 2020). During the last decades, developed countries have reported significant decreases in rates of FeLV infection. This has been mainly attributed to efforts in cat vaccination, continuous testing in vet clinics, and improved measures to reduce virus circulation in cat shelters such as testing prior to animal adoption, and elimination or isolation of progressively infected cats (Hardy et al. 1976b, Hofmann-Lehmann and Hartmann 2020, Westman, Malik, and Norris 2019).

For individual cats the main risk factors for FeLV have been associated with outdoor access, male sex and younger cats (Hardy et al. 1976b, Little et al. 2009, Gleich, Krieger, and Hartmann 2009). However, there are more factors involved in FeLV prevalence including: populations of stray cats, multi-cat household, shelters without hygiene measures, irresponsible breeders (Garigliany et al. 2016, Studer et al. 2019, Westman, Malik, and Norris 2019, Muz et al. 2021, Rungsuriyawiboon et al. 2022).

In a wider perspective, human social factors heavily affect FeLV prevalence. European and North American countries have more awareness about the disease, and there is a greater emphasis on animal welfare compared with other zones of the world (De Boo and Knight 2005). However, this also influences decisions that could increase the FeLV risk, for instance, leaving cats unneutered and protecting stray cat colonies (Spada et al. 2012, Studer et al. 2019). On the other hand, from an economic perspective, meta-analysis of FeLV prevalences, at the country level, has demonstrated a relationship between national gross domestic product (GDP) per country, through purchasing power parity (PPP), and FeLV prevalence (Ludwick and Clymer 2019, Studer et al. 2019). This has been explained by resources being available to finance

shelters, measures to control feral cats, programs for sterilization, and veterinary care by owners being affordable and available (Ludwick and Clymer 2019).

Multiple reports have described FeLV prevalence using different diagnostic methods. Some studies have performed comparisons between antigen and PCR methods. These studies have demonstrated that prevalence can vary considerable according to the method used. This is mainly explained by broader detection by PCR and hence higher prevalence with this method (Szilasi et al. 2020, Muz et al. 2021). In consequence, provirus detection is likely more adequate for examining virus exposure (Hartmann and Hofmann-Lehmann 2020, Little et al. 2020).

The prevalences described for detection of provirus (PCR based) in South-America have demonstrated high variability: In Brazil between 3% to 47.2% (Coelho et al. 2008, Lacerda et al. 2017); 11.82% in Argentina (Galdo Novo et al. 2016); 56% in Montevideo, Uruguay (Acevedo et al. 2020); 35% in areas of Guayaquil, Ecuador (Santana, Pozo, and Castañeda 2022); and between 23% to 59.4% in Colombia (Tique et al. 2009, Ortega, C. et al. 2020).

Although there is information about FeLV prevalence in other countries there are only a few studies of FeLV prevalence in Chile. Chile extends over 4,000km long, and 350 km wide (in the widest zone). Seventy-eight-point nine percent of people live in the central area and 87.9% live in urban areas. Regarding the cat population, there are estimated to be around 4 million owned cats, with approximately 86% of them living in urban zones (INE 2018). In domestic cat populations, FeLV prevalences between 3 to 33% were previously described (Bilbao 2008, Mora et al. 2015, Tabilo 2018, Sacristán et al. 2021a). However, two of these studies were conducted on a small group of animals, around 50 cats (Bilbao 2008, Tabilo 2018). Meanwhile, the other two studies were carried out in rural areas, to determine transmission of FeLV between domestic and non-domestic felids (Mora et al. 2015, Sacristán et al. 2021a). The aim of this study is to provide evidence about the prevalence of FeLV in Chilean domestic cats in Bio-Bio and Ñuble regions from Chile, and to evaluate risk factors commonly associated with the infection.

2.2.3 Methods

2.2.3.1 Sample collection for the prevalence study and molecular analysis.

A total of 182 blood samples from domestic cats were collected in EDTA, and kept at -20°C. The samples were collected over 2 years (2021-2022) from 16 veterinary clinics from 10 communes, located between the Bio-Bio and Nuble Region (Chile) (**Table 8** and **Figure 13**). All procedures and handling were performed by veterinarians during clinical diagnostics under the owner's consent.

Samples were processed in Haiken Laboratory in Concepcion (Chile). Nucleic acid extractions were performed using a commercial kit (Geneaid®) following manufacturer's instructions. PCR diagnosis specific for exogenous FeLV was performed to detect provirus using 3'LTR primers, Forward: 5'-CTACCCCAAAATTTAGCCAGCTACT-3' and Reverse 5' AAGACCCCCGAACTAGGTCTTC-3'), previously described by (Cattori et al. 2006).

2.2.3.2 Data collection for regression analysis

Overarching ethical approval for this study was granted by the University of Nottingham School of Veterinary Medicine and Science Committee for Animal Research and Ethics (CARE) (Number 3682 220923). Basic data were available for all cats (sex, age, sampling date and commune/location). Additionally, risk factors were retrospectively collected from each veterinary clinic, and any personal data was anonymised.

The survey was sent and all owner or cat identifying data was anonymised in compliance with General Data Protection Regulation (GDPR). The survey was completed by each cat's veterinary surgeon and included the following variables: breed, origin (rescued as stray cat or rehomed/purchased/born in-house), test reason (sick or prophylaxis), lifestyle (indoor-outdoor), reproductive status (neutered or intact), multi-household (single cat or multiple cats living in the same house), and FeLV vaccination status. Response was received for 182 cats. The dataset was managed using Excel Microsoft 365 (Version 2308).

2.2.3.3 Descriptive analysis

Descriptive analyses including prevalence were performed in STATA/SE 18 (StataCorp 2023). The Graph was created in GraphPad Prism software (GraphPad, San Diego, CA) and geographical visualization was done in Rstudio v4.1.3 (2023.09.1+494) (Rstudio Team 2020) using the “ggmap” (Kahle and Wickham 2013) and “rnaturalearth” package (Massicotte and South 2023).

2.2.3.4 Regression analysis

Protective and risk factors associated with positive PCR results for FeLV were evaluated by univariate analysis. The age variable was categorized for contingency analysis. This was grouped in four categories: Kittens (between 1 month to <6 months), young adult (>7 months to 2 years), adult (>2 years to 6 years) and old (over 6 years old). Univariate frequencies were compared in GraphPad with Fisher’s exact test, and the Baptista-Pike method to calculate odds ratio (OR) with 95% CI (confidence interval) was used. Afterwards, a multivariate logistic regression analysis was performed with variables with *p*-values <0.2.

Variables with the highest *p*-value were manually excluded and included to find the best model. Once the variables were selected, and if these were biologically relevant, these were evaluated for significance. The multivariate model suitability was assessed using Hosmer-Lemeshow goodness-of-fit test (Petrie and Watson 2006) and the limit of statistical significance was defined as $p<0.05$. Multivariate statistical analyses were conducted in Rstudio v4.1.3 using the packages “ResourceSelection” (Lele, Keim, and Solymos 2023), “broom” (Robinson 2014) and “caret” (Kuhn 2018).

2.2.4 Results

2.2.4.1 Description of the population

A total of 182 cats were assessed using PCR, 100 of them were positive for provirus (54.95%; 95%CI: 47-62%). Samples were collected during 2021 (26.37%) and 2022 (73.63%) from veterinary facilities from 10 communes, between the Bio-Bio and Nuble regions (**Figure 13**). The

residence sites for the cats were as follows: San Pedro (24.18%, n=44), Concepcion (43.4%, n=79), Chiguayante (7.1%, n=13), Talcahuano (9.8%, n=18), Chillan (6%, n=11), Hualpen (6.5%, n=12), and other locations (2.7%, n=5), which includes one case per location: Lota, Los Angeles, Penco, and Coronel (**Figure 13**)

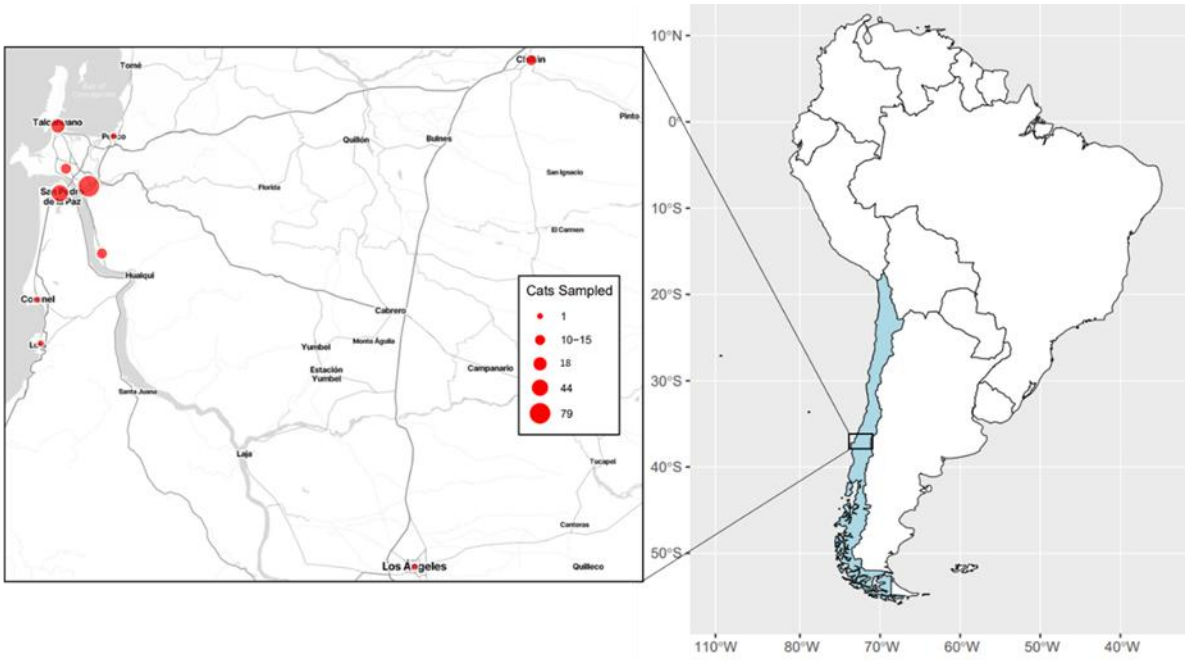

Figure 13 Right panel show map of South America and Chile geographical location (in light blue). The small square shows the area where FeLV provirus presence was assessed. The left panel show the map of sampling sites in Bio Bio and Nuble regions. Red circles sizes are proportional to the number of cats sampled and each circle indicate the sampling area. The sampling sites were, as follow: San Pedro (n=44), Concepcion (n=79), Chiguayante (n=13), Talcahuano (n=18), Chillan (n=11), Hualpen (n=12), Lota (n=1), Los Angeles (n=1), Penco (n=1), and Coronel (n=1).

The age distribution per year is shown in **Figure 14**. This category was grouped in 4 modalities for the logistic regression analysis: Kittens (11.54%) and old cats (10.44%) were the least represented, while Young Adult (29.67%) and Adult (48.35%) were the most represented (**Table 8**).

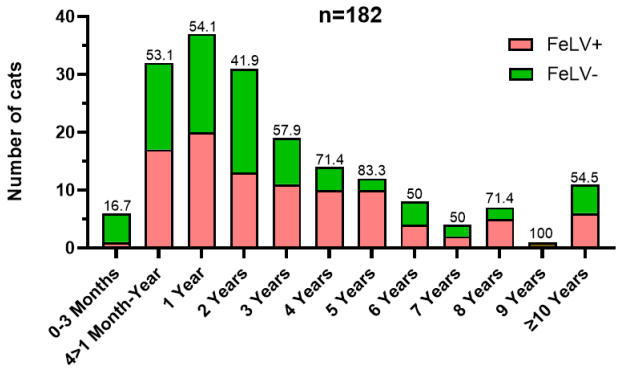

Figure 14 Age distribution per year of cats sampled. In light red are the Feline Leukemia Virus (FeLV) positive and in green are the negative cats. Numbers above bar indicate FeLV positive percentages.

Only slight differences were observed between clinically healthy cats (50.55%), and cats suspected of infection (49.95%). The cat's origin variable showed that most of cats had been born as stray cats, and they were rescued and resettled in a home (93.9%). Only a small number were non-stray cats, rehomed from breeders or other houses (6.04%). There was also a group for which it was not possible to determine where they had been born (11.54%). Regarding to the cat's sex, female (48.35%) cats were slightly less represented than male cats (51.65 %). Almost all samples were mixed breed cats (93.96%), contrasting with a few pure breed cats (6.04%). The pure breed cats were Abyssinian, Maine Coon, Persian, and Siamese. Reproductive status indicates that the majority of cats were neutered (86.81%), with only a few intact cats (11.54%) and some data missed (1.65%). The data about household composition was available for 161 cats, from them, near to half were multiple household cats (57.14%) and a third were single cats (31.32%). A similar situation in the access variable was observed. Most of the cats were indoor (69.23%), and close to a quarter were outdoor (27.47%), there were 6 (3.3%) cats for whom it was not possible retrieve this data. The vaccination rate was 20.33% and not vaccinated were 76.37% (**Table 8**).

Table 8 Sample characteristics and estimated odds ratios (OR) and p-values obtained in univariate analysis and Multivariate logistic Regression of the factors associated to provirus detection (n=182).

Variable	Modalities	Cat Sampled / FeLV$^+$	Univariate analysis		Multivariate Logistic Regression	
			***p*-value**	OR (95% CI)	***p*-value**	OR (95% CI)
Test Reason	Healthy	92 (50.55) / 47 (51.09)	Ref	-	-	-
	Suspect	90 (49.95) /53 (58.89)	0.30	1.37 (0.77-2.47)	-	-
Origin	House	11 (6.04) / 5 (45.45)	Ref	-	-	-
	Rescued	150 (82.42) / 84 (56)	0.54	1.52 (0.42-4.63)	-	-
	n/a	21 (11.54) / 11 (54.38)	>0.99	1.32 (0.32-6.03)	-	-
Sex	Female	88 (48.35) / 45(51.14)	Ref	-	-	-
	Male	94 (51.65) / 55(58.51)	0.37	1.34 (0.75-2.42)	-	-
Reproductive Status	Neutered	158 (86.81) / 88 (55.70)	Ref	-	-	-
	Intact	21 (11.54) / 9 (42.86)	0.35	0.59 (0.25-1.43)	-	-
	n/a	3 (1.65) / 3 (100)				
Age	Kitten	21 (11.54) / 9 (42.86)	Ref	-	-	-
	Young Adult	54 (29.67) / 29 (53.70)	0.44	1.54 (0.53-4.23)	-	-
	Adult	88 (48.35) / 50 (56.82)	0.33	1.75 (0.67-4.34)	-	-
	Old	19 (10.44) / 12 (63.16)	0.22	2.28 (0.68-7.73)	-	-
Breed	Pure	11 (6.04) / 5 (45.45)	Ref	-	-	-
	Mixed	171 (93.96) / 95 (55.56)	0.54	1.41 (0.55-3.87)	-	-
Household environment	Single	57 (31.32) / 27 (47.37)	Ref	-	-	-
	Multi	104 (57.14) / 57 (54.81)	0.41	1.34 (0.70-2.62)	-	-
	n/a	21 (11.54) / 16 (76.19)	0.038	3.55 (1.12-9.64)	-	-
Access	Indoor	126 (69.23) / 69 (54.76)	Ref	-	-	-
	Outdoor	50 (27.47) / 29 (58)	0.73	1.14 (0.58-2.18)	-	-
	n/a	6 (3.3) / 2 (33.33)	0.41	0.41 (0.07-1.83)	-	-
FeLV Vaccination	Yes	37 (20.33) / 9 (24.32)	Ref	-	-	-
	No	139 (76.37) / 88 (63.31)	<0.000	5.36 (2.24-12.75)	<0.00*	5.73 (2.3-15.8)
	n/a	6 (3.3) / 3 (50)	0.32	3.11 (0.61-14.78)	0.09	5.11 (0.70-37.2)
Year Sampled	2021	48 (26.37)/ 37 (77.08)	Ref	-	-	-
	2022	134 (73.63)/ 63 (47.01)	<0.000	3.791 (1.82-8.34)	0.002*	0.25 (0.10-0.60)
Area	Concepcion	79 (43.41) / 31 (39.24)	<0.000	0.166 (0.07-0.39)	0.001*	0.22 (0.08-0.56)
	San Pedro	44 (24.18) / 35 (79.55)	Ref	-	-	-
	Chiguayante	13 (7.14) / 8 (61.54)	0.14	2.47 (0.81-7.37)	0.71	0.75 (0.16-3.64)
	Talcahuano	18 (9.89) / 11 (61.11)	0.11	2.43 (0.89-6.31)	0.20	0.43 (0.12-1.59)
	Hualpen	12 (6.59) / 7 (58.33)	0.22	2.16 (0.65-6.68)	0.41	0.54 (0.12-2.45)
	Chillan	11 (6.04) / 7 (63.64)	0.19	2.71 (0.74-8.72)	0.24	0.40 (0.09-1.95)
	Others	5 (2.75) / 1 (20)	0.64	0.38 (0.03-2.56)	0.14	0.15 (0.00-1.49)

n/a: Not Available; Ref: Reference; Numbers between brackets indicate percentage; CI: Confidence interval.

Cats were tested for FeLV for two main reasons: rehoming or initial health checks for healthy animals or a suspicion of FeLV related disease. The age distribution categorised by the reason for testing is shown in **Figure 15**, as follows: 18 (85.7%) healthy and 3 (14.3%) suspect kittens; 26 (48.1%) healthy and 28 (51.9.3%) suspect young; 34 (38.6%) healthy and 54 (61.4%) suspect adults; and 14 (73.7%) healthy and 5 (26.3%) suspect old cats were sampled. Old cats

were primarily healthy animals being tested for rehoming and likely represent animals with regressive infections.

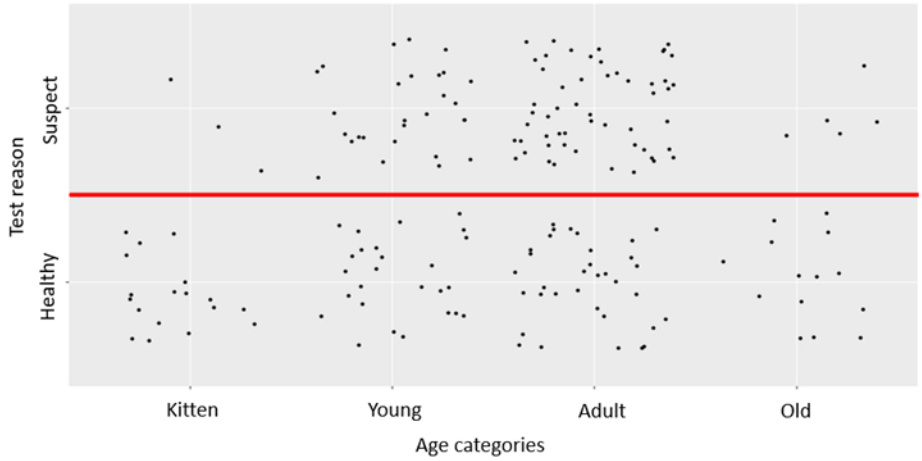

Figure 15 Graph of age distribution compared with test reason. Age categories are sorted by younger from the left to the older in the right. Black dots indicate number of cats sampled and red line divide between healthy from infection suspect cats.

2.2.4.2 Risk Factors and Regression Analysis

Each variable was evaluated through univariate analysis and those with *p*-value <0.20 were selected (Table 8). For the multivariate analysis vaccination status, year sampled, and area were considered. All of them showed at least one statistically significant modality (Table 8). Compared to San Pedro, all areas were considered as protective factor, but only Concepcion was statistically significant (OR= 0.22; 95% CI 0.08 to 0.56). Cats sampled during 2022 were less likely to be infected (OR= 0.25; 95% CI 0.1-0.6) and vaccination was a protective factor compared with not vaccinated cats (OR= 5.73; 95% CI 2.3-15.8). The Hosmer-Lemeshow goodness of fit test showed that the model fit the data acceptably (x^2=4.96, df = 3, p-value = 0.1747).

2.2.5 Discussion

The aim of this study was to determine the FeLV prevalence in a South-Central area of Chile. Although FeLV transmission has been successfully controlled in several countries, this is still a large problem for domestic cat health in Latin America. Comprehensive studies in North America

have shown a prevalence rate of 3.1% in healthy cats (Levy et al. 2006). In Europe the overall prevalence is low, 3.9% in sick cats and 1.6% in healthy cats (Studer et al. 2019). European countries with higher prevalence are Portugal, Hungary and Italy with prevalences between 5.7-11.8% (Studer et al. 2019, Szilasi et al. 2019). In Southeast Asia, low prevalences also have been described (~4%), except for Thailand with 18.5% (Capozza et al. 2021). However, FeLV still has higher prevalence in some areas of the world such as, Turkey, 69.7% of proviral DNA positivity in stray cats (Muz et al. 2021), 76% in Mexico (Ramírez et al. 2016), 56% in Uruguay (Acevedo et al. 2020), 59.4% in Colombia (Ortega, C. et al. 2020). In Brazil, there are studies describing of 3% and 4.5% prevalence in some areas of Brazil (Poffo et al. 2017, Lacerda et al. 2017), while there are higher rates such as 47.2% (Coelho et al. 2008), 22.3% in Santa Catarina (Cristo et al. 2019) or 31% in Rio Grande do Sul (da Costa et al. 2017).

This report has demonstrated a much higher prevalence rate (54.95%) in Chile, than previous reports. Two previous studies in one Chillan city (one of the cities evaluated here) have been conducted. These evaluated a limited number of cats (n=50), and both reports sampled cats from the same university's veterinary clinic, a decade apart. In the first study a 3% of prevalence using end-point PCR was described (Bilbao 2008), in the second study a 20% prevalence was reported using real-time PCR (Tabilo 2018). In Valdivia, a smaller and southern city in Chile, one study described 14.9% seroprevalence in 308 cats sampled (Arauna 2015). However, this cannot be compared directly with our results, as they used an antigen-based test, and higher prevalences are expected when provirus PCR diagnostics are used (Muz et al. 2021, Szilasi et al. 2020, Capozza et al. 2021, Giselbrecht et al. 2023). This study identified two risk factors in a univariate analysis: sick cats and no veterinary checks, but no factors in the multivariate analysis were statistically significant (Arauna 2015).

Additionally, two other studies in Chile have been conducted to evaluate risk transmission to native non-domestic felids. These sampled domestic cats from rural communities, adjacent to habitats of wild felids. They described a 33% prevalence in domestic cats from Chiloe Island (Mora et al. 2015), and a 20.6% prevalence in a Chilean central area (Sacristán et al. 2021a). However, 86% of Chilean cats live in urban areas and only 14% live in rural areas (Subdere 2022). We evaluated one of the most crowded cities in Chile (Concepcion) and transmission

dynamics in urban areas with higher cat density areas may change the transmission risk (Spada et al. 2012, Capozza et al. 2021).

Although the recruitment to our study was biased towards cats that veterinarians had a suspicion of FeLV or pre-vaccination test (hence presented for testing), the cat population sampled was consistent with the general population characteristics in the national pet survey (Subdere 2022). Multivariate analysis showed a statistical association between FeLV PCR positivity with vaccination rate, residence area, and year sampled. Notably in 2021, the Chilean government established movement restrictions for people during the COVID-19 pandemic and this likely influenced a reduction in preventive exams in healthy cats. The geographic location was another statistically significant factor in FeLV infection status. Cats living in Concepcion were less likely to be FeLV infected than cats in the other communes. This commune is wealthier and better pet care has been described than in other communes (Subdere 2022). FeLV status of the cats is likely associated with better owner socio-economic conditions and better veterinary care, linked to a higher ratio of vaccination and tested cats (Studer et al. 2019, Ludwick and Clymer 2019).

Vaccination as a protective factor was expected, according to our results, unvaccinated cats are approximately 5 times more likely to be infected by FeLV. These results confirm the efficacy of the current FeLV vaccines protecting against progressive infection (Little et al. 2020, Diesel et al. 2024). The vaccination rate in our results is still low compared with wealthier European countries (48%-81.5%) where FeLV circulation has been controlled (Studer et al. 2019). However, it can be considered better than Italy with 17.8%, Portugal with 14.2% (Studer et al. 2019) or Thailand with 19.96% (Rungsuriyawiboon et al. 2022). In South America, the FeLV vaccination rate is rarely described, for instance, in Santa Catarina (Brazil) 40.15% of cats have some type of vaccine and only 8.39% of cats were vaccinated against FeLV, in Buenos Aires (Argentina), according to estimations only 12% of cats have at least one vaccine, and only 1% are vaccinated against FeLV (Gómez 2016). In Peru only 7.8% of cats have received some type of vaccine (Gil, León, and Falcón 2022).

Although other variables such as origin, reproductive status or breed are commonly mentioned as risk factors in other studies, this was not the case in our multivariate model. We have only a small number of animals in these modalities, and it is possible that with increased numbers these factors might be statistically significant. It is also possible that concept of a fully indoor cat was not understood by the owners. Some indoor cats can occasionally have access to outside and have contact with other cats, but the owners may have declared these as indoor cats (with access to the exterior). We did not identify statistical significance between FeLV infection with multi-cat household status or age (Levy et al. 2006, Garigliany et al. 2016, Burling et al. 2017).

An unexpected result is the similar infection rate observed between older and younger cats. Infections in younger cats typically reduce survival time between 2-5 years post-infection in progressively infected animals. Consequently, a lower prevalence is expected in older cats due to early mortality (Spada et al. 2018), although a recent study in progressively infected cats has suggested that older cats could have longer survival times compared to younger cats (Westman et al. 2024).

It is also important to consider that high viral circulation may change the infection dynamics. The infection outcome is determined by host immune status (including previous immunity) and viral factors (as viral strain and loads), which can influence the proportion of infection types (abortive, regressive, progressive) across different populations and countries studied (Englert et al. 2012, Little et al. 2020, Giselbrecht et al. 2023, Diesel et al. 2024). In that respect, our other work on this population (currently in review) has demonstrated distinct geographical clustering of FeLV-A isolates from Chile, though we cannot assess pathogenicity based on sequence data alone (Castillo-Aliaga et al. 2023a).

Although, we cannot determine whether our cats were recently infected or they are undergoing a regressive infection, older cats were primarily healthy (66% were tested as part of preventative medicine examinations), with adult or young cats more likely to be tested for suspicion of disease (only 38.6% of adult and 48.8% of young cats were healthy at testing) We speculate that as we are measuring provirus FeLV exposure (whether progressive or regressive) and due to the high viral circulation, the cats are continuously exposed by outdoor access or cohabitation

during all life stages, and older cats are likely to have an increased cumulative lifetime risk of exposure (Englert et al. 2012, Little et al. 2020, Giselbrecht et al. 2023, Diesel et al. 2024). In a prospective study it would be ideal to also assess seroprevalence to gain a better idea of progressive vs regressive infection. However, cost constraints in Chilean primary veterinary practice mean that the in-house lateral flow diagnostic tests commonly used in developed countries are not widely available, hence no data on seroprevalence was accessible for this retrospective study.

The Chilean cat population has some good pet health indicators, for example the neutering rate of cats in this study was 86.81%, with 92.8% described in Chile (Salgado-Caxito et al. 2021), this is high when compared with other high FeLV prevalence areas such as: 32-48% cats neutered in Southeast Asia and Taiwan (Capozza et al. 2021, Rungsuriyawiboon et al. 2022), 63.5% in Peru (Gil, León, and Falcón 2022), and 54.38% in Brazil (Biezus et al. 2019). The Chilean rate of neutered cats is comparable to that in low FeLV prevalence countries where up to 85% of cats are neutered (Protection 2023). This may explain why the neutering status or gender were not risk factors for FeLV exposure in this study.

The problem in Chile, similarly to other countries in the region, is that cat owners frequently obtain stray cats without previous examination to incorporate into their house, therefore the other cats in the house are at risk of contracting diseases when new additions are not quarantined, or health checked. To our knowledge, in Chile there are no cat shelters with management and guidelines to control diseases such as there are working in developed countries. Although some shelters run by small animal welfare associations or a small number of city councils exist in Chile (Subdere 2021). All shelters in Chile lack resources and it is unlikely they have measures to control diseases, having poor sanitary conditions (Subdere 2021). In countries where FeLV has been controlled, breeders and shelters play a crucial role in controlling feline retroviruses. In these facilities routine pre-adoption screenings are performed and new cats are kept separated, FeLV immunizations are given as core vaccines, and isolation and euthanasia for progressively infected cats in poor condition is applied (Möstl et al. 2015, Westman, Malik, and Norris 2019b, Stone et al. 2020, Little et al. 2020). Chilean cat owners declare that the main route to obtaining a cat is through neighbours or social media, indeed

87.1% of cats owners in Chile reported that their cats never had offspring (Salgado-Caxito et al. 2021).

In the last decade, government programs for animal welfare and professional efforts have significantly improved the awareness of pet health. However, the prevention of FeLV in Chile are strongly veterinary practitioner and pet owner resources dependent, resulting in a limited number of animals being tested in veterinary hospitals. Further analysis, such as antigen testing or viral quantification, may help to elucidate the proportion of each infection type.

Future research should include strain typing, serology and PCR assessment, infection type classification, and prospective analysis with higher number of animals, to evaluate survival time in countries like Chile with higher FeLV prevalence rates. This knowledge would help to implement strategies for controlling roaming cat populations and improving domestic cat welfare.

Chapter 3 Molecular Analysis of *env* gene of Feline Leukaemia Virus in Chilean Domestic cats and Synonymous Site Conservation and RNA folding.

This section includes contributions from:

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Statement of contribution: This section of the work has been prepared for manuscript submission. Cristobal Castillo-Aliaga performed the bioinformatic and molecular analysis, wrote and edited the text. Adam Blanchard conducted Oxford Nanopore sequencing and bioinformatic analysis. Susana Castro-Seriche and Alonso Jerez-Morales collected samples, performed initial end-point PCR diagnosis. Ezequiel Hidalgo-Hermoso transported samples and edited the manuscript. Matthew Loose sequenced samples and edited the manuscript. Katherine Brown and Hazel Stewart conceptualised SynPlot2 analysis and performed RNA folding analysis. Rachael Tarlinton conceptualised whole study and edited the manuscript.

3.1 Comparison between Sanger, Illumina and Nanopore sequencing evidencing intra-host variation of Feline Leukemia Virus that infects domestic cats.

3.1.1 Abstract

Knowledge of Feline Leukemia Virus (FeLV) sequence variation has mainly been developed using Sanger sequencing methods. However, advances in next generation sequencing methods and their broad use in laboratories has been changing our understanding of viral genetics. FeLV sequencing has specific complications with the presence of both exogenous (exFeLV) and endogenous (enFeLV) virus with frequent recombination between them, limiting sequencing approaches. Here we report an FeLV-A and FeLV-B amplicon-based comparison between Sanger, Illumina, and Oxford Nanopore sequencing methods in Chilean domestic cats. We analysed the hypervariable envelope gene, where a higher number of variants as well as recombination with endogenous strains occurs. We detected multiple variants and viral quasispecies infecting the cats. We compared these three methods to evaluate the advantages and disadvantages between them. Although the Sanger method is highly reliable, it showed a high fail rate (many amplicons did not produce useable sequence) and the sequences obtained showed artificial sequence clustering when compared with the NGS methods. Illumina sequencing showed a lower error rate but could not discriminate between exogenous and endogenous viruses. Finally, Oxford Nanopore (MinION) sequencing could successfully detect low-abundance sequences and discriminate between FeLV-A and FeLV-B sequences, although its higher error-rate requires caution in interpretation of the results. Our results indicate advantages and disadvantages for each method, with the purpose of sequencing needing to be considered in the choice of method. Results of large viral phylogenetic trees combining sequences derived from mixed sequencing methods, such as those combining historical and contemporary sequencing need to be interpreted with some caution as artificial clustering by sequencing method may occur.

3.1.2 Introduction

Feline leukemia virus (FeLV) is an enveloped retrovirus that belongs to the family *Retroviridae*, genus *Gammaretrovirus* (Coffin et al. 2021). The FeLV genome consists of two copies of a single positive sense RNA around 8.4 kb in length. It encompasses three genes: *gag* (capsid proteins), *pol* (reverse transcriptase enzymes, protease, and integrase), and *env* (envelope proteins). These are flanked, 5' and 3', by two identical regulatory sequences called LTR (long terminal repeat). During infection, the RNA genome is copied into a DNA genome by the reverse transcriptase enzyme and is inserted as a provirus into the host genome (Willett and Hosie 2013).

FeLV has a world-wide distribution in domestic cat populations and is one of the most important pathogens for their health (Hartmann and Hofmann-Lehmann 2020). The virus causes clinical signs such as immune and bone marrow suppression, lymphadenopathy, lymphoma, leukaemia (Willett and Hosie 2013). The virus is excreted in saliva, urine, faeces, milk and nasal secretions (Torres, Mathiason, and Hoover 2005). Social activities like grooming, sharing food and water dishes, nursing and bites during fights are the main transmission routes. It can also be transmitted via blood contact (blood transfusion or contaminated instruments) or sharing of litter trays (Little et al. 2020). Another important route of transmission is vertically, where the queen cat transmits virus to her neonatal kittens, either transplacentally, during parturition or through nursing (Hardy et al. 1976a). FeLV also has importance in wild felid health and conservation, causing severe disease and mortality (Cunningham et al. 2008, Meli et al. 2010a, Chiu et al. 2019). This has been notably harmful in genetically bottle-necked populations (Chiu et al. 2019). Although the virus can persist in wild species, domestic cats have been the infection source for non-domestic animals in the majority of cases (Brown et al. 2008, Meli et al. 2010, Petch et al. 2022).

On the other hand, endogenous FeLV (enFeLV) are retroviruses that have integrated over millions of years into the cat genome. EnFeLV were acquired during the divergence of the Felidae and are present in domestic cat chromosomes and their close relatives in the *Felis* genus (Benveniste and Todaro 1975). They are transmitted from parents to descendants

through chromosomes (Polani et al. 2010). Exogenous and endogenous viruses share 86% similarity at the nucleotide level with the main variation points occurring in *gag* and *env* (Chiu, Hoover, and Vandewoude 2018a). The enFeLV in cat populations have multiple variations, influenced by the integration site, viral transposition events and several independent integrations during evolutionary history. Even the cat gender may influence the enFeLV quantity, due to an increased load on the Y chromosome compared to the X chromosome (Roca et al. 2005, Tandon et al. 2008, Powers et al. 2018). Recently studies have associated higher loads of enFeLV with protective factors against exFeLV infections (Powers et al. 2018, Chiu and VandeWoude 2020). Conversely the presence of enFeLV can result in recombination events with exFeLV and produce FeLV-B variants which are related to cases of lymphoma and leukaemia (Ahmad and Levy 2010, Biezus et al. 2023).

Several groups of FeLV have been described, whether naturally or experimentally detected (Miyake et al. 2019, Anai et al. 2012, Erbeck et al. 2021). These arise from recombination between exogenous and endogenous viruses or mutation of exogenous viruses (Willett and Hosie 2013). The majority of the variation occurs in the *env* gene, which is the binding point between the virus and host cell (Cano-Ortiz et al. 2022). The *env* gene encodes the Surface unit (SU) and Transmembrane proteins (TM). SU includes three regions from 5' to 3: Receptor binding domain (RBD), Proline rich region (PRR) and C-Domain. The RBD and C-Domain are involved in cellular tropism, therefore changes in these regions can modify cell receptor usage and so change clinical progression (Faix et al. 2002, Cano-Ortiz et al. 2022b). More specifically, there are three main Variable Regions (VRA, VRB, VRC) identified, along with specific amino acid changes which are associated with changes in receptor usage (Boomer, Sarah et al. 1997, Tailor and Kabat 1997, Cano-Ortiz et al. 2022b).

FeLV-A is the most widespread group that can be horizontally transmitted and often described as the least pathogenic subtype (Ahmad and Levy 2010, Biezus et al. 2023). FeLV-B is the second most common subgroup, being detected in approximately half of all FeLV-A cases (Powers et al. 2018). FeLV-B is a recombinant between FeLV-A and enFeLV viruses and it is associated with higher numbers of neoplastic malignancies, like thymic lymphoma and multicentric lymphoma, and is commonly associated with worse clinical signs and prognosis

(Ahmad and Levy 2010, Tsatsanis et al. 1994, Sheets et al. 1993, Roy-Burman 1995, Coelho et al. 2008). Although FeLV-B has historically been described as unable to be horizontally transmitted without FeLV-A, there are documented cases suggesting horizontal transmission of FeLV-B without FeLV-A (Stewart et al. 2013, Chiu et al. 2019). FeLV-C, FeLV-E and FeLV-T arise from FeLV-A mutation. In general, these subtypes are associated with increased virulence but have diminished transmissibility (Overbaugh 2000, Riedel et al. 1986, Miyake et al. 2016). FeLV-C is an infrequent subgroup causing aplastic anaemia (Riedel et al. 1986), while FeLV-T is a T-cytopathic virus which has been related to lymphoid depletion and immunodeficiency disease (Overbaugh 2000). FeLV-E has been solely described in a natural thymic lymphoma in one cat (Miyake et al. 2016). FeLV-D is the most distant FeLV, because it is a recombinant between FeLV-A and another phylogenetically distinct endogenous retrovirus (not enFeLV), called domestic cat endogenous retrovirus (ERV-DC) (Anai et al. 2012).

FeLV could be considered as a highly conserved virus, with less than 2% of variation in *env* genes (Overbaugh and Bangham 2001) when compared with other retroviruses such as lentiviruses like human immunodeficiency virus (HIV) or feline immunodeficiency virus (Perelson et al. 1996, Bendinelli et al. 1995). FeLV can vary through small insertions, variation points and acquiring sequences from ERVs (Overbaugh and Bangham 2001), thus multiple FeLV variants can be detected in a cat population or a single cat (Watanabe et al. 2013, Erbeck et al. 2021). To detect viral quasispecies variations, next-generation sequencing has been broadly used in HIV to evaluate virus variations related to drug-resistance (Teo et al. 2022). This contrasts with FeLV studies, where the majority of *env* gene analyses have been performed using plasmid libraries and Sanger sequencing to evaluate the viral diversity (Watanabe et al. 2013, Erbeck et al. 2021, Petch et al. 2022, Biezus et al. 2023). To our knowledge, NGS in FeLV has been used in only two studies; these were: Roche 454 sequencing of an FeLV outbreak in Iberian Lynx in Spain (Geret et al. 2011) and one study using Illumina sequencing demonstrating decreased enFeLV expression in lymphomas from exFeLV negative cats (Krunic et al. 2015).

FeLV epidemiology is related to multiple factors including household conditions, cat population management and vaccination rates; even the purchasing power parity per capita of owners can influence prevalence rates (Ludwick and Clymer 2019). Thus, developed countries generally

have a controlled situation with FeLV. The current prevalence in Europe has been described as between 0.7%-5.5% (Studer et al. 2019), with a 3.1% FeLV prevalence in North America (Burling et al. 2017). Conversely, the situation in South America is highly variable, ranging from 3% in north-eastern Brazil (Lacerda et al. 2017) up to 59.44% in Colombia (Ortega, C. et al. 2020), whilst in Chile, the prevalence has been described as between 20.2%-33% in rural areas (Mora et al. 2015, Sacristán et al. 2021a).

This study was developed in Chilean domestic cats, with the aim of evaluating the diversity of the *env* gene in FeLV proviruses, using the traditional (Sanger) sequencing method and comparing with short (Illumina) and long read (Oxford Nanopore) next generation sequencing methods (Illumina and Nanopore) in order to examine sequence diversity and recombination between exogenous and endogenous FeLV with higher precision than previously possible.

3.1.3 Methods

3.1.3.1 Samples and DNA extraction

Twenty domestic cat blood samples were collected in EDTA by jugular venipuncture and kept at -20°C. All procedures and handling were done by veterinarians during clinical diagnostics in the city of Concepcion under the owner's consent. Overarching ethical approval for this study was granted by the University of Nottingham School of Veterinary Medicine and Science Committee for Animal Research and Ethics (CARE) from the number 3672 220923.

Nucleic acid extractions were processed at the Haiken Laboratory, Concepcion, using a Geneaid DNA Isolation Kit™ (Geneaid Biotech Ltd, Taiwan) extraction following manufacturer's instructions. PCR diagnosis of FeLV was made using 3'LTR primers, Forward: 5'-CTACCCCAAAATTTAGCCAGCTACT-3' and Reverse 5' AAGACCCCCGAACTAGGTCTTC-3') described by (Cattori et al. 2006) as a first screen at Haiken Laboratory. Genomic DNA was then shipped to the School of Veterinary Medicine and Science, University of Nottingham United Kingdom.

3.1.3.2 End-point PCR and Sample Preparation

End-point PCR using two primer pairs following methods from Erbeck *et al*, (2020) was used to amplify the envelope gene hypervariable region and 3'LTR region from FeLV-A and FeLV-B. PCR was performed using GoTaq® Long PCR Master mix (Promega) and each reaction consisted of: 12.5µl of GoTaq® Long PCR Master mix; 0.5µl (10µM) Forward primer; 0.5µl (10µM) Reverse primer; 9.5µl of Nuclease-Free water; 2µl of template DNA to reach a final volume of 25µl per reaction. The same protocol was used for each pair of primers and was performed in triplicate to confirm results. Thermo-cycler conditions used a Gene-Touch Thermal-cycler, from Bioer Technology® following the Erbeck *et al*., (2020) protocol with some modifications according to the polymerase requirements. The protocol was: initial denaturation at 94ºC for 2 min followed by 45 cycles of denaturation at 95ºC for 30 s; annealing at 63 ºC for 30 s; extension at 72ºC for 2 mins and a final extension at 72ºC at 2 mins. The thermo-cycler conditions were tested with a gradient PCR to select the optimal annealing temperature and every set of PCR reactions included a negative control with no template (DNA free water) and a positive control (FeLV-A Glasgow strain) which was kindly donated by Dr. Margaret Hosie, University of Glasgow. The PCR products were run by electrophoresis on a 1% agarose TAE (Fisher scientific®) gel at 80V, 400 mA for 60 minutes. Bands of interest were excised, and DNA purification was performed from agarose gel using the Nucleospin® Gel and PCR Clean-up extract kit (Macherey-Nagel®, Germany), according to manufacturer's instructions.

The fragments were cloned into pGEM®-T Easy Vector Systems following the manufacturer's instructions (Promega, the United States) and five of these colonies were re-amplified from the respective plasmid using the same primers as described above using FeLV-A and FeLV-B primers.

DNA quantification was performed with a Qubit 4 Fluorometer (Thermo Fisher Scientific®). NGS sequencing requires high quantities of DNA as input, therefore FeLV-A and FeLV-B amplicons of high enough quality and quantity (>50 ng/ul) were selected for Illumina and Nanopore sequencing.

Of the twenty cats sampled, eight cats were selected for sequencing based on band quality, sample volume, DNA quantity. Samples of poor quality after Sanger sequencing were discarded. The details of the sequenced samples provided in **Table 9**.

Table 9 Sequencing method performed in each Chilean domestic cat.

Cat name	Sequencing Method	Amplicon sequenced and number of long sequences	Age	Sex	Genbank Accession
1. Omali	Sanger	FeLV-A (1) / -B (1)	n/a	n/a	OR513915 /OR513914
2. Maqui	Sanger	FeLV-A (1)	n/a	n/a	OR513916
3. Coco	Sanger / Plasmid	FeLV-A (6) / -B (5)	2 y	Female	OR513918
4. Leon	Sanger	FeLV-A (1)	5 y	Male	OR513917
5. Polo	Nanopore	FeLV-A (3) / -B (5)	11 y	Male	
6. Adonis	Nanopore	FeLV-A (8) / -B (2)	n/a	n/a	
7. Cuchi	Illumina / Nanopore / Plasmid	FeLV-A (9) / -B (3)	3 y	Male	SRA: PRJNA1014909
8. Felix	Illumina / Nanopore	FeLV-A (5) / -B (2)	6 y	Male	

n/a: Not available; y: year

3.1.3.3 Genome Sequencing

Amplicons from 8 domestic cats were sequenced by three different methods detailed in Table 9 and all sequencing results were submitted to GenBank. 18 amplicons were sequenced by Sanger method in a commercial laboratory (Eurofins Inc., Germany), including 5 from direct amplicons, and 13 from plasmid cloning of amplicons (**Figure 16**).

For Illumina sequencing, amplicons of FeLV-A and FeLV-B were pooled and sequenced using the Illumina platform (HiSeq4000) with 300-bp paired-end distances using a coverage of 30x by Novogene Europe, Cambridge, United Kingdom.

The libraries for Nanopore sequencing were processed following the Native Barcoding Kit 24 V14 protocol (Oxford Nanopore Technologies) with FeLV-A and FeLV-B amplicons individually barcoded. Library Quantification was carried out using a Qubit fluorometer and the Qubit dsDNA HS Assay kit (Thermofisher). 20 fmol of DNA library was loaded onto a R10.4.1 flow cell and sequenced using an Oxford Nanopore MinION Mk1C.

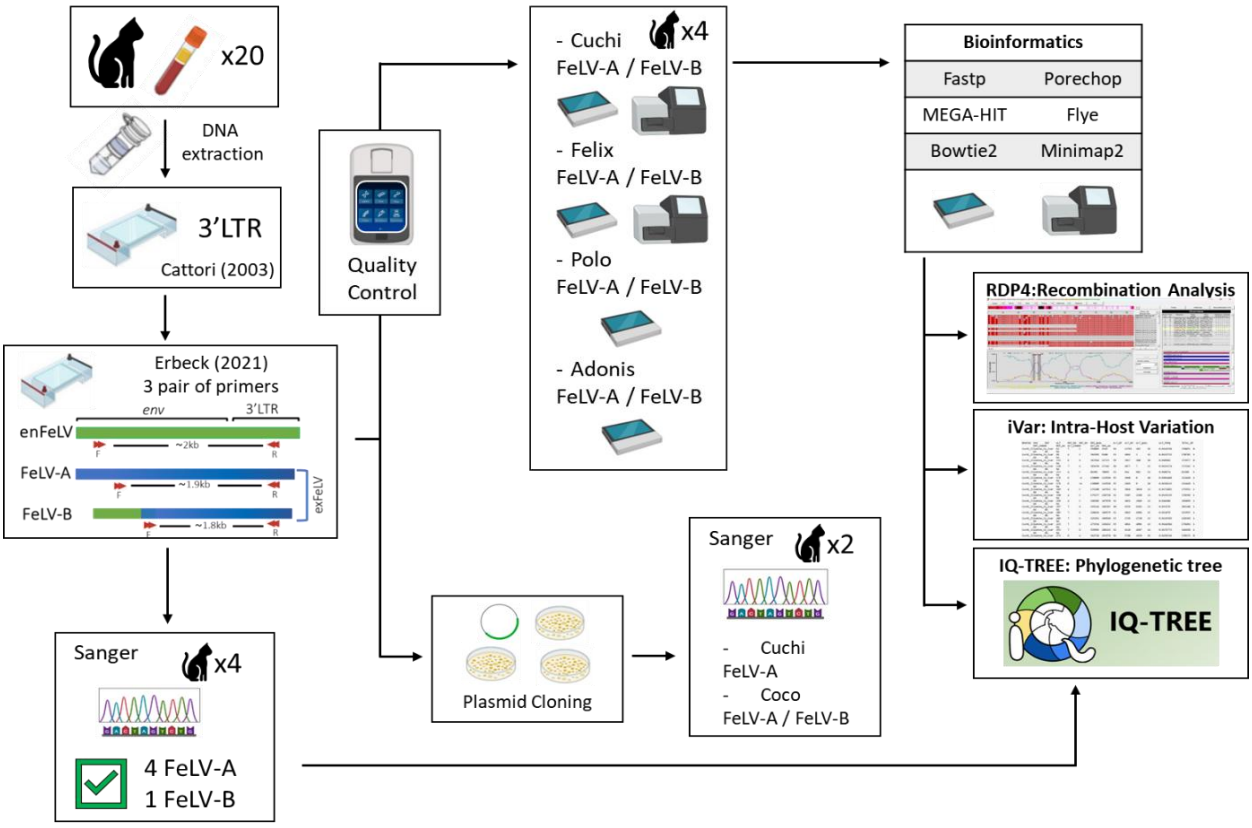


Figure 16 Workflow of Sequencing methods and bioinformatics tools used.

3.1.3.4 Read Processing, Assembly and Mapping

Illumina reads were trimmed and adaptors removed using FastP v0.23.1 (Chen et al. 2018). The Nanopore raw reads were base called using Guppy v6.4 and super accurate model was used. and Nanopore adaptors were eliminated using Porechop v0.2.4 (Wick et al. 2017), NanoFilt v2.6.0 (De Coster et al. 2018) was used to filter and discard reads shorter than 500 and longer than 4,000 bp with a quality of <Q10.

De Novo Assembly from Illumina reads was performed using MEGAHIT (Li et al. 2015) and for Nanopore reads, Flye (Lin et al. 2016) was used for FeLV-A barcodes. FeLV-B barcodes did not assemble reliably in Flye due to a mix of endogenous and exogenous sequences.

The contigs generated by MEGAHIT and raw reads from Nanopore, were mapped to FeLV-A FAIDS (M18247), enFeLV (M25425, LC196053), FeLV-T (M89997); FeLV-C (M14331), (MT302119, MT302153 and MT681671), Gardner-Arnstein FeLV-B (V01172) and FeLV-A and FeLV-B Sanger sequences from Chilean cats. Illumina sequences were mapped using Bowtie2 (Langmead and Salzberg 2012). Nanopore reads were mapped using Minimap2 (Li 2018a) with two preset options: the first was all raw reads by the more sensitive preset options “Oxford nanopore reads” (map-ont). The second approach was mapping to specific variations of interest (Chilean Sanger, FeLV-T, and US FeLV-B) with raw reads and using the preset options for “long assembly increased the specificity up to 5% sequence divergence” (asm5) to consider those under-represented sequences that could be missed by Flye. Reads were considered in the second approach if there were at least 30 reads with similar variations to generate a consensus sequence.

All contigs and consensus sequence were extracted and imported to Geneious v2023.0.4 (Biomatters Inc., Newark, NJ) to annotate and continue phylogenetic analysis. All bioinformatics pipelines are described at: https://github.com/cristobalsecast/Feline-Leukemia-Virus/tree/main.

3.1.3.5 Phylogenetic analysis

The dataset was constructed in Geneious (Biomatters Inc., Newark, NJ) using all available complete *env* gene FeLV related sequences and were annotated and aligned with MAFFT (Katoh and Standley 2013). This was exported and used to construct phylogenetic trees and evaluate recombination between FeLV-A and enFeLV sequences. Recombinant sequences were not removed to clearly demonstrate the phylogenetic associations of enFeLV, FeLV-B and FeLV-A.

The alignment was sent to the IQTREE web server (Trifinopoulos et al. 2016), where a maximum likelihood tree was inferred using the ModelFinder (Kalyaanamoorthy et al. 2017) to select the best-fitted model nucleotide substitution. The model chosen was general time reversible (GTR+F+G4), branch support analysis was ultrafast with 1,000 bootstraps (Hoang et al. 2018). EnFeLV was used as an outgroup to root the tree, and the phylogenetic tree was visualized in FigTree v1.4.4 (Rambaut 2018).

3.1.3.6 Intra-host Variation Analysis and Recombination Analysis

The intra-host single nucleotide variation (iSNV) was done using BWA-MEM (Li 2013) and iVar (Grubaugh et al. 2019). All libraries were mapped to the FeLV-A reference sequence (M18247) using BWA-MEM and a consensus sequences were generated using iVar consensus command for each library. All reference sequences generated by iVar were cut at nucleotide 66 (M18247), to initiate at the same nucleotide position. The output was used to call single nucleotide variants and indels in iVar. The minimum quality parameter was <Q10 and the minimum frequency selected was 0.01 and was considered as true only those statistically significative ($p < 0.05$).

Recombination analysis was carried out in RDP v4.101 (Martin et al. 2015) using six different methods and default parameters. The methods used were RDP (Martin et al. 2015), GENECONV (Padidam, Sawyer, and Fauquet 1999), Chimaera (Posada and Crandall 2001), MaxChi (Smith 1992), Bootscan (Martin and Rybicki 2000), 3Seq (Boni, Posada, and Feldman 2007) and Siscan (Gibbs, Armstrong, and Gibbs 2000). Recombinants were considered if at least three methods detected breakpoints. The analysis included the reference sequences used previously and all sequences obtained by the three methods. Determination of enFeLV and exogenous FeLV was based on the first portion of 3'LTR.

3.1.3.7 Synonymous Site Conservation (SSC) and RNA folding.

Alignments generated from Illumina and Nanopore were used and aligned using ClustalW (Larkin et al. 2007). FeLV-A and FeLV-B analyses were carried out including sequences available from GenBank, and these were classified into *env* gene. Due to the sequences having different lengths, two alignments for FeLV-A were generated. To increase statistical significance, the shorter alignment included a greater number of sequences, whereas the larger alignment included fewer sequences. Each nucleotide alignment was translated and aligned by codon using "Codon Alignment tool v2.1.0" from HCV (HCV 2024).

SynPlot22 software (Firth 2014) was used to analyse synonymous site variability in the codon alignments. The reference sequences used were FeLV-A FAIDS (M18247) and FeLV-B Garner-Arnstein (V01172) in each case. The results over the threshold level (dotted line) indicate that

the genome is maintaining either a standard ORF or a structural element. RNA folding was performed in RNAalifold (Gruber et al. 2008), in the new version 2.6.3 with RIBOSUM scoring and default parameters (Bernhart et al. 2008) in “RNAalifold WebServer”.

3.1.4 Results

Amplicons for FeLV-A PCR showed lengths around the expected amplicon size (~1.9kb) with some products showing multiple bands. All FeLV-B PCR amplicons had similar lengths to the expected amplicon (~1.8 kb). Additionally, some FeLV-B PCR products showed the presence of an extra band below 1.8 kb (these were identified as truncated enFeLV in the NGS results).

Twenty Sanger sequences (10 of FeLV-A and 10 of FeLV-B) of samples were performed to confirm PCR product identity. Only four samples of FeLV-A were successfully Sanger sequenced (Omali, Maqui, Coco and Leon) with around 1.8 kb of sequence obtained, these DNA samples however did not have enough volume remaining to sequence with the other methods. BLASTn analysis from NCBI showed 97-99% identity with FeLV-FAIDS (M18247.1) sequence. Only one FeLV-B sample (Omali) was sequenced successfully (1,793bp) by the Sanger method, showing 98% identity with FeLV-B sequences from the USA (MT302107.1). Most amplicons failed to produce good quality results with the Sanger method, due to high interference from the presence of multiple variants. Addtionally, amplicons from Cuchi_FeLV-A, Coco_FeLV-A, and Coco_FeLV-B were also sequenced by Sanger from cloned plasmids.

For the amplicons sequenced by Illumina, one long sequence per cat was assembled with multiple small contigs in different gene sections. Four long sequences between FeLV-A and FeLV-B were obtained and were considered in the alignment for further phylogenetic analysis. For Nanopore sequencing, two contigs per cat were obtained by the first approach (De Novo assembly using Flye). These contigs were approximately 1,900bp in length. Afterwards, using the second preset options in Minimap2, 13 consensus sequences were obtained (6 sequences from Adonis; 4 from Cuchi; 5 from Felix; 1 from Polo). These were between 1,764bp to 1,602bp in length, including insertions, deletions and missed nucleotides in the sequence start.

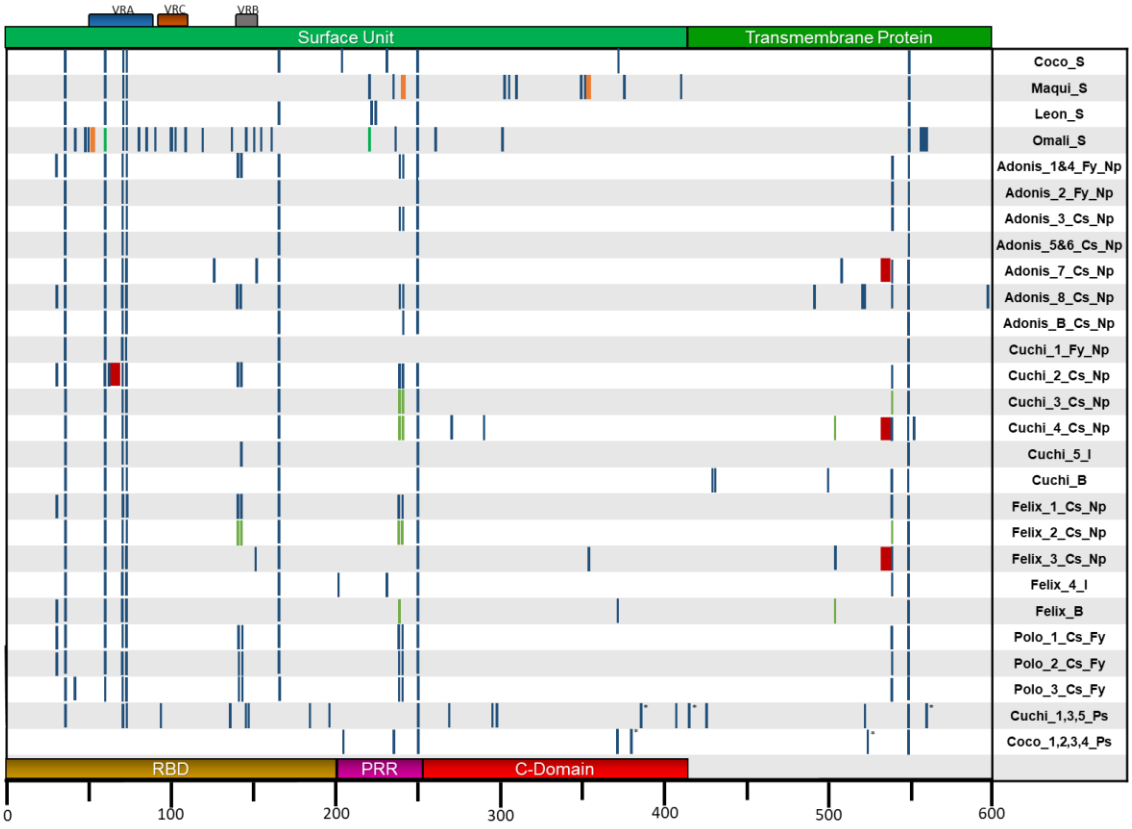
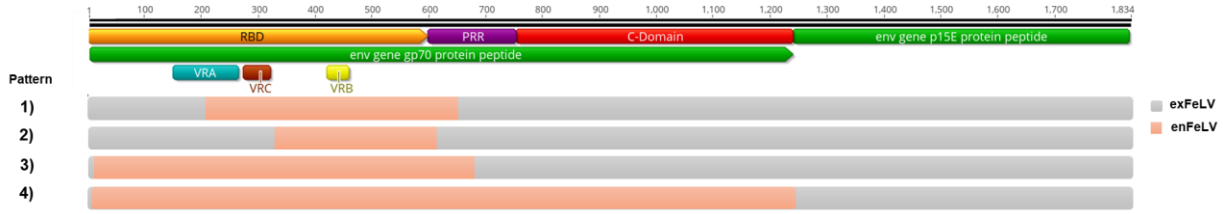


Figure 17 Scheme of alignment of envelope gene showing amino acid changes positions in FeLV-A sequences obtained from Sanger (S), Illumina (I), and Oxford Nanopore (Np) method. The coloured horizontal bars show the variable regions (VRA, VRC and VRB), the surface unit (light green), Transmembrane protein (green), Receptor binding domain (RBD) (yellow), Proline rich region (purple) and the C-Domain (red). Each blue vertical bar shows a single amino acid variation compared to the reference sequence; broader bar indicates two or more adjacent variation. Red vertical bars are deletions; Orange bars are insertion; Green bars alternate amino acid in the same position. Sequences are labelled by cat name, sequencing methods, assembly method, consensus (Cs) or De novo assembly in Flye (Fy).

The alignment of all these FeLV-A included 37 sequences belonging to 8 different cats. The alignment shows a similarity between 100-94.8% at nucleotide level. Amino acid changes were mainly identified in the SU (**Figure 17**). Seven variations were present in almost all Chilean sequences; 5 variations showed 2 possible amino acids. Other variations were less often detected; these were mainly present in Sanger sequences. The most distant sequence was a Sanger sequence (Omali_S). Illumina amino acid changes were consistent with Nanopore results, except for some minor changes in Felix_5_I in positions 202 and 230. The main amino acid insertions were detected in Maqui (Sanger). Deletions were observed in the SU region from

nanopore sequences generated by consensus in Felix (5_Cs_Np), Cuchi (4_Cs_Np) and Adonis (7_Cs_Np). Although plasmid clones were mostly consistent with other methods, several random nucleotide variations were identified, causing non-synonymous variations identified by this method, these were sample-specific (**Figure 17**).

The FeLV-B amplicon obtained by Illumina sequencing only produced one long sequence and multiple small fragments concordant with FeLV-B or enFeLV. The longest sequence was obtained from Cuchi. From Nanopore FeLV-B amplicons, 17 sequences were generated, 4 of which were considered to be FeLV-A. The other sequences were more closely related to enFeLV. The longest sequence was 1,818bp (represented by plasmid cloning) and the shortest was 1,474bp (Nanopore). These short sequences were missing nucleotides at the beginning of the sequence (5' end). The percentage similarity was 99.5% to 82.3%. The plasmid cloning sequence (Coco cat) was the most distant phylogenetically, more closely related to enFeLV sequences (**Figure 19**).



Pattern detected	Number of events detected	Sequence Name
1	10	Omali_FeLV-B_S; Adonis_FeLV-B_2_Cs_Np; Adonis_FeLV-B_4_Cs_Np; Felix_FeLV-B_3_Cs_Np; Felix_FeLV-B_4_Cs_Np; Cuchi_FeLV-B_1_Cs_Np; Polo_FeLV-B_1_Cs_Np; Polo_FeLV-B_2_Cs_Np; Polo_FeLV-B_3_Cs_Np; Polo_FeLV-B_4_Cs_Np
2	2	Cuchi_FeLV-B_3_Cs_Np and Polo_FeLV-B_5_Cs_Np
3	1	Cuchi_FeLV-B_I
4	5	Coco_FeLV-B_1_Ps, Coco_FeLV-B_2_Ps, Coco_FeLV-B_3_Ps, Coco_FeLV-B_4_Ps, Coco_FeLV-B_5_Ps

Figure 18 Scheme of recombinant patterns from RDP4 analysis. The reference sequence is the envelope gene from FeLV-A_FAIDS (M18247) sequence annotated in Geneious 2023.1.1. All sequences were considered in the analysis, but recombination was only detected in FeLV-B amplicons. Sequences from Sanger (S), Plasmid sanger (Ps) Illumina (I), and Nanopore (Np) are shown. The gray bar colour is sequences related to exogenous FeLV (exFeLV) and orange is for endogenous FeLV (enFeLV). VRA, VRC and VRB are the variable region A, B and C. RBD is the receptor binding domain, PRR: Proline rich region. The table enumerates the detected patterns, the number of sequences in which recombination events were identified, and the names of the sequences where these events occurred.

The recombination analysis performed in RDP4 showed four recombination breakpoint sites which were unique in the FeLV-B amplicons. All recombination breakpoints were derived from enFeLV genes with variable length inserted in the SU. The longest enFeLV insertion was in all Coco_FeLV-B_Ps which encompassed the whole RBD, PRR, and C domain. While the second pattern was a smaller portion of enFeLV inserted in the end of RBD and PRR (**Figure 18**).

A total of 174 sequences were considered in the phylogenetic analysis, including all *env* complete sequences from GenBank and a second tree was constructed including 104 sequences (**Figure 19**), including phylogenetically closer sequences and representative clades. The tree is rooted in enFeLV, being subdivided into two major clades, enFeLV and FeLV-A. The Chilean FeLV-A clade is clearly identified next to the US (United States of America) sequences, and the cats are homogeneously represented through the Chilean clade. Sanger sequences are more related to each other than the rest of the NGS sequences. In contrast to the FeLV-A Chilean clade, the FeLV-B sequences are not closely related in a unique clade, where the biggest nanopore group are located next to Sanger sequences and the Illumina sequence is closer to Gardner-Arnstein (V01172) with a third clade formed by Cuchi_FeLV-B_3_Cs_Np and Polo_FeLV-B_5_Cs_Np, closer to FeLV-A. This third clade also demonstrated the second recombination breakpoint pattern with a shorter enFeLV portion.

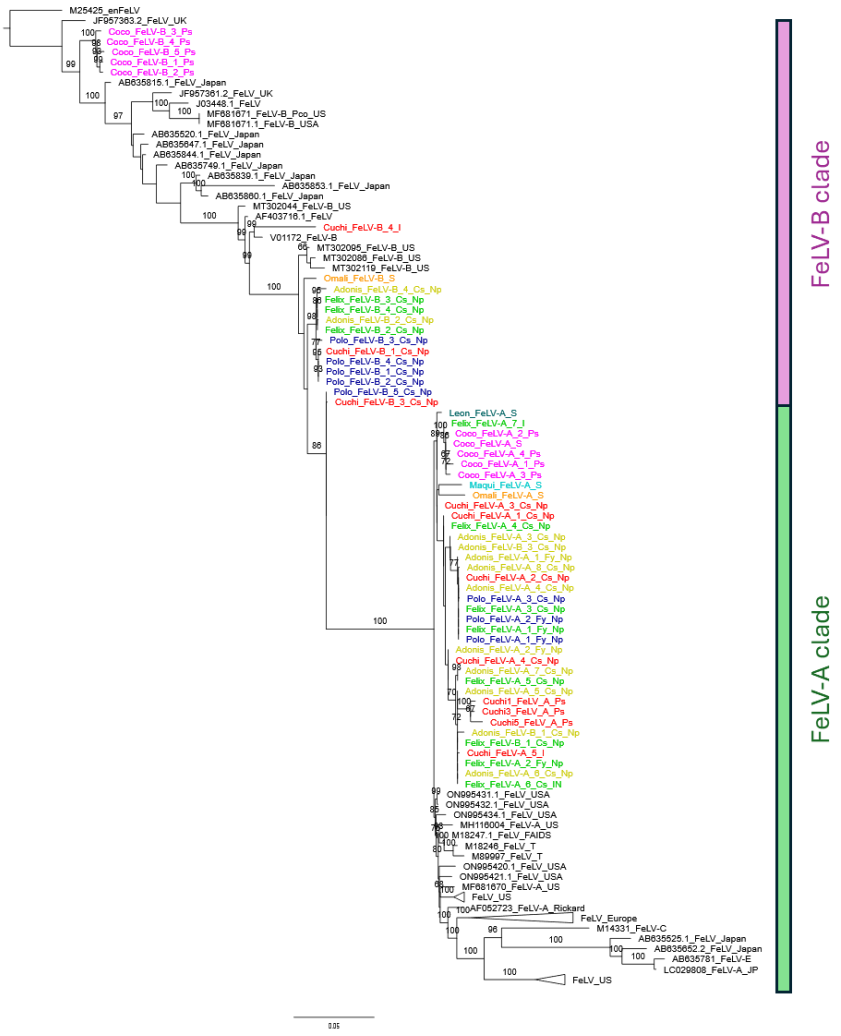


Figure 19 Phylogenetic tree of nucleotide env gene constructed with 1,000 bootstrap approximation and rooted in enFeLV (M25425). Eighty-four FeLV sequences were considered, including references sequences of FeLV-A, FeLV-B, FeLV-C, FeLV-T and FeLV-E. Clades from the US, Europe, and Japan have been collapsed for clarity (horizontal triangles). The sequence name colour indicates the cat sampled. Sequences obtained from GenBank were called according to its reference number, type of FeLV (if is known it), species (if was required), and country of origin. The sequences obtained in this study were called according to name; FeLV amplicon obtained; number sequence per cat; sequence obtained by consensus (Cs) or de novo assembly in Flye (Fy); and sequencing method, Illumina (I), Nanopore (Np), Plasmid clone (Ps) or Sanger (S).

iVar intra-host variation analysis was applied to all NGS results, comparing the two different NGS methods (Cuchi and Felix) and comparing FeLV-A and B amplicons from nanopore results

(**Figure 20**). Differences between the methods and amplicons were observed. In general, the nanopore results in FeLV-A amplicons detected around 400 variation events, but only a small portion of these were statistically significant (less than hundred). Similarly, in FeLV-B above 800 variation events were detected, but less than 200 on average were statistically significant. This contrasted with the Illumina results, where less than 100 variations were observed and half of them were considered as true. All Illumina variations were detected in low frequencies and were located from the end of RBD and beginning of PRR. Nanopore results showed a longer distribution of variation through all the *env* gene from FeLV-A amplicons. The cat "Felix" FeLV-A amplicon showed high frequencies of variations in the RBD, but these were highly similar to the FeLV-B amplicon from the same cat. All FeLV-B amplicons showed a high number of variations in the first portion of RBD and a minor group of changes in TM (**Figure 20**).

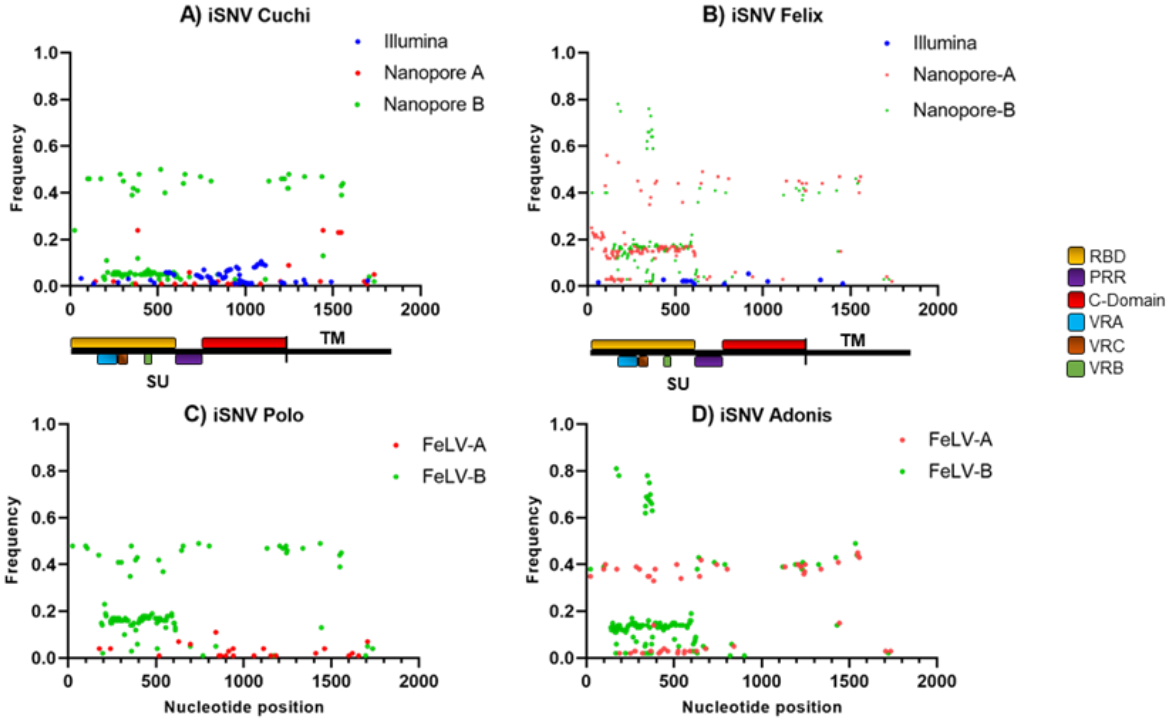


Figure 20 iSNV sites detected in env gene amplicons from NGS results. iVar software was used to generate a consensus sequence per library and to be used as reference sequence (~1,800bp). In the middle are schemes of FeLV-A (M18247) showing gene positions and main variation sites (RBD: Receptor Binding Domain; PRR: Proline Rich Region; VRA, VRC, and VRB are variable region A, C, and B respectively. Each graph (A, B, C and D) represents the cat sampled and frequency and nucleotide

position classified by amplicon and sequencing method are compared. The blue points correspond to variation points by the Illumina method (in A and B graph), the red points are variation points in the FeLV-A amplicon and green points are the FeLV-B amplicon variations (all graph).

With regards to nucleotide variation (**Figure 21**) compared to the reference sequence (1765bp), its frequency is led by adenine (29.35%) and cytosine (27.20%), followed by thymine (21.53%) and guanine (21.93%). The most common variation in Cuchi and Polo (all libraries) were A→G and T→C. Similarly, Felix-I has higher variation to A→G and T→C, although nanopore results had a similar variation percentage between A→G and T→C with C→T and G→A. Adonis, in both nanopore libraries, showed an increased percentage of C→T and G→A mutations.

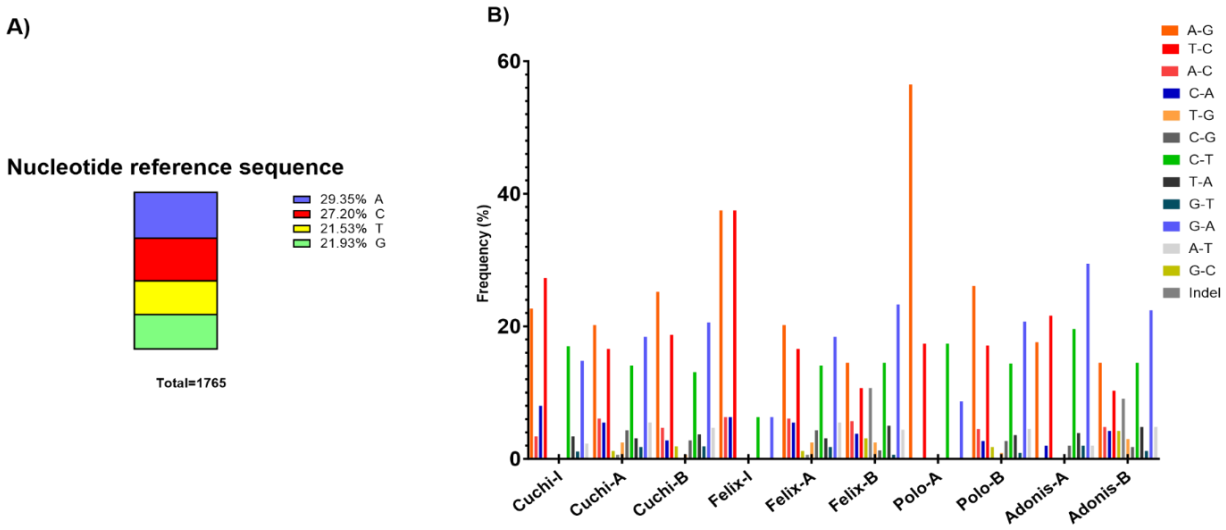


Figure 21 Nucleotide variation in sequences obtained from NGS sequencing. A) Graph of nucleotide frequencies in reference sequence. B) Bar graph showing nucleotide change frequency. Each colour represents one nucleotide change and there are grouped by cat and amplicon obtained. Nanopore results are pointed out the cat and from which amplicon was obtained, FeLV-A (A) or FeLV-B (B). Illumina results are represented with the cat's name and "I".

3.1.4.1 SSC and RNA folding.

FeLV-A SynPlot2 analysis was conducted using two alignments for FeLV-A sequences. The longer alignment (with a smaller number of sequences) included 90 sequences and 1,758bp of length. The shorter alignment included 127 sequences and 1,479bp of length (**Figure 22**). The longer alignment (with fewer sequences) showed two peaks close to the threshold in codon 250

and 350, but increasing the number of sequences, the codon in 350 (in C-Domain) overcame the threshold and another peak in codon 220 (PRR) was confirmed.

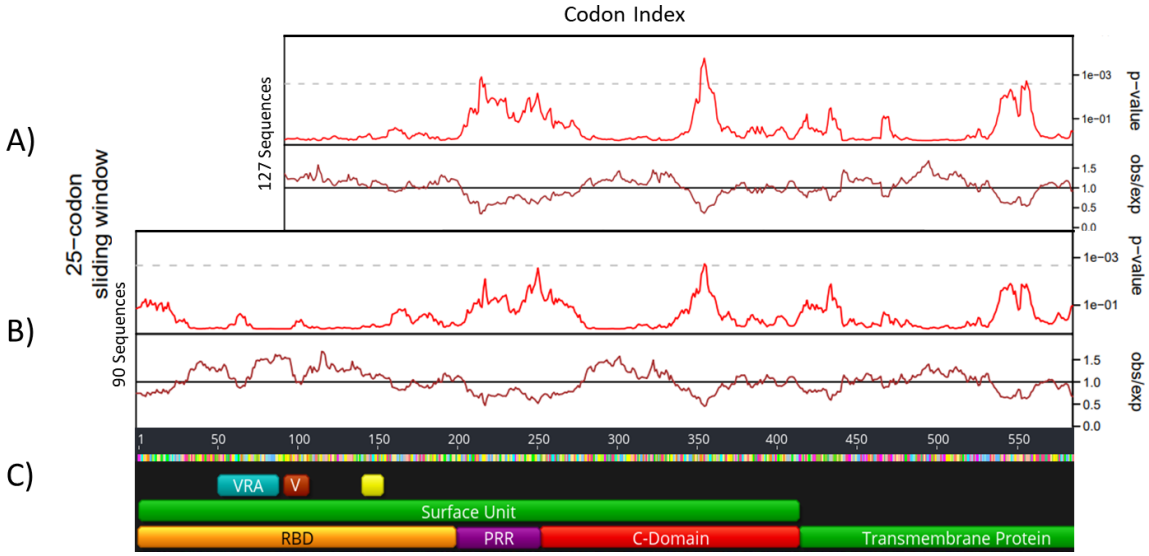


Figure 22 FeLV-A envelope gene synonymous site conservation analysis performed in SynPlot2 software. Red peaks over the threshold (dotted line) indicate statistical significance. A) Graph of SynPlot2 using an alignment of 90 sequences and 1,758bp (586 amino acid) of length. B) Graph of SynPlot2 using an alignment of 127 sequences and 1,479bp of length. C) envelope gene scheme generated in Geneious. In emerald, brown and light yellow are shown the variable regions, VRA, VRC and VRB, respectively. Receptor binding domain (RBD) is represented in yellow, Proline rich region in purple and C-Domain in red.

FeLV-B SynPlot2 analysis was carried out using an alignment of 85 sequences available in GenBank, and 1,653bp of length (**Figure 21**). The SynPlot2 analysis showed a smaller peak at the beginning sequence (codon number 1 to 20) and a broad peak from codon 40 to 190.

Figure 23 FeLV-B envelope gene synonymous site conservation analysis performed in SynPlot2 software. Red peaks over the threshold (dotted line) indicate statistical significance. A) Graph of SynPlot2 using an alignment of 85 sequences and 1,653bp (551 amino acid) of length. B) envelope gene scheme generated in Geneious. In brown and light yellow are shown the variable regions, VRC and VRB, respectively. Receptor binding domain (RBD) is represented in yellow, Proline rich region in purple and C-Domain in red.

The RNA structure analysis of the *env* gene showed that FeLV-A is a complex and highly branched structure with a central core of folding (**Figure 24**). This complex structure may suggest a less stable and more flexible structure, which may present dynamic regulation and an adaptable strain. The core of the image represents a multibranch loop, at the right area the image shows a hairpin with terminal loop, at the opposite site, an extended stem-loop. The lower area of the image, this shows a dense loop cluster. The end of the image shows the end of *env* gene with loops and bulges representation.

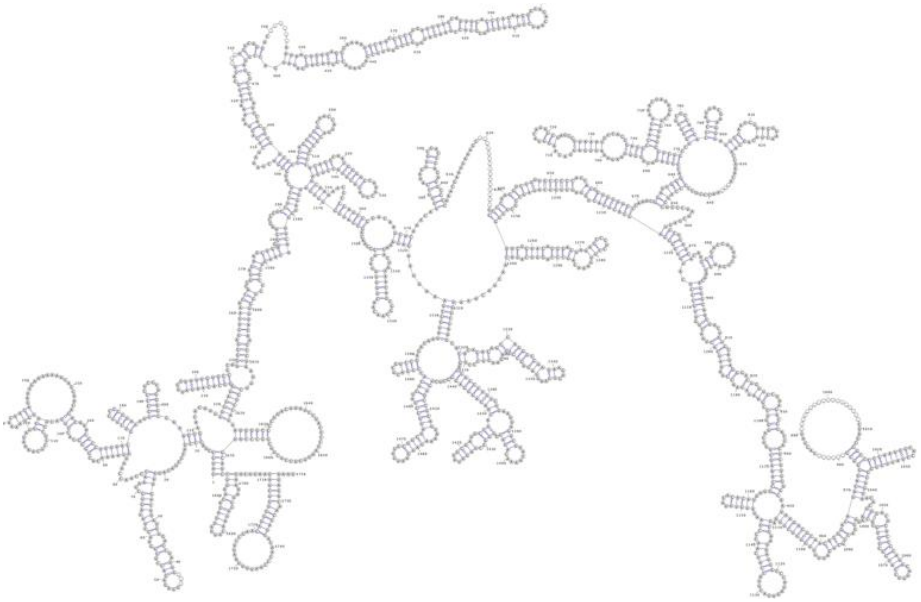


Figure 24 RNA folding from FeLV-A structure. Predicted RNA folding was generated using RNAalifold.

The FeLV-B RNA structure showed shorter loop lengths, fewer larger bulges and a continuous structure compared with FeLV-A (**Figure 25**). It suggests that the FeLV-B *env* region is more stable compared to FeLV-A. The structure shows multiple short multibranch loops, with short hairpins towards both ends. This structure is caused by recombination and is possibly more adapted to the host.

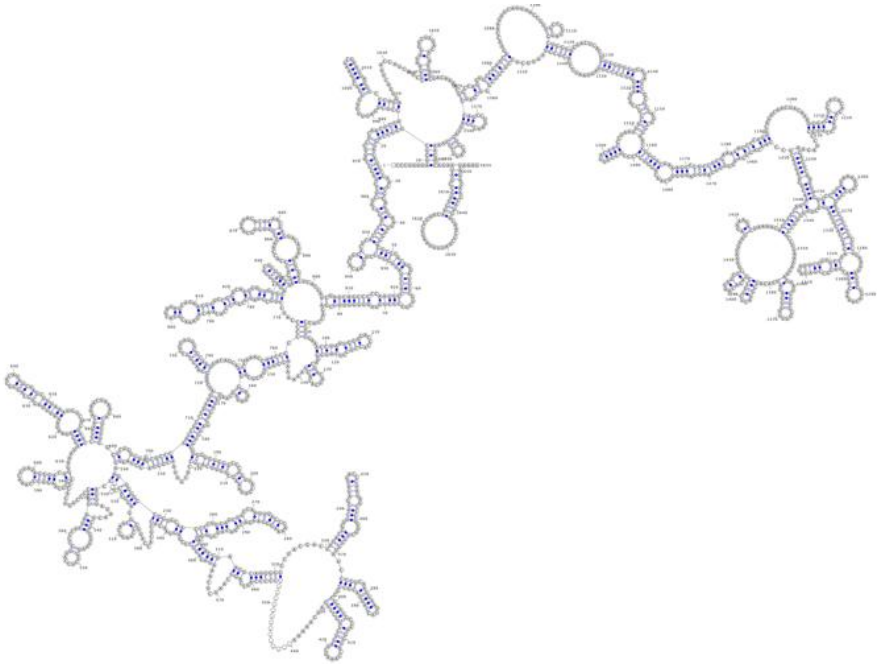


Figure 25 RNA folding from FeLV-B structure. Predicted RNA folding was generated using RNAalifold.

3.1.5 Discussion

Originally, FeLV variant characterization has been studied by viral interference assays in cell culture, but genetic advances have facilitated the evaluation of specific genes and correlates with functional proteins (Chiu, Hoover, and Vandewoude 2018). The most common genetic analysis in FeLV has been based in the LTR due to its high conservation (Cattori et al. 2006, Tandon et al. 2008). Nonetheless, the importance of viral recombination has driven analysis of the envelope gene. Studies using molecular analysis based in *env* are available only in a few countries: Japan (Miyake et al. 2016), the US (Erbeck et al. 2021), the UK (Stewart et al. 2013), Spain (Geret et al. 2011) and Brazil (Cano-Ortiz et al. 2022, Biezus et al. 2023). This is mainly because the work is laborious, with multiple steps such as end-point PCR or nested PCR, plasmid cloning and Sanger sequencing (Watanabe et al. 2013, Erbeck et al. 2021). Although NGS has had a rapid advancement, these techniques are still new in FeLV diagnosis, where the main problem is the high conservation of sequence identity between enFeLV and exogenous

FeLV (Chiu, Hoover, Vandewoude 2018). All our sequencing results were broadly consistent, however there were differences detected depending on the sequencing method and clustering by sequencing method. Here we documented the comparison of *env* gene amplicon-based sequencing using three methods and evidencing advantages and limitations of each technique.

Sanger sequencing has been the most widely used method in *env* analysis, it is highly reliable but only can generate only one single read per amplicon. To obtain clear reads in a mixed population (like a retroviral quasispecies), it is usually necessary to create plasmid clones and sequence these individually (Watanabe et al. 2013, Erbeck et al. 2021). Our initial results were performed directly from the PCR amplicon because it was not the aim of this study and, as expected, most sequences did not show good quality. Only those with clear high-quality reads were considered in this methods comparison. Cloning of PCR products into plasmids (*Escherichia coli*) and then sequencing of the plasmids produced more sequences of good quality but did not increase the number of variants detected per cat. Similarly, FeLV-B amplicons had worse quality due to the presence of FeLV-A and enFeLV in the same amplicon (as was confirmed by the NGS results). The main advantage of the Sanger method is the lower error rate, described as 0.001% (Hoff 2009), however added to this error rate the multiple steps, PCR amplifications, plasmid cloning and sequencing should be considered (Cheng, Fei, and Xiao 2023). Studies based on HIV have shown that Sanger sequencing has a limited capacity to detect low-abundance intra-host variants when these are under a threshold of 20% of frequencies. This could mean missing information about less-representative but still clinically important sequences (Ávila-Ríos et al. 2020). Our FeLV-A Sanger sequences (whether direct or from plasmid clones) clustered closer together than sequences from the other two methods. These sequences, however, had a higher number of SNPs and indels per sequence than contigs obtained by other methods. Plasmid clone “Coco” were closely related to the original direct Sanger sequence, indicating that sequencing without cloning was reliable. However, “Omali”, at a SNP level, showed the highest number of amino acid variations. This likely reflects the consensus nature of the Sanger sequences, but also there was a high rate of failure for Sanger sequencing (only 5 of 20 sequences attempted showed good quality), likely reflecting the sequence diversity of the PCR amplicons, failing to produce a clean enough sequence to

read. Notably, the “Coco” FeLV-B, showed a longer enFeLV insertion not observed on the rest of NGS sequences. These sequences were closer to almost whole *env* genes from enFeLV described in Japan and the UK (Watanabe et al. 2013, Stewart et al. 2013).

The Illumina short-read method is described as the most reliable method for detecting iSNVs, showing only slightly higher base-pair error than Sanger sequencing, between 0.26%-0.8% (van Dijk et al. 2014). However, it was not possible to assemble many long contigs from the Illumina short read data. In order to increase the number of contigs assembled, various bioinformatics modifications were tried, for instance reducing the filtering options in FastP and using SPAdes as an assembler, but this did not make a substantial difference to contig recovery. Only one Illumina FeLV-B sequence was therefore available for recombinant analysis, this sequence showed a different structure to those detected by the Sanger or Nanopore methods. Due to a failure to generate long contigs, it was only possible evaluate SNVs using the iVar software comparing reads mapped to reference sequences of FeLV. Using iVar software a reduction in the minimum frequency threshold to include under-represented reads was required, because a high number of artefacts from endogenous FeLV from the cat genome and FeLV-B recombinants were detected as well.

Oxford Nanopore was a successful tool to discriminate between FeLV-B and FeLV-A and to determine the structure of the enFeLV and exFeLV recombinants in the FeLV-B PCRs, but it is a still developing tool and its error rate is described as between 5-15% (Rang, Kloosterman, and de Ridder 2018). Although successive MinION versions have been decreasing this error rate. At the iSNVs level, its results should be cautiously treated in order to minimize the error (Grubaugh et al. 2019). This is reflected in the sequences retrieved in this study by the number of sequences with X (uncertain amino acid) reads. The Nanopore long read method is however much better suited to detecting large indels in sequences and this is reflected in this study by its better success at retrieving and mapping FeLV-B recombinants. Similarly to the main FeLV-B recombination pattern previously described (Cano-Ortiz et al. 2022), the majority of sequences obtained included RBD and a portion of PRR derived from enFeLV. Nonetheless, we used a conservative criterion to generate consensus sequences. Some were in fact exogenous reads with a longer portion derived from enFeLV, where the exogenous sequence had an endogenous

insertion encompassing the RBD up to almost the whole C-Domain, but these were detected in a small number of reads.

Regarding amino acid and nucleotide variations, some of them were present in all Chilean reads when compared to the reference sequence (a US isolate from 1988) and are clearly features of Chilean strains. Other iSNVs were present in only a small number of sequences at a low frequency and probably represent minor variants within the quasispecies mix. Interestingly many of these iSNVs were outside what are usually regarded as the more variable regions of the *env* gene. Different recombination patterns are also evident in the sequences. A comprehensive FeLV recombination review (Cano-Ortiz et al. 2022) showed multiple recombination patterns between exogenous and endogenous FeLV. In our data recombination's between enFeLV and exFeLV were frequent in the RBD with very few in the C-Domain. This is reflected in the lower number of SNPs in the C-Domain with most related to exFeLV variations (as few FeLV-B fragments contain enFeLV sequence in this domain). Our results confirm the previous data with the RBD (the most important region for binding to the receptor) the most variable site and the site of most frequent recombination with enFeLV. The C-Domain appears to be the most highly conserved region of the virus. This domain is known to play a role during viral infection as a secondary receptor, particularly for the FeLV-C strain, and appears to be particularly important in tropism for particular receptors/cell types (Boomer, S et al. 1997, Ramsey, Spibey, and Jarrett 1998, Gwynn et al. 2000, Rey, Prasad, and Tailor 2008). The lack of sequence variation in this study indicates that conservation in this domain is clearly critical to the virus.

The SynPlot2 analysis also confirmed the high conservation and importance of C-Domain. The SynPlot2 indicates that the genome is conserving two elements: the standard ORF and another element that may be an overlapping ORF or an RNA structural element. Generally, narrow peaks indicate pseudoknots, hairpins or RNA regulatory elements, while broader peaks indicate overlapping ORFs present (Firth 2014).

In the SynPlot2 analysis in FeLV-A, two peaks were observed in the PRR and in the C-Domain. The C-Domain has been recognized a highly conserved site in recombinant analysis, its role has not been completely understood, although it is suggested that it may work as secondary

receptor-binding domain and improve the binding efficiency of the env protein and receptors (Rey, Prasad, and Tailor 2008, Cano-Ortiz et al. 2022).

On the other hand, the PRR of type-C retroviruses in mammals is known to conduct conformational modifications to facilitate the virus access to the host cell. Among these changes are the conformation of the RBD, stability of the SU-TM association and interactions between amino- and carboxy- terminal domains in the SU region of the env protein (Lavillette et al. 1998). In FeLV, one study demonstrated that PRR folds into a polyproline β-turn helix, which is an ordered rigid structure capable of self-assembly into complex ordered multimers (Fontenot et al. 1994, Lavillette et al. 1998). Therefore, the SynPlot2 peak observed in the PRR in the plot is likely a result of this structure characteristics.

The broad peak observed in the FeLV-B SynPlot2 analysis is probably an artefact caused by the recombinant nature of this strain. This may be caused because the first portion of gene is derived from endogenous virus, which has a slow mutation rate (the host cat genome mutation rate), whereas the FeLV-A portion is rapidly mutating as occurs in RNA viruses (Drake and Holland 1999). Additionally, the limited number of FeLV-B sequences available in GenBank mostly come from sequences of intra-host mutations, and lower variability is expected (Watanabe et al. 2013, Erbeck et al. 2021). In consequence, a greater number of sequences are necessary to reach statistical significance for highly conserved areas like enFeLV. Thus, SynPlot2 analysis in enFeLV was inaccurate, due to fewer sequences being available for the analysis, and all of them being obtained from very specific cat populations and recombinant viruses. Although the SynPlot2 were not reliable for FeLV-B, the RNA folding was consistent, showing FeLV-B as a more stable molecule. The modification of this RNA structure modifies the receptor usage but also its stability (Tailor and Kabat 1997). Intriguingly, we obtained a general structure of FeLV-B, but the recombination patterns determine influence of enFeLV on the general structure of FeLV-B. Therefore, further studies could determine the influence of the portion of enFeLV on RNA folding structure, in consequence it could determine higher or lower pathogenicity of this recombinant virus.

The NGS results also showed that neither the PCR primers nor the mapping software completely discriminated between FeLV-A and FeLV-B sequences. FeLV-A sequences were generated from FeLV-B amplicons, and during the iSNV analysis, all sequences were mapped to a consensus generated from FeLV-A reads, however, in Adonis-A and Felix-A, these showed a group of high frequency iSNVs closely related to FeLV-B. The pattern of nucleotide change (A→G, T→C, and A→T) is similar to that in a study of FeLV cats infected with tumours (Rohn, J L et al. 1994). This type of mutation signature is commonly caused by the innate anti-viral APOBEC protein system, one of the main host responses in retrovirus infection (Stavrou and Ross 2015). These proteins generate hypermutated viral genomes with an increased number of G→A mutations to produce non-infective virions (Terry et al. 2017). Our results using NGS consistent with previous findings, further confirming a low impact of APOBEC-mediated hypermutation in FeLV (Münk et al. 2008, Stewart et al. 2012).

Each sequencing method produced different results even for the same animal and this needs to be considered when comparing large phylogenetic trees generated from amplicons derived from different sequencing methods. Each sequencing method has advantages and disadvantages, but phylogenies based on Sangar sequencing probably overestimate viral variation and may result in artificial sequence clustering when compared with the NGS methods. Moreover, other factors, like the input DNA amounts and concentrations used need to be considered in interpreting viral sequence variation. Our Illumina analysis was done using the minimum DNA requirement (around 5µg) and using 30x of coverage input, conversely, we used the maximum allowed DNA input for nanopore (around 250µg), this may have influenced the sequencing quality and quantity of under-represented sequences in Illumina as has been reported before (Illingworth et al. 2017) contrasting to the higher diversity detected in nanopore, although similarly to other reports, minority reads were masked and diluted by a greater number of sequences during consensus read construction (Mori et al. 2022).

Overall, the different methods were useful for different purposes with Sanger and Nanopore less reliable for iSNVs detection than Illumina. On the other hand, Nanopore was more successful for recombinant FeLV-B detection. With the growth in sequencing studies using different technologies, it will become increasingly important to consider which sequencing method was

used in interpreting geographic and temporal variations in viral sequences, as well as designing and standardizing appropriate pipeline analysis to improve the quality of sequence, data interpretation, and prevent sequence dilution effects, a practice already being implemented in diagnostic HIV sequencing (Ávila-Ríos et al. 2020, Lee et al. 2020, Mori et al. 2022). Artificial clustering and estimation of sequencing variation due to sequencing artifact may complicate the interpretation of large viral phylogenies, particularly in viruses like FeLV where there are high virus circulation and recombination. Due to this being the first study conducted with NGS sequencing strategies for, there are no previous comparable data. Future works should include a greater number of animals and geographically distant FeLV strains, to analyse the viral diversity in domestic and non-domestic felids.

Chapter 4: Endogenous Gamma retroviruses in Chilean Domestic Cats

This section includes contributions from:
Ezequiel Hidalgo-Hermoso[1], Constanza Napolitano[2], Camila Stuardo[2].

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Statement of contribution: Cristobal Castillo-Aliaga performed Nanopore sequencing, bioinformatic and molecular analysis, wrote and edited the text. Adam Blanchard contributed conditioning, setting up laptop, and real-time sequencing. Ezequiel Hidalgo-Hermoso transported samples and edited the manuscript. Constanza Napolitano and Camila Stuardo contributed sampling domestic cats, the samples selection and submission of the samples. Rachael Tarlinton conceptualised whole study and edited the manuscript.

4.1.1 Abstract

Endogenous retroviruses are viral sequences that have been inserted into the host genome during its evolutionary history. Some ERVs have been “domesticated” to assume cellular functions in the host. Domestic cats possess an unusually high abundance of gamma retroviruses compared to other carnivores. The age of these ERVs is associated with inactivation by host defence mechanisms. In domestic cats, Fc-gamma4 is considered one of the oldest ERVs, estimated to have integrated around 5.7 Mya, whereas ERV-DC and enFeLV are believed to have integrated between 2.5 to 3 Mya. The most recently endogenized element is the RDRS, which may have been incorporated into the genome during or even after the divergence of modern cat breeds as a result of reactivation of RD114.

Understanding the functional consequences of ERV insertions remains challenging due to their varied effects on host gene expression and their interaction with diverse regulatory pathways. A novel NGS sequencing approach called Nanopore Adaptive Sampling (NAS) was used in this

study. NAS generates a bioinformatic enrichment, which allows targeting in real-time a specific sequence to filter out unmatched sequences during the sequencing. This method was employed to enrich gamma retroviruses from genomic DNA samples of Chilean domestic cats.

Using NAS, the study successfully detected enFeLV, Fc-gamma3, exFeLV, RDRS, and ERV-DC. Although sequencing depth was insufficient for robust SNP analysis, LTR sequences were mapped against the cat genome to identify insertion sites. The highest number of common integration sites (CIS) shared was observed for the older Fc-gamma4 and ERV-DC, while enFeLV insertions appeared more randomly distributed, consistent with previous studies. RDRS insertions were detected at low frequency, with only 20% of sites shared among multiple individuals.

To assess potential genomic influence, the genomic context of each insertion was evaluated by counting protein-coding genes within ±1Mbp of each IS. RDRS insertions were found in closer proximity to genes, with an average of 12.8 genes per site, followed by enFeLV (7.2 genes), ERV-DC (5.5 genes) and Fc-gamma4 (4.5 genes). These patterns align with the proposed timeline of endogenization, where older endogenization events occur farther from encoding genes. These findings demonstrate the utility of NAS for detecting ERV insertions and exploring their evolutionary and functional dynamics in the domestic cat genome.

4.1.2 Introduction

The evolutionary history of mammals has led to multiple viral insertions into host genomes. Retroviruses that have become integrated into the host genome are known as Endogenous Retroviruses (ERVs). In species such as humans, mice and felids, ERVs are estimated to occupy between 8-12% of the host genome (Gifford and Tristem 2003). Throughout evolutionary history, viral and host genomes have co-evolved and ERVs have been domesticated to assume some cellular processes such as immune modulation, placentation, and oncogenesis regulation (Johnson 2019). Although most ERVs have been inactivated to allow coexistence with the host, some of them can be reactivated by recombination with exogenous (horizontally infectious) retrovirus (XRV) counterparts. The most common recombination site between XRVs and ERVs

is located in the envelope (*env*) gene, which is the gene responsible for cellular receptor interference and cellular entry binding sites (Chabukswar et al. 2023).

The ERV-DC, like the enFeLV, are a group of endogenous gamma retroviruses with an integration into the cat genome that likely occurred around 2.8 million years ago (Mya) (Anai et al. 2012). Thirteen different loci have been reported throughout the cat genome (Anai et al. 2012, Kuse et al. 2016, Pramono et al. 2024), although 17 loci have been estimated to exist across cat populations (Kawasaki and Nishigaki 2018).

Phylogenetic classifications divide ERV-DCs into three genotypes (GI, GII, and GIII), reflecting different integration events and distinct impacts on the feline genome (Anai et al. 2012). Among them, ERV-DC8, DC14, DC10 and ERV-DC18 have intact ORFs and can produce infectious viral particles (Anai et al. 2012, Pramono et al. 2024).

ERV-DC are also classified based on a *cis*-acting nucleotide variation within the LTR promoter region. This variation may have arisen during the endogenization processes, with the T-type likely derived from the A-type as an evolutionary strategy. This is based on the idea that the T-type LTRs attenuated promoter activity may help to evade the host immune system (Kuse et al. 2016).

Genotype I (GI) is the largest group and is known to recombine with exogenous FeLV. FeLV-D is a recombinant form of FeLV that has acquired its *env* gene from GI ERV-DCs, and this recombination occurs *de novo* in each cat (Anai et al. 2012). GII represents a domesticated group of ERVs, that have been disrupted by mutations and deletions in the *pol* and *env* genes, encoding Refrex-1, which is an antiviral factor inhibiting ERV-DC GI and FeLV-D binding to the host receptor (Ito et al. 2013, Pramono et al. 2024). GIII includes ERV-DC6, DC10 and DC18; although ERV-DC18 is probably a reintegration, reinfection, or transposition of ERV-DC10, as both differ by only single nucleotide variation in the primer-binding site of their full-length proviral genomes (Ngo et al. 2019).

During endogenization, ERVs undergo host control modifications such as CpG methylation patterns in 5’ LTRs, which happens downstream of the TATA box reducing the ERV replication activity. Thus the 5’LTRs of GIII of ERVs are hypermethylated (ranging from 84 to 98%

methylated), contrasted to the GII which are hypomethylated, showing higher expression levels (Kuse et al. 2016).

The FcERV-gamma4 is the oldest group of gamma retrovirus ERVs in the cat genome, these were inserted approximately 5.7 Mya (Kawasaki et al. 2017). Among them, Gamma4-E2 is estimated to be the oldest insertion, whereas gamma4-X2 may represent a younger insertion in the cat genome, with variations in prevalence between Japanese domestic cat populations (Kawasaki et al. 2017). Recombinants between FeLV and a portion of the 5'-leader sequence and the *gag* gene of FcERV-gamma4 have been described which may increase the viral replication and these cases were associated with hematopoietic tumours (Kawasaki and Nishigaki 2018, Ngo et al. 2024).

RD-114 is an ERV that is replication competent. RD-114 uses ASCT2/SLC1A5 and secondly ASCT1 (SLC1A4) as a cellular receptor. The RD-114 group is closely related to other ERVs (RDRSs), although these have been described as non-replicative (Shimode, Nakagawa, and Miyazawa 2015). Reports have described between 8 to 13 distinct loci. Four of the six proviruses (RDRS A2, E3, D4, C2b) were probably recently integrated into the cat genome (<0.2Mya), as there is no evidence of LTR-LTR recombination (Shimode, Nakagawa, and Miyazawa 2015).

The RDRS C2a is the oldest related provirus and closely related to RD-114 (95.7%). This has been proposed as the ancestral virus that infected domestic cats around 1.6 Mya. These group of RDRS can vary in their prevalence, for example most Asian cats breed lack RDRSs and RDRS_E3 is limited to certain cat breeds, supporting the theory of recent invasion of RDRSs into the cat genome (Shimode, Nakagawa, and Miyazawa 2015).

The effects of insertion sites are highly complex to understand. There is more evidence on exogenous retroviral insertion site effects. These can significantly contribute to pathogenesis, modifying host gene regulation to cause dysfunction and subsequently, disease. For instance, avian leukosis virus (ALV), FeLV and murine leukaemia virus oncogenesis involves preferential integration at sites near to protooncogenes such as c-myc, resulting in overexpression (Hayward, Neel, and Astrin 1981, Corcoran et al. 1984, Fujino et al. 2009). This dysregulation

works through complex mechanisms, such as upregulating Bmi-1, a repressor of apoptosis genes, thereby involving tumour suppressor pathways (Jacobs et al. 1999).

Although insertion site preference is not a strict rule, oncogenic retroviruses often preferentially insert into actively transcribed chromatin, where open chromatin may facilitate the integration. For example, MLV tends to integrate near to active enhancer sequences just upstream of gene start regions, slightly preferring active genes, while HIV-1 shows a strong preference for CpG islands and actively transcribed genes in both primary cells and transformed cell lines (Mitchell et al. 2004, LaFave et al. 2014). In contrast, ASLV and human T-cell leukemia virus (HTLV) exhibit a more random integration pattern (Mitchell et al. 2004, Derse et al. 2007).

The LTR by itself, can also drive and regulate retroviral transcription. Each LTR consists of three main elements: U5, which assists in reverse transcription; R, which contains transcription start and termination sites; and U3, which harbours enhancer and promoter elements (Uren et al. 2005). The U3 region includes critical regulatory sequences such as the TATA box and GC-rich motifs, which are crucial for recruiting the basal transcriptional machinery (Denner 2010). Consequently, mutations or structural modifications within the U3 segment can influence the spectrum of insertion sites, which may be related to predisposition to lymphoma and enhance viral pathogenesis in retroviral infection (Nielsen et al. 2005).

Proviral insertions can disrupt normal cellular gene function through several mechanisms. These include truncation of host gene transcripts, enhancement of gene expression, or the formation of chimeric transcripts (Uren et al. 2005). Furthermore, these effects can occur not only at the site of insertion but can also cause effects over long genomic distances via chromatin loop interactions (West and Fraser 2005). The influence of LTRs in gene enhancers could be up to 1 Mbp from the insertion point (Pennacchio et al. 2013, Chisholm, Busch, and Crowder 2019, Chiu et al. 2024, West and Fraser 2005). In such cases, looping factors are recruited to facilitate the formation of chromatin loops, bringing enhancer elements closer to the target gene (Krivega and Dean 2012). Also, proteins such as CTCF and cohesin proteins help to mediate enhancer-promoter interactions. Typically, when the provirus integrates in antisense orientation, its

regulatory effects are upstream of host genes, whereas in sense orientation, it commonly influences genes downstream of the insertion site (Uren et al. 2005).

While initial ERV insertion is largely random there are some patterns in IS, particularly in older ERVS. In some cases, ERV insertions are concentrated in specific chromosomes, such as the Y chromosome, where they tend to be more abundant. This may be due to common recombination events and the absence of structural rearrangements during mitosis and meiosis in this chromosome as it does not pair completely with the X chromosome (Medstrand, Van De Lagemaat, and Mager 2002). Additionally, retroelements often show preferences for insertion near to CpG islands and GC-rich regions. Moreover, older and domesticated ERVs may be found farther from genes when under selective pressure, contrasting to younger LTR class II elements, which are more likely to be inserted more evenly and near or in genes. Older ERVs also tend to be closer to AT-rich regions, whereas younger ERVs have a preference for GC-rich DNA (Medstrand, Van De Lagemaat, and Mager 2002).

Genetic enhancers are *cis*-acting DNA elements that increase gene transcription. Nonetheless, understanding their effects has several complications. First, enhancers are located in non-coding regions or intron areas, also their target can be highly variable, affecting upstream or downstream genes (Pennacchio et al. 2013). Additionally, enhancers can bypass the nearest promoters to regulate more distant genes, also a single enhancer may control multiple genes (Mohrs et al. 2001). Furthermore, enhancer activity can be restricted to specific maturity stages, physiological context, pathological situations, or even environmental contexts (Pennacchio et al. 2013).

Retroviral genomes can undergo recombination between their 5’ and 3’ LTRs, resulting in the formation of solo-LTRs. These elements can conserve their functional effects or can be mitigated by epigenetic mechanisms, including DNA methylation, histone lysine methylation, and small noncoding RNAs (Thompson, Macfarlan, and Lorincz 2016). LTRs may conserve their regulatory regions required for transcription, such as transcription factor binding sites, which possess the capacity to recruit cellular machinery and enhance proviral mRNA transcription (Thompson, Macfarlan, and Lorincz 2016). In mice, LTR insertion sites participate in

overexpression of APOBEC3, enhancing the anti-viral activity of this gene (Sanville et al. 2010). LTR expression can be also dependant on the host cell maturation stage, with some elements becoming inactivated during maturation. In some cases, host genomes have even adapted and repurposed ERVs for novel biological functions such as Syncytin 1 and Syncytin 2 derived from HERVs which play a role during placentation (Blond et al. 2000, Blaise et al. 2004); the Arc (activity-regulated cytoskeleton-associated) gene derived from Ty3/gypsy retrotransposon associated with cognitive functions (Kedrov, Durymanov, and Anokhin 2019); or LTRs from MER41 which can enhance immune system gene transcription (Nataf, Uriagereka, and Benitez-Burraco 2019).

Moreover, proviral insertions within coding regions can disrupt normal gene function by inactivating regulatory elements or causing transcript truncation. During integration, the viral promoter replaces the cellular promoter, using transcriptional cellular machinery to generate chimeric transcripts (Wotton et al. 2002, Uren et al. 2005), This process can lead to oncogenic modifications like upregulating tumour suppressors such as p53 and NF1 (Uren et al. 2005), or production of truncated proteins, with oncogenic properties, like *Notch*1 and *Tpl*-2 (Uren et al. 2005, Lund et al. 2002, Weng et al. 2004).

While certain LTRs can maintain their promoter or enhancer functions for host genes, none of the known enFeLV have demonstrated activity in gene functions (Ghosh, Roy-Burman, and Faller 2000, Chiu et al. 2024). This lack may relate to host genomic regulatory mechanisms, particularly the APOBEC antiviral system (Terry et al. 2017).

EnFeLV has between 8-12 copies per cell (Kumar, Berry, and Roy-Burman 1989, Polani et al. 2010a, Roca et al. 2005) and more than 80 copies per cell of solo-LTRs (Powers et al. 2018, Chiu and VandeWoude 2020). Multiple endogenization events of enFeLV have occurred (Kumar, Berry, and Roy-Burman 1989, Polani et al. 2010, Roca et al. 2005), and general enFeLV insertions points are randomly located in the cat genome (Chiu et al. 2024).

Additionally, some specific factors have been related to the number of enFeLV IS. Higher numbers of IS have been associated with inbred populations, whereas more mixed populations may have fewer insertions in their genome (Chiu et al. 2024). Male cats have as well shown

higher copy numbers compared with females. This has also been associated with resistance to exogenous FeLV when there are higher copies of enFeLV (Tandon et al. 2008, Powers et al. 2018).

Initial methods for identifying insertion points primarily involved Southern blot analysis combined with genomic library screening (Reik, Weiher, and Jaenisch 1985). Afterwards, PCR-based genome walking methods were developed for a diverse range of organisms to enrich for insertion site sequences (Bushman et al. 2005, Uren et al. 2005). These include inverse PCR, which employs primers to amplify target regions from a previously circularized, restriction-digested DNA template (Ochman, Gerber, and Hartl 1988).

Most PCR-based approaches, whether involving plasmid clones, gel extraction of fragments, capillary electrophoresis, or fluorescent *in situ* hybridization (FISH), are laborious and require multiple processing steps to achieve high-quality sequences; these steps can also increase bias in sequence representation (Uren et al. 2005, Fujino 2003). Inverse PCR in particular has limitations, among them, needing efficient DNA circularization, with shorter restriction fragments often failing to circularize, resulting in reduced amplification efficiency (Fujino, Ohno, and Tsujimoto 2008). FISH is another widely used technique that enables the visualization of specific DNA and RNA sequences within cellular or chromosomal preparations. The method employs fluorescently labelled nucleic acid probes that hybridize to complementary target sequences, allowing their detection and spatial localization under fluorescence microscopy. A disadvantage, as with many probe or PCR based techniques, is that it requires prior knowledge of the target sequences for design (Gozzetti and Le Beau 2000, Fujino, Ohno, and Tsujimoto 2008, Chiu et al. 2024, Fujino 2003).

Nowadays, advances in NGS and bioinformatics through whole chromosome resequencing have facilitated these types of analysis opening a new approach to determining the insertion sites of variable loci like ERVs (McEwen et al. 2021, Chiu et al. 2024).

Oxford Nanopore Technologies (ONT) have developed a sequencing method through proteins that analyse molecules and sequencing in real-time. The sequencer produces electrical current as the DNA molecules pass through the protein pore. The measure of this electrical current

determines signals that are recognized as specific nucleotide base in contact with the pore (https://nanoporetech.com/). It is able to sequence long reads with an average read length of 20kb, and it also can produce single reads over 1 Mb in length. The channels can be managed independently, and it is possible to reverse the voltage across the pore, rejecting individual DNA molecules that do not match a set selection of sequences to generate selective sequencing, reducing the sequencing capacity wasted on non-target sequence. This procedure was firstly called “Read Until” (Loose, Malla, and Stout 2016). Afterwards, this system has been called “Adaptive Sampling” (NAS) and a fast and rapid method for sequencing (Payne et al. 2021, Cheng, Fei, and Xiao 2023, Ulrich et al. 2024). The NAS uses a GPU and “ReadFish” (Payne et al. 2021) software to manage channels independently. The main advantage is that this allows a reduction in the effort and cost of sequencing and sample preparation. This facilitates only paying attention to long-read sequences in specific pathogen or host regions in the sample (Cheng, Fei, and Xiao 2023).

ReadFish is a configurable toolkit which connects the flow cell with bidirectional communication using the Read Until application programming interface. As reads are being sequenced, a brief chunk of signal is used for base-calling using Guppy or Dorado. This is mapped against an index (.mmi) using minimap2 (Li 2018). The velocity is 0.4-s read approximately 180 bases, and it can be modified by increasing the time to read longer chunks. Each read may map in one site, multiple sites or not map at all. The user can modify the read selection using “unblock” to reject a read, “proceed” to acquire more data for that read, or “stop receiving” to stop receiving data for the rest of that read. Thus, different settings can be used for different experimental purposes, such as host depletion, targeted sequencing, target coverage depth, and low-abundance enrichment (Payne et al. 2021).

NAS has been used for a variety of purposes such as: to detect Short Tandem Repeats (STR) causing neuromuscular diseases (Stevanovski et al. 2022), human vaginal microbiome (Marquet et al. 2022), antimicrobial resistance genes (Cheng et al. 2022), analysis of DNA accessibility and methylation of plant genomes, monitoring of wild species from environmental samples (Urban et al. 2023), Mycobacterium detection (Su et al. 2023), RNA enrichment in transcriptome

(Wang et al. 2024) and metagenomic surveillance of bacterial tick-borne pathogens (Kipp et al. 2023).

This study explores the use of NAS to sequence ERV from exFeLV infected Chilean cats to characterise viral insertion sites of both ERV and XRV as well as recombinant variants of these in these cats.

4.1.3 Methods

Nine domestic cat (*Felis catus)* (BC01, BC02, BC03, BC04, BC5, BC6, BC7, BC8, BC9) blood samples were collected in EDTA tubes by jugular venipuncture and kept at -20°C in Chile. All procedures and handling were done by veterinarians during clinical diagnosis of retroviral infections. Nucleic acid extractions were done using a commercial kit following the manufacturer instructions (DNeasy Blood & Tissue kit, Qiagen®) and this genomic DNA was used for all workflow analysis (**Figure 26**). Overarching ethical approval for this study was granted by the University of Nottingham School of Veterinary Medicine and Science Committee for Animal Research and Ethics (CARE) from the number 3672 220923.

Quality control before library preparation was carried out using a TapeStation 4200 (Agilent Technologies Inc, USA), using the Genomic DNA Screen Tape Assay to assess the DNA average fragment size at the DeepSeq, Centre for Genetics and Genomics (University of Nottingham). DNA quantification was done before and during library preparation, using the high-sensitivity dsDNA assay kit (Thermo Fisher) on a Qubit 4 Fluorometer (Thermo Fisher Scientific®).

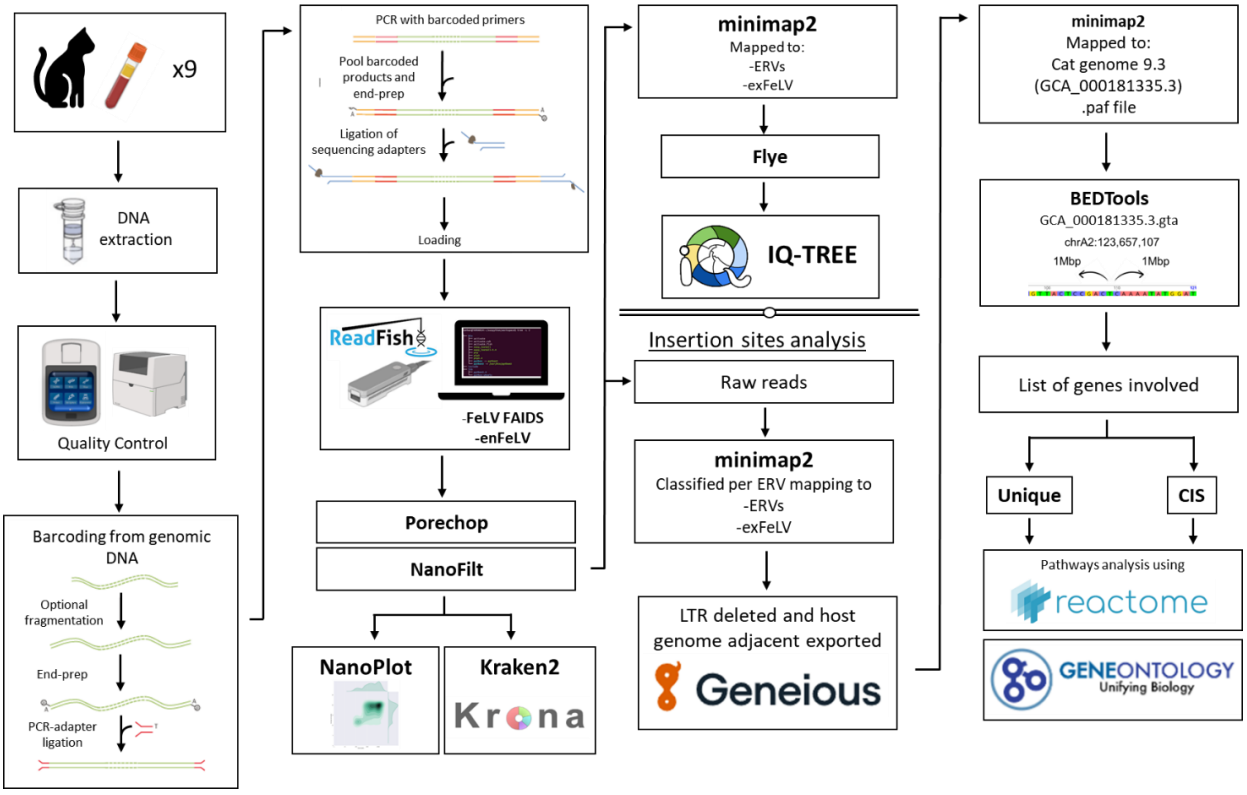

Figure 26 Workflow of methodologies for Nanopore Adaptive Sampling and bioinformatics analysis.

Genomic DNA was extracted from Chilean domestic cats for library preparation. Oxford Nanopore libraries were processed using the Ligation Sequencing Kit V14 (SQK-LSK114) protocol (Oxford Nanopore Technologies), including the PCR Barcoding Expansion Kit (EXP-PBC001) for samples with low DNA input. End-preparation was conducted, both before and after PCR using the NEBNext® Ultra II End Repair and dA-tailing Module (New England Biolabs®), following the manufacturer's recommendations.

PCR adapters ligation and amplification were done using LongAmp Taq 2x Master Mix (New England Biolabs®) and primers with unique barcodes provided by the ONT kit. Thermocycler conditions were as follows: initial denaturation at 95°C for 3 mins, followed by 18 cycles of denaturation at 95°C at 15 seconds; annealing at 62°C for 15 seconds; extension at 65°C for 5 mins and a final extension at 65°C at 15 mins.

Adapter ligation and clean-up were completed using the NEBNext® Quick Ligation Reaction Module (New England Biolabs®) and Long Fragment Buffer (ONT). For priming and loading step, 20 fmol of the DNA library was loaded onto a R10.4.1v flow cell and run on an Oxford Nanopore MinION Mk1B device.

Adaptive basecalling and data acquisition were operated in MinKNOW software (v23.11.5), using Bream 7.8.2, and Dorado 7.2.13. NAS was performed using the “selection” TOML file from the ReadFish software package (Payne et al. 2021). Sequencing was conducted for 24 hours targeting the *env* gene of FeLV-A FAIDS (M18247), and 48 hours using the whole genome of enFeLV (AY364318) and FeLV-A FAIDS (M18247) as target sequences. NAS processing demands high computational resources, conducting basecalling and data analysis (alignment and variant calling) at the same time. The system was carried out in a MacBook Pro M3 Max, which has 16-core CPU (12 performance cores and 4 efficiency cores), 40-core GPU, and 16-core Neural Engine.

Read processing, Assembly and Mapping

Nanopore adaptors were eliminated using Porechop v0.2.4 (Wick et al. 2017). NanoFilt v2.6.0 was used to filter for quality of <Q10 and NanoPlot to generate quality plots (De Coster et al. 2018). Nanopore reads were assembled using Flye (Lin et al. 2016). Longer raw reads were used for insertion sites analysis (>100 bp).

Kraken2 was used to assign taxonomic labels to the raw reads (Wood and Salzberg 2014). This was run on the European Galaxy server (Abueg et al. 2024) using the “core-nt (2024)” and “Viral Genomes (2019)” databases available on the Galaxy server. The report obtained from Kraken2 was visualized on Krona (Ondov, Bergman, and Phillippy 2011, Cuccuru et al. 2014) on the Galaxy server.

The contigs generated by Flye and raw reads from Nanopore were extracted and imported to Geneious v2023.0.4 (Biomatters Inc., Newark, NJ). Sequences were mapped to FeLV-A FAIDS (M18247), enFeLV (AY364318), RDRS (LC005745-LC005748), RD-114 (AB705392-AB70539293) and ERV-DC and FcERV-gamma4 (LC176791- LC176800) using Minimap2 (Li 2018) with the more sensitive preset options “Oxford nanopore reads” (map-ont).

Phylogenetic Analysis

The dataset for phylogenetic analysis was constructed in Geneious (Biomatters Inc., Newark, NJ) using reference sequences for each group and the Flye output of whole genome or *env* sequencing. Datasets were aligned with MAFFT (Katoh and Standley 2013). Both alignments were exported and used to construct phylogenetic trees.

The alignment was sent to the IQtree web server (Trifinopoulos et al. 2016), where a maximum likelihood tree was inferred using the ModelFinder (Kalyaanamoorthy et al. 2017) to select the best-fitted model nucleotide substitution. The model chosen was general time reversible (GTR+F+G4), branch support analysis was ultrafast with 1,000 bootstraps (Hoang et al. 2018). Murine Leukemia Virus (AY818896) was used as an outgroup to root the tree, and the phylogenetic tree was visualized in FigTree v1.4.4 (Rambaut 2018).

Insertion Site Analysis

To evaluate insertion sites (IS), all reads were mapped to enFeLV (AY364318), FcERV-gamma4 (LC176791- LC176800) and ERV-DC (AB674439- AB674451) using minimap2 in Geneious software. Reads were classified as solo-LTR or viral-LTR (attached to *env* or *gag* genes). In all cases, LTRs were manually deleted, and the adjacent host genomic sequences were extracted and exported in FASTA format.

Reads of host genomic sequences were mapped to the female Abyssinian *Felis catus* whole genome 9.0 (GCA_000181335.3) with the addition of the domestic cat Y-chromosome (KP081775), however only low-quality mapping and inconsistent match were identified. Minimap2 with the more sensitive preset options “map-ont” was used. The output was exported in PAF format to generate a table of IS, including mapping quality, strand, start alignment and end positions. The dataset was filtered based on >100bp in length and mapping quality >5 was retained.

The first half of the LTR was used to classify between enFeLV and exFeLV. IS were categorized as unique IS (detected in one cat only) or common insertion site (CIS), if it was shared between

two or more cats. It was only possible to compare enFeLV IS with previous reports (Chiu et al. 2024) as comparable data does not exist for the other ERV types.

Genes associated with IS were identified using the “intersect” function of BEDTools (Quinlan and Hall 2010), based on the annotation of the current version of the reference cat genome (GCA_000181335.3) using the “GTF” annotation file. Gene datasets were characterised by if they were inserted within a gene, located within 1Mbp downstream or upstream of a gene, and if they belonged to CIS or unique insertions. All bioinformatics pipelines are described at: https://github.com/cristobalsecast/Feline-Leukemia-Virus/tree/main.

The gene lists generated from the IS datasets were submitted to the Reactome Pathway Database for pathway enrichment analysis and visualization, using the Reactome analysis tool (Milacic et al. 2024).

4.1.4 Results

Nine samples were quality assessed using the Agilent TapeStation and Qubit™ dsDNA HS Assay Kit in Qubit 4 Fluorometer. The average DNA fragments length ranged from 17,812 to 57,691bp (**Table 10**), and these measurements were used to prepare the nanopore libraries.

Table 10 Sample Quality Control including average length and DNA quantity before library preparation.

	Sample	TapeStation	DNA Quantity
1)	BC01	17,812	80.2 ng/µl
2)	BC02	33,945	52 ng/µl
3)	BC03	49,588	1.16 ng/µl
4)	BC04	16,865	56.8 ng/µl
5)	BC05	30,686	10.8 ng/µl
6)	BC06	54,276	96.8 ng/µl
7)	BC07	55,366	118 ng/µl
8)	BC08	57,691	4.8 ng/µl
9)	BC09	34,585	118 ng/µl

Base-calling was carried out for 47 hours, resulting in a total output of 246.6 Gb and approximately 6 million reads (**Table 11**). Among the barcode samples, BC01 and BC06

generated the highest data yields, followed by BC02, BC04, and BC07. BC05, BC08 and BC09 produced lower read volumes. BC03 yielded an insufficient amount of data for analysis.

Table 11 Total reads base-called from the nine different barcodes of Chilean domestic cats

	Barcode	Total bases (Mb)	Passed bases (%)	Total reads (k)	Passed reads (%)
1)	BC01	3665.99	93.4	1039.958	93.3
2)	BC02	2481.896	93.8	569.646	93.7
3)	BC03	6.112	87.9	3.738	85.4
4)	BC04	2459.637	94.1	752.743	94
5)	BC05	1433.178	91.9	423.733	91.9
6)	BC06	3685.478	93.5	844.714	93.4
7)	BC07	2108.03	93.6	783.684	93.6
8)	BC08	1515.664	93.9	436.191	93.6
9)	BC09	1386.14	92.7	518.767	92.7

Data were visualized using NanoPlot prior to trimming (**Figure 27**). The reads generated for sample BC03 were highly fragmented and exhibited the lowest quality scores (Q<10), therefore this sample was excluded from further analysis. Overall, the read quality across samples ranged from Q8-14, with BC08 and BC09 showing the longest read lengths.

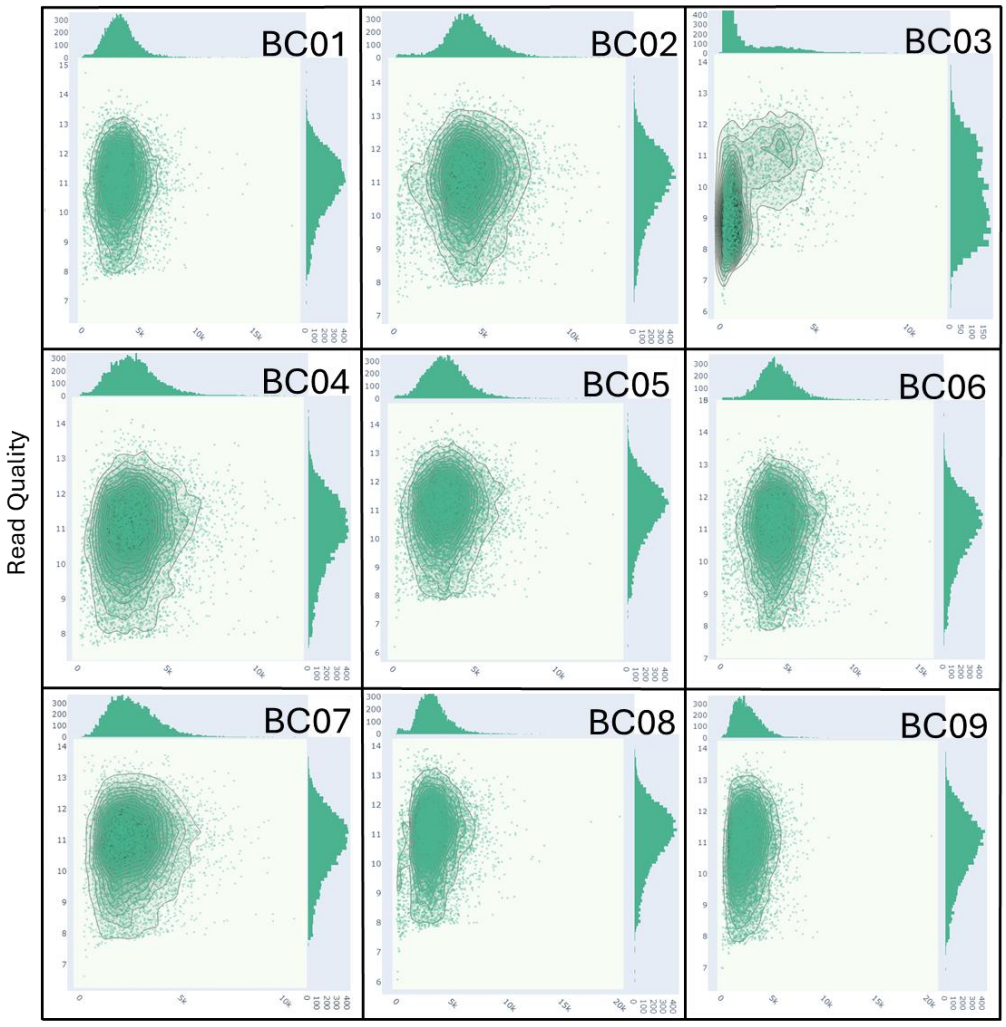

Figure 27 NanoPlot graph for the nine barcodes showing read length (X-axis) and read quality (Y-axis).

Krona visualizations generated from the Kraken2 report using the full database (core_nt) showed that majority of reads were assigned to the felid genome. Around 2% of the total reads were assigned to bacteria, while 0.02% were assigned to viral sequences. Of the viral reads, 9% were assigned to the Retroviridae family, specifically to FeLV.

The Kraken2 report using “Virus database”, identified 0.06% of reads assigned to Caudovirales, followed by 0.04% under Gamma retrovirus. Within the Gamma retrovirus group, 74% of these

reads corresponded to RD-114, 14% to FeLV, and 12% to other retroviral sequences (**Figure 28**).

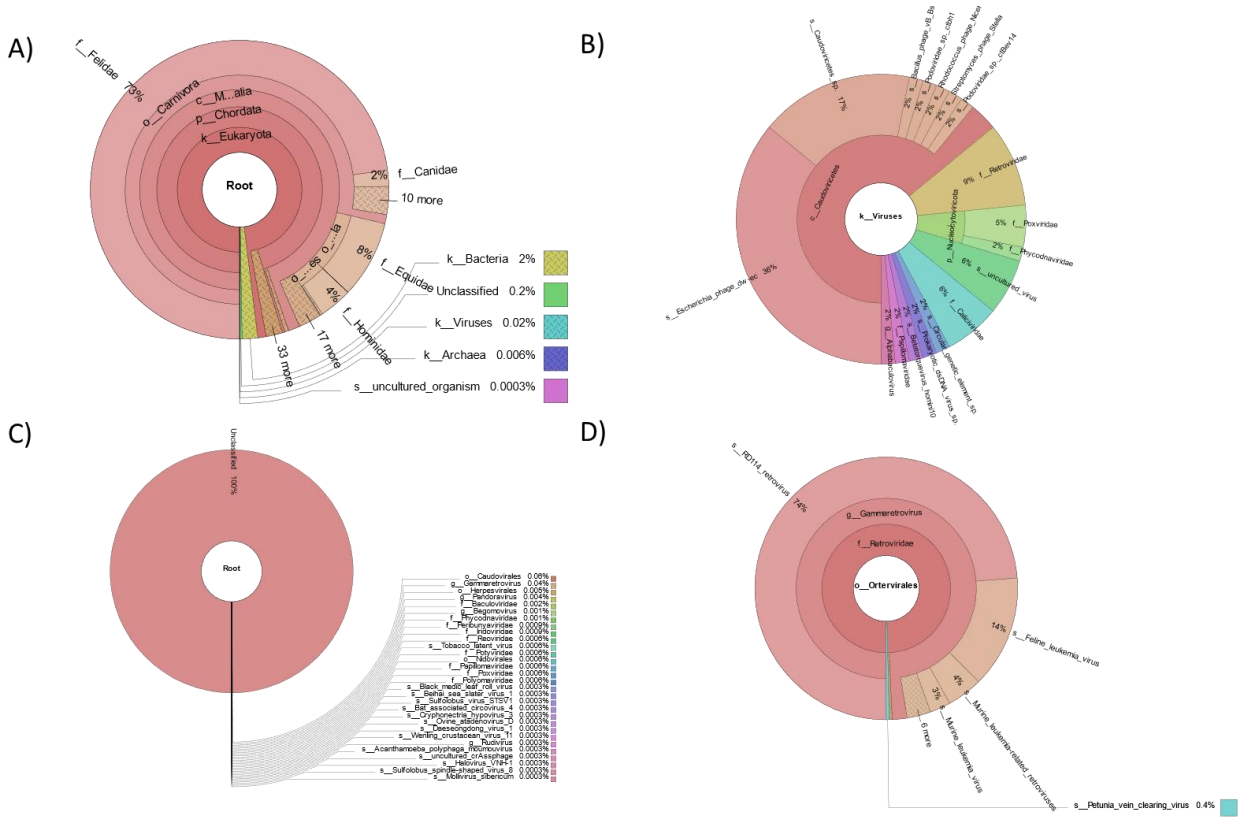

Figure 28 Krona pie chart from an individual cat as example. Panel A) shows Kraken2 classification using the complete database and B) shows a zoom into the viruses' classifications. Panel C) shows classification with the "Viruses" database and D) shows a zoom into the Retrovirus classification.

Raw reads were mapped against representative reference sequences of ERVs and exFeLV. These alignments were used to generate *de novo* assemblies to build a phylogenetic tree. Clades were identified around endogenous and exogenous retrovirus reference sequences

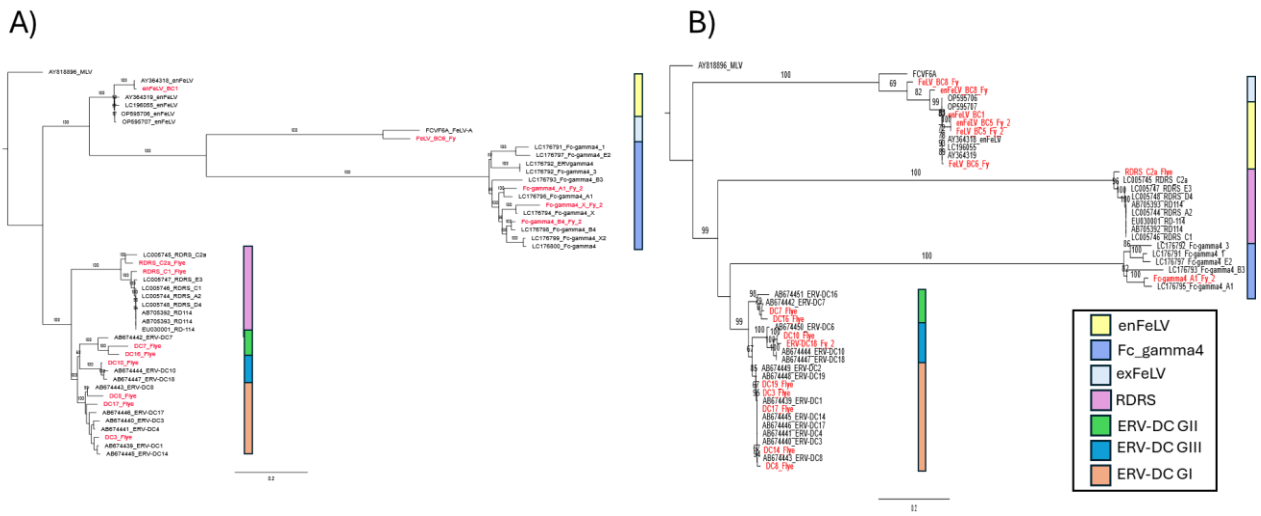

Figure 29 Phylogenetic tree of de novo assembled sequences and reference sequences of 4 main groups of felid ERVs. A) Phylogenetic tree of whole genome and B) *env* gene phylogenetic tree. Both were constructed with 1,000 bootstrap approximation and rooted in MLV. Nodes in red indicates samples obtained in this study.

Two phylogenetic trees (whole genome and *env* gene) were constructed using *De Novo* contigs and references sequence classified as ERVs or exFeLV. Four main clades were identified, enFeLV, Fc_gamm4, exFeLV, RDRS, and ERV-DC. The ERV-DC clades were also subclassified in three clades GI, GII, and GIII (**Figure 29**). exFeLV were identifiable by their LTR sequence and were excluded from insertion site analysis as these are expected to be individual to each cat.

Insertion Sites.

A total of 441 enFeLV-related IS were identified, including both solo-LTR and viral-LTR elements. Among these, 213 IS represented a unique integration site, as shown in **Table 12** and **Figure 28**. Of these, 124 (58.8%) were detected in an individual cat, and 89 (42.2%) were classified as CIS (i.e. shared by more than one cat or previously described in Chiu et al. (2024). Overall, 126 IS (59.7%) were identified as solo-LTRs, and 87 (41.2%) as viral-LTRs (**Figure 30**).

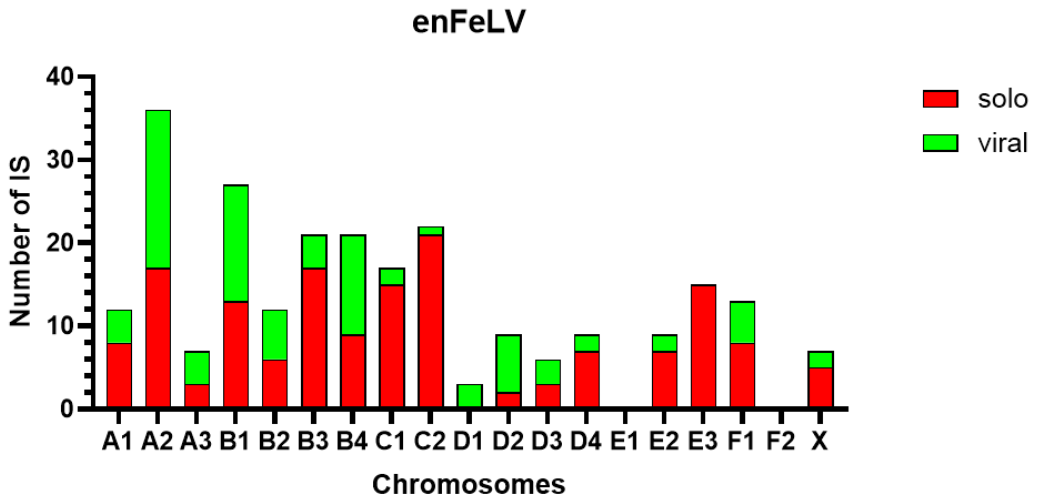

Figure 30 Insertion Sites of enFeLV identified as solo-LTR or viral-LTR (LTR attached to virus genomes) by chromosome

The distribution of IS was across all chromosomes (**Figure 29**). Among the cats, the number of IS was variable between the cats. Cat01, 02, 05, 06, 08 had between 30-38 IS, whereas Cat07 had 21 IS, and Cat04 had 14 IS and Cat09 had only 5 IS (**Figure 31**).

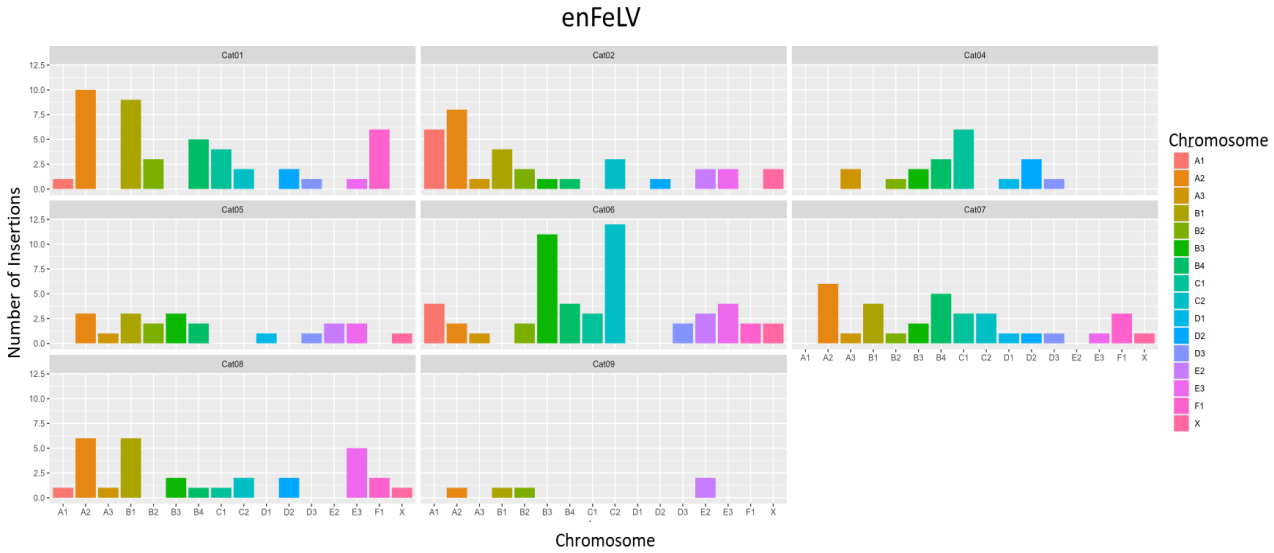

Figure 31 Bar graph showing number of insertion sites of enFeLV per chromosome and cat. Each graph represents one cat

Gene analysis showed that 15 IS were located within protein coding genes. These IS were identified either in a single individual or in multiple cats, including IS previously described (**Table 12**). The full gene analysis, including regions 1Mbp upstream and downstream of each IS, identified a total of 1,542 protein-coding genes. When comparing single cat IS against CIS. IS found in single cats were associated with 1,330 genes (10.7 genes per IS in average). In contrast, CIS were associated with 212 genes (2.3 genes per IS in average).

Reactome pathway analysis was performed separately for single cat IS and CIS. With regards to single cat IS, the 12 most significant pathways ($p<0.05$) were identified, however, none of them were associated with cancer nor immune system regulation. Similarly, the CIS analysis did not reveal any cancer or immune system regulations among its top 12 significant results. However, the pathway FBXW7 mutants and loss of function and NOTCH1 signalling approached significance for cancer related processes ($p=0.053$).

IS located within genes were individually assessed for associations with neoplasia or the immune system. Only one, KIAA1211 was found to be directly involved in cancer pathways. KIAA1211 is an alias for Capping Protein Inhibiting Regulator of Actin Dynamics (CRACD).

Table 12 enFeLV insertion sites detected within a gene

Insertion	Gene	Cat
1) ChrA2:127,342,100	DPY19L1	BC07
2) ChrA3:76,390,625	ZBTB46	BC08/B05/Chiu
3) ChrA3:72,162,706	NRXN1	BC06
4) ChrB1:31,380,841	ADRA1A	BC01
5) ChrB1:119,202,419	INTS12	BC08
6) ChrB1:162,545,622	KIAA1211	BC01,02,05,07,08
7) ChrB2:55,690,560	HMGCLL1	BC01,02,08
8) ChrB3:49,906,318	ZNF280D	BC01, 05, 08
9) ChrB4:64,792,601	TMTC1	BC01, 04,07, Chiu
10) ChrC2:131,951,593	TMEM108	BC06, Chiu
11) ChrD2:12,595,540	LGALS8	BC04, Chiu
12) ChrD2:60,627,129	CUTC	BC01,02, 04, 05,07, 08
13) ChrD3:26,724,052	GRK3	BC07, Chiu
14) ChrF1:11,603,826	FMN2	BC01, 02, 05, 07, Chiu
15) ChrF1:44,636,999	GOLT1A	BC01, 06, 07, 08, Chiu

4.1.4.1 Insertion sites ERV-DC

The ERV-DC reads that matched with cat genome positions were at 697 sites. Of these 389 unique positions were identified, with 162 (41.6%) positions identified only in individual cats, and 227 (58%) CIS (**Table 13**). A total of 201 (51.7%) IS were associated with viral-LTRs and 188 (48.3%) withs solo-LTRs.

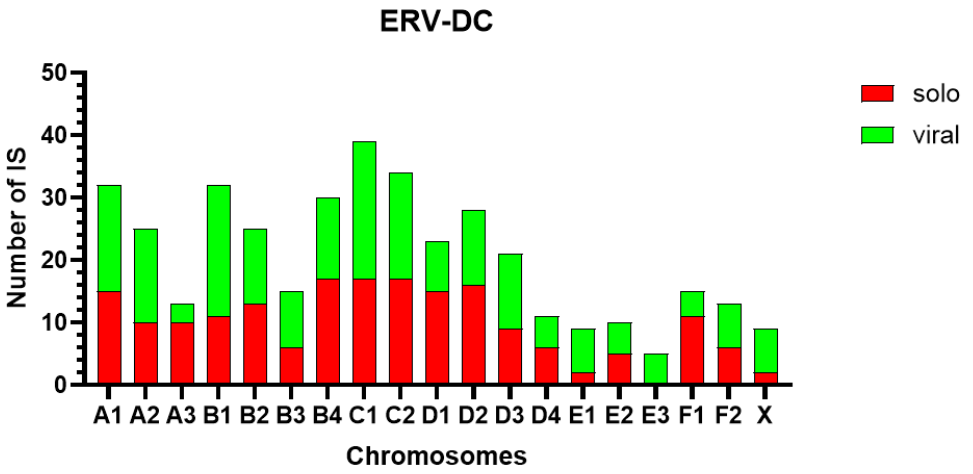

Figure 32 Insertion Sites of ERV-DC identified as solo-LTR or viral-LTR (LTR attached to virus genomes)

With regards to IS distribution across the cats and chromosomes, ERV-DC showed more insertions in Cat01, 02, 04, and 06 (**Figure 33**). Cat 04 and 06 showed the highest number of insertions of ERV-DC with 66 and 65, respectively. Followed by Cat02 and Cat01, with 58 and 52 IS. Cat07,08, and 09 had between 34 to 45 IS. Cat05 showed the lowest number of IS with 29 IS (**Figure 33**).

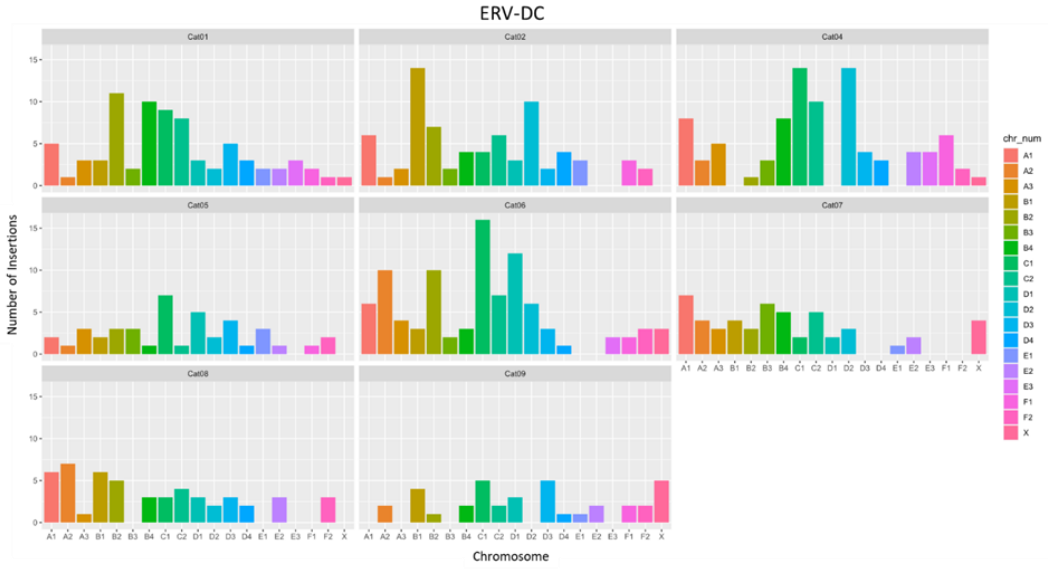

Figure 33 Graph bar showing number of insertion sites of ERV-DC per chromosome and cat.

Only viral-LTR were classified into GI, GII, and GIII. Most IS were associated with GI, followed by GIII and GII. There were only a minor number of reads labelled as GII (**Figure 34**).

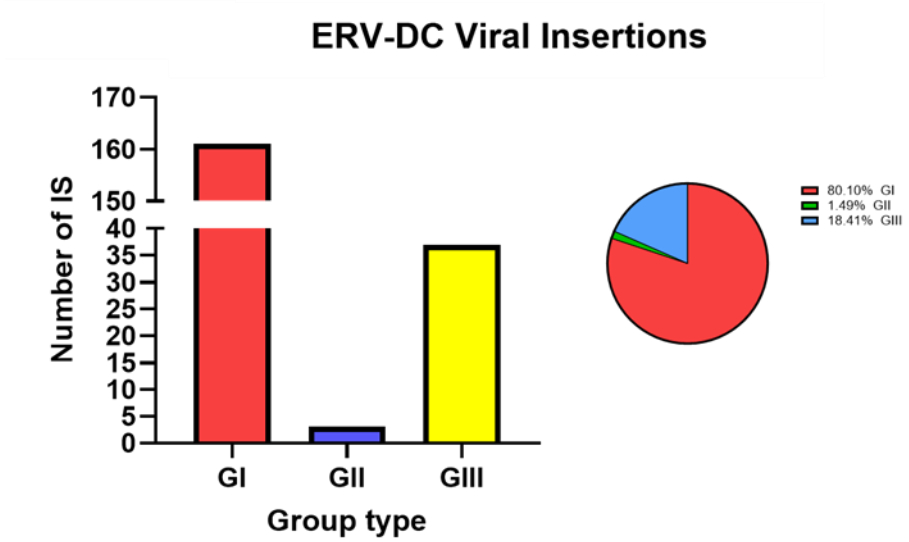

Figure 34 Number of insertion sites classified by ERV-DC group.

The gene analysis showed that 31 IS were located within genes, and the full analysis identified 2186 genes were in the possible area of influences of these IS.

4.1.4.2 ERV-DC Fcgamma4

A total of 937 FcERV-gamma4-related IS were identified, including both solo-LTR and viral-LTR elements. Among these, 302 IS represented a unique integration site, as shown in **Table 13**. Of these, 104 (34.4%) were detected in an individual cat, and 85 (65.6%) were classified as CIS. Of total sequences analysed, 267 were solo-LTR (88.4%), and only 32 (10.6%) were viral-LTRs (**Figure 35**).

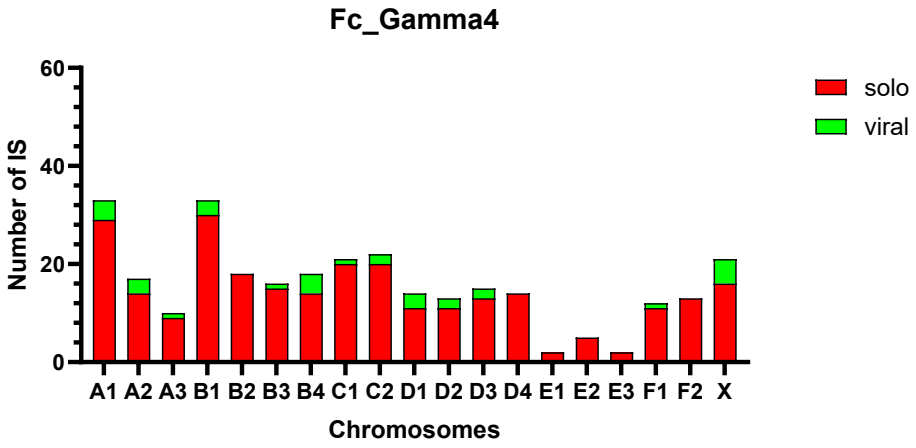

Figure 35 Graph bar showing number of insertion sites of Fc-ERVgamma4 identified as solo-LTR or viral-LTR.

Most of the insertions of FcERV-gamma4 were in Cat01 and Cat05 (75 and 68 IS).

Cat02,06,07,08, and 09, showed between 21 to 34 IS. Cat04 showed only 4 IS.

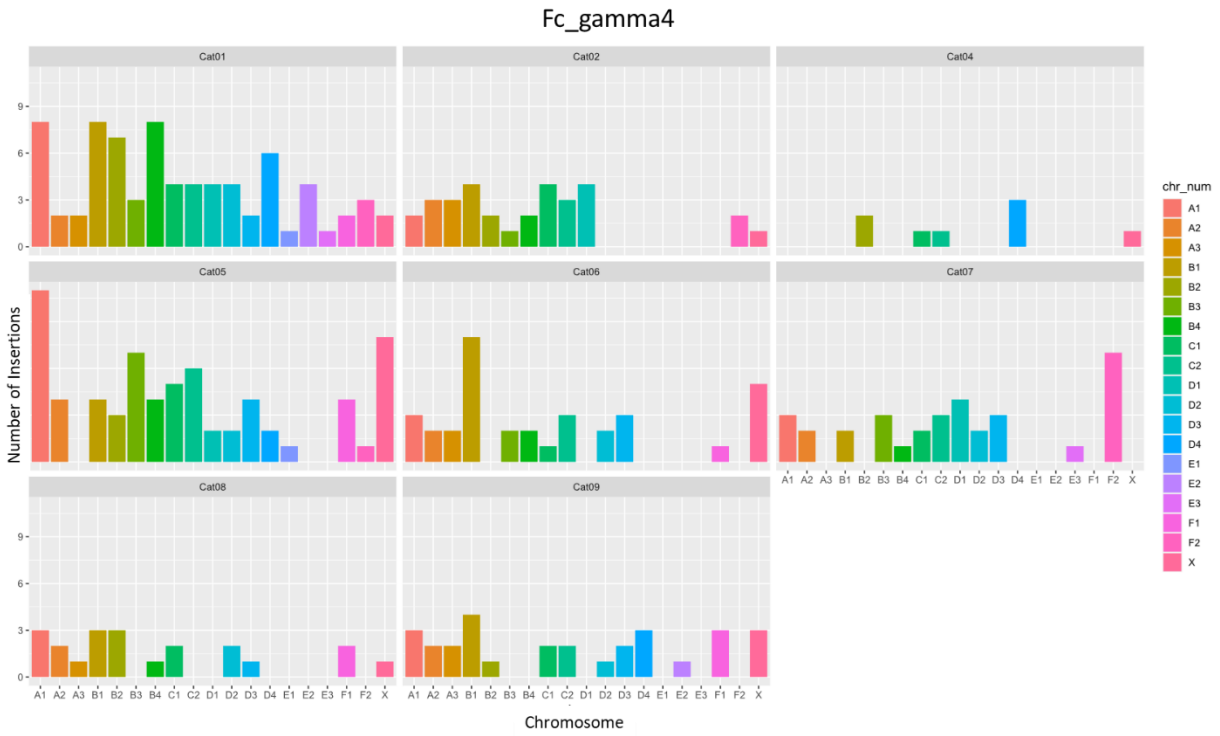

Figure 36 Graph bar showing number of insertion sites of Fc-ERVgamma4 per chromosome and cat.

Gene analysis showed that 29 (6.9%) IS were located within protein coding genes. These IS were identified either in a single individual or in multiple cats, including IS previously described (**Table 13**). The full gene analysis, including regions 1Mbp upstream and downstream of each IS, identified a total of 1,362 protein-coding genes (4.5 genes per IS in average).

4.1.4.3 RDRS

RDRS insertions sites were the least represented. Only 31 matches were identified. Among these, 24 IS represented a unique integration site, as shown. Of these, 19 (79.2%) were detected in an individual cat, and 5 (20.8%) were classified as CIS (i.e. shared by more than one cat), as shown in **Table 13**. All sequences were identified as viral-LTR. RDRS sequences were detected in only 6 cats. Cat01 and Cat02 showed a higher number of insertions, compared to Cat04 and Cat05 with one and two insertions respectively (**Figure 37**).

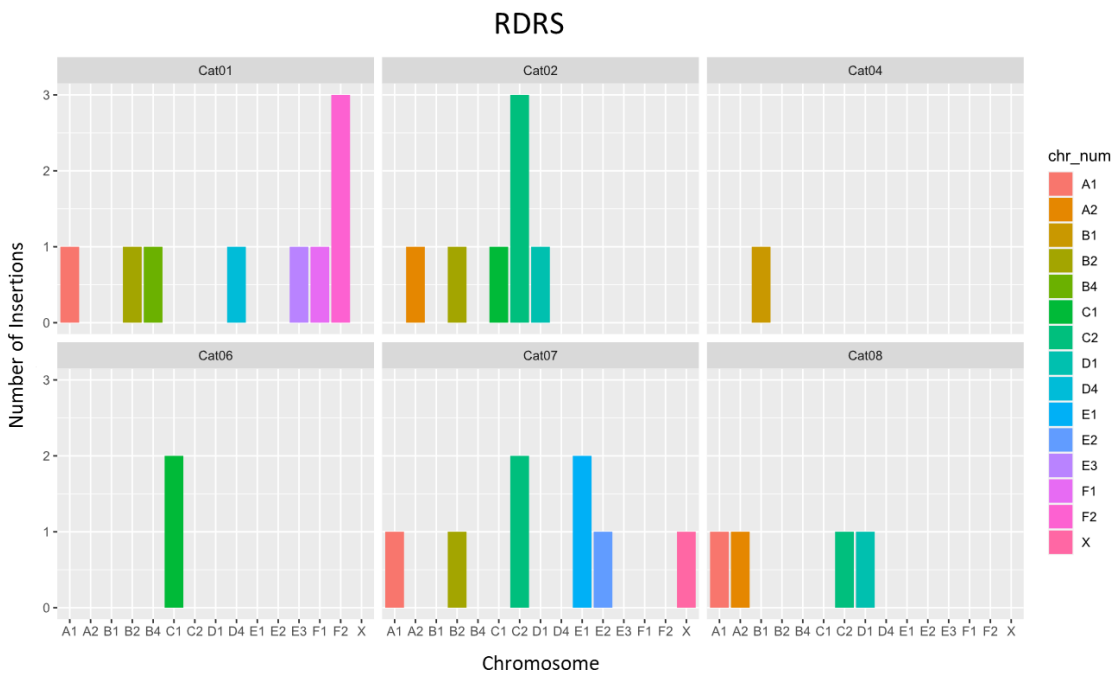

Figure 37 Graph bar showing number of insertion sites of RDRS per chromosome and cat.

Gene analysis showed that 5 IS were located within protein coding genes. The full gene analysis, including regions 1Mbp upstream and downstream of each IS, identified a total of 308 protein-coding genes (10.7 genes per IS in average) (**Table 13**).

Table 13 Summary of insertion sites across the enFeLV, ERV-DC, RDRS, and Fc-gamma4

ERV	Insertion Sites	Genes 1Mbp	Genes per site	Within gene
enFeLV	213 (89 CIS)	1542	7.2	15 (7%)
ERV-DC	389 (227 CIS)	2186	5.5	31 (7.9%)
RDRS	24 (5 CIS)	308	12.8	5 (20%)
Fc-gamma4	302 (198 CIS)	1362	4.5	29 (6.9%)

4.1.5 Discussion

NAS has reduced the time and cost for sequencing genomic material, enabling a focus on long-read sequencing of specific regions to address biological questions (Loose, Malla, and Stout 2016). This technique was successfully applied here in a single step to Chilean domestic cats infected by FeLV, identifying a wide range of feline gamma-retroviruses (endogenous and exogenous) insertions without any prior enrichment. While this single step method reduces the

error rates compared to amplicon-based method approaches (Grubaugh et al. 2019, Cheng, Fel, and Xiao 2023), it obtained lower coverage compared with amplicon-based methods (Castillo-Aliaga et al. 2023). In consequence, achieving better quality and efficiency is needed for NAS.

According to the Oxford Nanopore guidelines, optimal sequencing quality and efficient pore usage require that the flow-cell not be loaded more than its capacity (200 fmol). The volume loaded depends on the molecular weight and concentration of genetic material in the sample. For amplicon-based methods, fragment length is known and usually shorter than genomic DNA. However, it was estimated using an Agilent Tape Station fragment analyser. In the initial attempt, large sample volumes of sample were needed because they showed long fragments (the average was between 20,000 to 50,000bp). Shorter fragments reduce the volume required to load libraries onto the flow-cell, as molarity increases with decreasing fragment size. Additionally, higher amounts of DNA increase the number of strands rejected, which makes pore blockage more likely. Consequently, sequencing shorter fragments improves flow cell longevity and increases coverage of the target sequence. Another recommendation from ONT is to include multiple flow cell washes throughout the run and reload the library (https://nanoporetech.com/document/adaptive-sampling). Hence future attempts at this methodology should use sheared DNA of shorter average sequence length to increase sequencing efficiency.

Bioinformatic tools, software settings and other parameters are important considerations when running NAS. More studies have been conducted to describe the microbiome. While depletion mode can identify a greater number of pathogens, it produces less total as well as more host contamination (from 34.73% from depletion to 8.29% with the enrichment mode) (Marquet et al. 2022). Another report using NAS demonstrated that depletion was 2.7-fold compared with normal sequencing, with 30x of coverage achieved within four hours (Cheng et al. 2022).

The bioinformatic tool used to run NAS is also important. In a comparison between ReadFish and UNCALLED to evaluate *Mycobacterium tuberculosis* (TB) under simulated conditions, ReadFish using enrichment method increased the coverage and fold enrichment. ReadFish

reached 3.07-fold enrichment, with ~9.5x of coverage, while UNCALLED reached 1.98-fold enrichment with ~3.5x of coverage (Su et al. 2023).

Analysing retroviruses can be even more challenging than microbiome NAS with higher bacterial concentrations. Exogenous retroviruses are typically present in very low quantities, depending on the viral replication and infection conditions (Powers et al. 2018, Beall et al. 2019). In this case, cats were uniquely selected by a previous diagnostic LTR end-point PCR (Sacristán et al. 2021). These were however clinical samples collected from domestic cats several years ago, which may have led to some degradation in DNA quality over time. Therefore, although retroviral coverage was low, it was considered acceptable when compared with previously described methods for this type of study (Marquet et al. 2022, Su et al. 2023, Cheng et al. 2022). An example to be compared was the approach of whole genome sequencing using the Illumina method was tested in Maedi-visna identifying ~6 times less of retroviruses than our study here, with only 5% of reads associated to Maedi-visna (Jones et al. 2021). Furthermore, this analysis was conducted directly from lymph nodes with clinical lesions. Maedi-visna, similar to FeLV, typically shows higher viral concentrations in lymphoid tissue (Zhang et al. 2000, Helfer-Hungerbuehler et al. 2015). Another example involved a lynx (*Lynx lynx*) presenting intestinal lesions, where whole genome sequencing using Illumina was attempted. However, it was unsuccessful at recovering FeLV whole genome, only partial regions of the virus were amplified (Gregor et al. 2024).

Additionally, the target sequence is critical for NAS performance, as the system reads an initial portion of each fragment to decide whether to continue or reject sequencing. In this study, we initially targeted a specific region, the *env* gene of exFeLV, and later a broader perspective including full genomes of exFeLV and enFeLV. All gamma retroviruses detected in this analysis share some degree of sequence similarity. For example, the LTRs of FeLV-related and FcERVgamma4 show close phylogenetic relationships (Kawasaki et al. 2017), while the *env* gene is more closely related between ERV-DC and enFeLV (Anai et al. 2012, Pramono et al. 2024a). Hence while our primary target sequence was FeLV variants many related gamma retroviruses were recovered.

According to previous work, NAS can tolerate up to 30% sequence divergence (Horsfield et al. 2025), nonetheless its efficiency depends on several factors, including the length of the fragment used to make the decision. Longer time for initial sequencing allows for a more accurate sequence or reject decision, while shorter fragments speed up the process and reduce the damage on pores, but may decrease specificity (Payne et al. 2021).

Insertion sites

Except for enFeLV, endogenous retroviruses have been considered less important due to their older/ancient insertion into the cat genome, having undergone a longer period of domestication and inactivation, particularly in cases such as ERV-DC and FcERV-gamm4 (Song et al. 2013). However, recent studies have described recombination events involving these ERVs and suggested that their recombination frequency may be higher than previously described (Anai et al. 2012, Pramono et al. 2024, Ngo et al. 2024).

Our NAS results indicate important differences in ERV-DC and FcERV-gamma4 compared with the available reference sequences. Different insertion sites as well as high divergence with reference sequences were identified. However, direct comparisons are difficult due to the lower base-level accuracy of Nanopore sequencing relative to Sanger or Illumina platforms.

The functionality and biological effects of ERVs descriptions are often determined at SNP level. For example, SNPs have been used to distinguish replication-competent ERV-DCs, and to classify different ERV-DC types (Anai et al. 2012). Most available reports include a small number of animals and rely on PCR and Sanger sequencing methods methods (Chiu et al. 2024, Pramono et al. 2024a, Shimode, Nakagawa, and Miyazawa 2015, Anai et al. 2012), which may increase biases during ERVs characterization. The effects of ERV insertion on the host genome are poorly understood due to the limited data available. However, in mice a substantial proportion of underlying genetic variation between strains is due to variation in ERV loci and copy number with recently integrated ERVS such as those studied here forming an under-appreciated source of genetic variability for the host (Nellåker et al. 2012).To better understand the complex consequences of ERVs, knowledge of their genomic insertion and location is also important and this is easier to determine with long read sequencing methods as used here.

Ideally, to fully understand the potential impacts and functionality of these ERVs, it would be useful to integrate data both from short and long read sequencing methods at greater depth and to analyse a larger number of individuals per study (Uren et al. 2005).

A well-studied example of ERV endogenization is koala retrovirus (KoRV), which is currently undergoing integration into the koala genome and is estimated to be in the early stages of endogenization (~50,000 years) (McEwen et al. 2021). As a young ERV, KoRV is not fixed in the population, with approximately 80-100 proviral copies identified in individual germlines. CIS are largely associated with the geographical proximity between koala populations. These geographic patterns have also been associated with increased susceptibility to developing particular types of neoplasia, as certain CIS are associated with upregulation of proto-oncogenes predisposing animals to lymphoma or osteosarcoma (McEwen et al. 2021).

In comparison, the situation in cats is likely more complex, due to both a longer history of domestication and interactions with a wider range of closely related species. Since the emergence of the *Felis* lineage, multiple ERV endogenization events have occurred, potentially driven by shifts in prey species and interspecies interactions that facilitated new viral exchanges and subsequent proviral insertions (Benveniste and Todaro 1975, Song et al. 2013, Shimode, Nakagawa, and Miyazawa 2015, Ngo et al. 2019). Moreover, a crucial point was cat domestication, around 10,000 years ago in the Middle East. From there, cats spread eastward into Asia via the Silk-Road, and westward into Europe and the Americas through Viking expeditions and trade ships (Driscoll et al. 2009). These ancient migration routes have played an important role in shaping the current distribution and diversity of feline ERVs (Kawasaki and Nishigaki 2018).

Comparative studies of ERV-DCs in domestic cats from different regions of the world have revealed varying levels of endogenization. For example, the prevalence of ERV-DC8 in domestic cats is 77.5% in Japan and 65.7% in Spain (Ngo et al. 2019). In other countries, prevalence ranges from 52.6% to 85.8%, with the highest observed in Tanzania and the lowest in Vietnam and South Korea (Pramono et al. 2024). ERV-DC18 has solely been described in Japanese cats (Ngo et al. 2019). The introduction of domestic cats to Latin America largely occurred through

Portuguese and Spanish colonization, indeed there is a low relationship between British breeds and Latin-American cats. Specifically in Chile, historical records indicate that cats were primarily brought via routes connecting southern Spain with Peru, Bolivia and Chile (Ruiz-García and Alvarez 2008).

In our study, the distribution of ERV-DC groups differs from previous reports. GI was more prevalent, representing over 74% of the sequences identified. In contrast, GII and GIII were underrepresented compared to the previously reported frequencies of approximately 6% and 20%, respectively (Ito et al. 2013, Kuse et al. 2016). Although the number of IS is not always directly linked to proviral replication (Ito et al. 2013). GII is known as the domesticated group and has been associated with resistance to exogenous retrovirus infections. Meanwhile GI is thought to have a higher potential to recombine with exogenous FeLV (Kawasaki and Nishigaki 2018, Miyake et al. 2022).

The RDRS clade, considered one of the most recent ERV endogenization events in cats, has been mostly described in European breeds (British shorthair), contrasting to American breeds (American Shorthair and American Curl) which lack some RDRS variants (Shimode, Nakagawa, and Miyazawa 2015). In Chilean cats, only the original proviruses (RDRS_C2a and RDRS_C1) were identified.

Most existing knowledge on ERV diversity in cats originates from studies conducted in Asia, Europe, and the United States (Kumar et al., 1989; Polani et al., 2010; Roca et al., 2005). In addition, the Chilean cat population consists largely of mixed breed individuals (Castillo-Aliaga et al. 2024, Salgado-Caxito et al. 2021).

Two studies using NGS tools to analyse ERVs in domestic cats have been used, the first one determined a smaller expression of enFeLV in cats with lymphoma using RNA-sequencing (Krunic et al. 2015). The second one used PCR enrichment and Illumina short read sequencing and focused insertion sites of enFeLV (Chiu et al. 2024). Chiu et al. (2024) reported a higher number of insertion sites per cat (38.5) compared to our findings using NAS (26.62 per cat). However, Chiu et al. (2024) also noted discrepancies between their NGS and qPCR results and observed that certain individual cats exhibited an unusually high number of insertions. While

Chiu et al (2024) reported that 53.9% of insertions were CIS, our study identified 41% as CIS. Also, 39% of these CIS were shared between both studies. This difference may reflect the genetic and geographical diversity of the sampled cats. Our study involved unrelated mixed breed cats from various regions across Chile. Since CIS tend to be more prevalent among closely related individuals as is described for enFeLV and KoRV (McEwen et al. 2021, Chiu et al. 2024), therefore lower levels of CIS are expected. The sequencing method may also influence these results. Oxford Nanopore, while useful for long-read sequencing, has lower accuracy and coverage compared to Illumina platforms, which may impact CIS detection.

Chiu et al (2024) identified three CIS that were present in all cats in their cohort (chrA3:3,320,238, chrB2:55,690,560, chrB2:146,684,232). In our Chilean cat samples, only the first two were detected: The first one was found in Cat02, Cat05, and Cat08, while the second was in Cat01 and Cat02. Another notable common insertion site in our dataset was chrA2:7,199,456, which appeared in 6 out of our 8 cats.

Regarding the potential influence of ERVs on host gene expression, previous studies suggest that enFeLV does not influence host gene expression (Ghosh, Roy-Burman, and Faller 2000, Chiu et al. 2024). However, these conclusions are based on experimental models or small sample sizes. Although a comprehensive analysis of the numerous genes potentially affected by ERV insertions is difficult, Chiu et al (2024) primarily focused on immune-relate gene impacts.

In our study, we performed gene influence analysis using two datasets: single-cat insertion sites and CIS. This distinction is based on the idea that shared insertions may represent older loci and which in other species are more likely to be inserted in areas of inactive genomic regions (Medstrand, Van De Lagemaat, and Mager 2002). We found that the 124 single insertions were associated with 1,330 genes (10.7 genes per site), whereas the CIS were associated with only 212 genes (2.3 genes per site). These findings support the theory that older ERVs tend to be located in areas with less transcriptional activity. There were no notable cellular functional pathways identified in the Reactome pathway analysis, conducted using the CIS dataset. Though the cancer associated NOTCH1 signalling pathway, approached significance (p=0.053).

Some individual ERV insertions in specific genes have a potential role in oncogenesis, for example, ChrB1:119,202,419 (shared by 5 cats) was inserted into the KIAA1211 gene. In humans, this gene, also known as CRAD (capping protein inhibiting regulator of actin dynamics), has been implicated in tumour suppression pathways, including lung cancer and carcinoma (Kim et al. 2024, Cui et al. 2020). Another insertion of interest was located at chrD3: 26,724,052. Though found in only one cat in our study, it was also reported by Chiu et al (2024). This insertion was identified in the GRK3 gene, which has recently been identified as a with tumour proliferation promoter (Naderinezhad et al. 2023, Jiang et al. 2017). While this needs more directed study than this initial methods exploration it is possible that underlying genetic propensity to cancers in cats may be dependent on their ERV complement as has been recently demonstrated for koalas (Tarlinton et al. 2022). This may even underly the high rates of non-exFeLV lymphoma seen in domestic cats compared to other species and the association with specific breeds (Meichner et al. 2012, Bennett, Williamson, and Taylor 2024).

We compared the gene influence area per insertion site in relation to the estimated age of ERV endogenization. For example, FcERV-gamma4, the oldest insertion, was associated with an average of 4.5 genes per site. In contrast, enFeLV and ERV-DC, which have a similar estimated insertion time (2.5-2.8 Mya) were associated with 7.2 and 5.5 genes per site, respectively. The most recent ERV clade, RDRS showed the highest gene association, with 12.8 genes per site. This suggest that RDRS may have experienced less evolutionary pressure from the host genome and is consistent with results from other species where ERV location and association with genes is dependent on how recently endogenization occurred.

These variations may in turn be linked to interactions with exogenous retroviruses. Therefore, local ERVs may influence the pathogenesis of ongoing infections and may explain differences in disease progression. This is not something we have explored in this study but there are well described examples in other species, such as mice and chickens, where specific ERV loci either enhance or inhibit (usually via receptor blockade) exogenous retrovirus infection and replication (Ngo et al. 2024).

4.1.6 Conclusion

The NAS enrichment of ERVS here was successful in demonstrating detailed variation in the location of gammaretroviral ERVs between individual cats. Nevertheless, further work is required to be done to optimize the method in terms of fragment length and sequencing depth to ensure optimal recovery of ERV loci. Furthermore, interpreting the functional impact of viral insertions on gene expression is highly complex and loci variations should be considered for this type of analysis. Therefore, further studies with a higher number of individuals analysed is needed to understand the consequences of ERVs in domestic cats. Despite these limitations, this method make contributions to understand the consequences of ERV loci to the genetic predisposition to neoplasia in cats potentially more cost effective and feasible going forward.

Chapter 5: Cross-species transmission of Feline Leukaemia Virus between domestic felids and Guigna.

This section includes contributions from:
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Statement of contribution: Cristobal Castillo-Aliaga performed bioinformatic and molecular analysis, wrote and edited the text. Ezequiel Hidalgo-Hermoso contributed with animal sampling and edited the manuscript. Constanza Napolitano and Camila Stuardo contributed animal sampling and molecular analysis. Rachael Tarlinton conceptualised whole study and edited the manuscript.

5.1 Abstract

FeLV is distributed worldwide among domestic cats and non-domestic felids. Frequent spillover events from domestic cats to wild felids have resulted in fatal conditions in individual animals and outbreaks in free-range populations. The main consequences of FeLV infection have been hematopoietic disorders, enteritis and neoplasia cases. Domestic cats have both endogenous (copies of virus in the cat genome) and exogenous variants of FeLV, these frequently recombine producing variants with recombinant envelope genes and alternate receptor usage, while non-domestic species, except for wildcats (Felis silvestris) do not have the endogenous viruses.

This study applied amplification of the envelope gene of FeLV with Illumina sequencing of PCR products to determine the envelope gene diversity and transmission dynamics of FeLV variants in a non-domestic species, guigna (Leopardus Guigna), small felids native to Chile and Argentina. Although the *env* gene PCR produced amplicons for both FeLV-B and FeLV-A, sequencing of samples from captive animals revealed non-specific amplification of felid genomic DNA. In contrast, true FeLV-A sequences were successfully obtained from five wild animals.

The Illumina sequencing results confirmed the origin of infection was from Chilean domestic cats. However, a distinct guigna cluster was identified, characterized by unique variations that differentiate it from domestic cat sequences and may indicate that transmission is also occurring between guigna independent of domestic cats. Some of these variations have also been previously reported in both domestic and non-domestic species from other regions. Additionally, intra-host variation analysis revealed greater genetic diversity and a higher number of non-synonymous mutations across the env gene in guigna. Unlike the variation patterns typically observed in domestic cats, where mutations in the env gene are predominantly located in the SU region, particularly within the RBD. Here, a substantial proportion of mutation in guigna were found in the TM region. The pattern of mutations suggests an influence of the APOBEC antiviral immune system on guigna isolates.

Our findings provided evidence of intra-species transmission within guigna populations, demonstrated using Illumina sequencing, a method not commonly applied in this context. Thus, this study offers a novel perspective on the disease dynamics of transmission between domestic and non-domestic felids.

5.2 Introduction

FeLV is a globally distributed pathogen affecting domestic cats, and several species of non-domestic felids. In Africa, two reports of seropositive free-ranging cheetahs (*Acinonyx jubatus*) have been described (Thalwitzer et al. 2010, Krengel et al. 2015), along with a case of multicentric lymphoma in a captive cheetah (Marker et al. 2003). In Asia, seropositive FeLV cases have been reported in Pallas's cat (*Otocolobus manul*), Amur leopards (*Panthera pardus orientalis*), and leopard cats (*Prionailurus bengalensis)* in Russia (Naidenko, Pavlova, and Kirilyuk 2014, Naidenko et al. 2018); Sand cat (*Felis margarita*) in Saudi Arabia (Phane Ostrowski et al. 2003); and clouded leopard (*Neofelis nebulosa*), leopard (*Panthera pardus*), tigers (*Panthera tigris*) and fishing cats (*Prionaulurus viverrinus*) in captivity in Thailand. Additionally, FeLV has been reported in a Tsushima leopard cat (*Prionailurus bengalensis euptilura*) in Japan (Kusuda et al. 2022).

In South America, molecular detection of FeLV has been reported in guignas (*Leopardus guigna*) from Chile (Mora et al. 2015, Sacristán et al. 2021), ocelots (*Leopardus pardalis*) from Ecuador (Villalba-Briones et al. 2022), and multiple species from Brazil, including ocelots, oncillas (*Leopardus tigrinus*), jaguarundis (*Puma yagouaroundi*) (Filoni et al. 2017), and jaguars (*Panthera onca*) (Guimaraes et al. 2009, Silva et al. 2016). In North America, FeLV has been associated with clinical signs and pathological findings in a captive bobcat (*Lynx rufus*) in the USA (Sleeman et al. 2001), as well as outbreaks in pumas (*Puma concolor coryi*) and some cases in bobcats (Petch et al. 2022, Chiu et al. 2019, Brown et al. 2008, Cunningham et al. 2008).

In Europe, wildcats (*Felis silvestris*) and Iberian Lynx (*Lynx pardinus*) are the most frequently affected species. Multiple studies have reported FeLV in wildcat populations in Scotland (Daniels et al. 1999), Portugal (Duarte et al. 2012), France (Fromont et al. 2000), Luxembourg (Heddergott et al. 2018), and Germany (Leutenegger et al. 1999). Recently, the Eurasian lynx (*Lynx lynx*) was added to the list of FeLV infected wild felids. A recent case study reported necropsy findings consistent with FeLV infection and used NGS (Whole Genome Sequencing by Illumina) on gut tissue for molecular characterization. However, the whole viral genome was not recovered using Illumina, and PCR amplifications followed by Sanger sequencing was required to cover regions lacking sufficient coverage (Gregor et al. 2024).

The most emblematic examples of FeLV impact in non-domestic felids are the Florida Panther and Iberian Lynx. As previously mentioned in Chapter 1, one of the main consequences of FeLV introduction in these species has been the exacerbation of genetic bottlenecks in already vulnerable populations (Palomares et al. 2010, Meli et al. 2010, Chiu et al. 2019, Nájera et al. 2024). The most recent FeLV survey in North American included 641 pumas and 212 bobcats with 304 domestic cats. FeLV prevalence was 3.12% in pumas, 0.47% of bobcats, and 6.25% in domestic cats. A total of 20 transmission events was inferred, three cat to puma spillovers, three confirmed puma-to-puma transmissions, and 14 events likely to have originated from domestic cats (Petch et al. 2022).

The Iberian lynx has also been severely affected by FeLV (López et al. 2014). Risk factors in this population resemble those observed in domestic cats, with older male lynxes exhibiting higher prevalence, probably due to aggressive interactions (Nájera et al. 2024). Between 2003-2007, at the beginning of the outbreak, 21% of animals tested positive for FeLV, and 6 individuals died from FeLV-related disease (Meli et al. 2009). Similarly, three of ten Florida panther deaths were attributed to FeLV-related disease (Brown et al. 2008). As a consequence, intensive management strategies have been implemented in both species, including vaccination campaigns, isolation of progressively infected animals, and translocations to decrease the inbreeding rates and disease risk (Nájera et al. 2021, 2024, Palomares et al. 2010, Chiu et al. 2019, Cunningham et al. 2008).

After more than a decade of intervention, the epidemiological situation has shifted from acute mortality (Cunningham et al. 2008, Brown et al. 2008, Luaces et al. 2008, Meli et al. 2010) to more controlled scenarios that allow continuous surveillance. This has deepened the understanding of FeLV dynamics in endangered species. However, changes in population density and reduced habitat availability have led to greater overlapping areas and higher tolerance of intra-species interactions, facilitating puma-to-puma transmission (Cunningham et al. 2008, Petch et al. 2022, Chiu et al. 2019). Despite this, domestic cats remain the most likely source of initial infection (Meli et al. 2009, Chiu et al. 2019, Petch et al. 2022), although there is a risk that some viral variants may become more adapted to different species such as FeLV-B in pumas (Chiu et al. 2019). Additionally, reports of enteritis associated with FeLV have been described (Gregor et al. 2024, Filoni et al. 2017), expanding the traditional profile of FeLV as the cause of tumoral and immunosuppressive disease.

The guigna has been catalogued as “Vulnerable” for over a decade, with a continually decreasing population trend due to severe fragmentation of its habitat (Rodríguez and Calzada 2020). Like other felids, guignas are solitary animals, except during mating (Sanderson, Sunquist, and W. Iriarte 2002). In contrast to the managed FeLV outbreaks in the USA and Spain, guignas in Chile have shown even higher FeLV prevalence (>20%) than observed in Florida panthers or Iberian lynxes (Mora et al. 2015, Sacristán et al. 2021). This could reflect, 1) increased interactions with domestic cats due to habitat disruption, or 2) more frequent guigna-

to-guigna contact. Moreover, FeLV remains uncontrolled among domestic cats in Chile (Castillo-Aliaga et al. 2024), similarly to other Latin American countries (Ortega, C. et al. 2020, Acevedo et al. 2020, Santana, Pozo, and Castañeda 2022).

5.3 Methods

Captive felid samples were collected in a Zoological Park (Santiago, Chile) by veterinarians during preventive medicine management of the animals. Free-range guigna samples were collected for previous studies with permission from the Chilean Agriculture and Livestock Service (Capture permits 814/13 2008, 109/9 2009, 1220/22 2010,1708/26 2010, 7624/2015, 2288/2016, 2185/2017, 4072/2018) and approved by the Animal Ethics Committee (Institute of Ecology and Biodiversity, resolution 20 November 2015).

Sixteen blood samples were collected from a range of species of captive felids from a Zoological Park in Santiago city. These samples comprised: 4 samples from Pumas (*Puma concolor*), 2 from Jaguars (*Panthera onca*), 5 from Lions (*Panthera leo*), 1 from a Caracal (*Caracal caracal*), 1 from a Snow leopard (*Panthera uncia*), 1 from a Tiger (*Panthera tigris*), 1 Ocelot (*Leopardus pardalis*) and 1 Guigna (*Leopardus guigna*).

Table 14 Detail of samples used for further analysis

	Species	Origin	Number of Animal
1)	Puma	Captive	4
2)	Tiger	Captive	1
3)	Lion	Captive	5
4)	Caracal	Captive	1
5)	Jaguar	Captive	2
6)	Snow Leopard	Captive	1
7)	Ocelot	Captive	1
8)	Guigna	1 Captive / 10 Free-range	11

Ten blood samples were collected from free ranging guignas collected between the cities of Talca and Chiloe, Between 2010 to 2016. All these samples were previously diagnosed by nPCR

detection of the U3-LTR gene, and these results were previously published by Sacristán et al. (2021a).

The samples from captive animals were processed at the Laboratorio de Virologia Animal, de la Facultad de Ciencias Veterinarias, Universidad de Concepcion. The samples from free ranging guigna were processed at the Laboratorio de Virologia Animal, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile. In all cases, DNA extraction was performed using the commercial QIAGEN DNeasy Blood and Tissue kit following manufacturer's instructions (Qiagen, Valencia, California, USA).

End-point PCR using two primer pairs following methods from Erbeck *et al*, (2020) was used to amplify the envelope gene hypervariable region and 3'LTR region from FeLV-A and FeLV-B. PCR was performed using GoTaq® Long PCR Master mix (Promega) and each reaction consisted of: 12.5µl of GoTaq® Long PCR Master mix; 0.5µl (10µM) Forward primer; 0.5µl (10µM) Reverse primer; 9.5µl of Nuclease-Free water; 2µl of template DNA to reach a final volume of 25µl per reaction. Captive animals end-point PCR was conducted at the University of Nottingham, whereas that for free-range guignas was conducted at the Universidad de los Lagos, Osorno, Chile.

The same protocol was used for each pair of primers. Thermo-cycler conditions used followed the Erbeck *et al*., (2020) protocol with some modifications according to the polymerase requirements. The protocol was: initial denaturation at 94ºC for 2 min followed by 45 cycles of denaturation at 95ºC for 30 s; annealing at 63 ºC for 30 s; extension at 72ºC for 2 mins and a final extension at 72ºC at 2 mins. The thermo-cycler conditions were tested with a gradient PCR to select the optimal annealing temperature, and every set of PCR reactions included a negative control with no template (DNA free water) and a positive control (FeLV-A DNA). The PCR products were run by electrophoresis on a 1% agarose TAE (Fisher scientific®) gel at 80V, 400 mA for 60 minutes. DNA purification used the Nucleospin® extract II kit (Macherey-Nagel®), according to the manufacturer's instructions.

DNA quantification was performed with a Qubit 4 Fluorometer (Thermo Fisher Scientific®). FeLV-A and FeLV-B amplicons of high enough quality and quantity were selected for Illumina and Nanopore sequencing.

5.3.1 Nested PCR U3-LTR to *gag* fragment.

To confirm the presence of exogenous FeLV, a second round of amplifications was performed for a shorter amplicon. The method was a nested PCR in a highly conserved gene, U3-LTR to *gag* gene, with the aim of confirming the viral diagnosis. Here, specific primers for exFeLV were used. These changes were made to increase the sensitivity, targeting a smaller amplicon (707bp outer primers and 601bp inner primers), and to increase the specificity, targeting only exFeLV copies. Samples of Chilean domestic cats were used as controls to standardize the technique.

The primers spanned the U3-LTR (5'end) to the *gag* gene in exogenous FeLV. The primers were F: U3-LTR (5'-ACAGCAGAAGTTTCAAGGCC-3') and R: gag (5'-GACCAGTGATCAAGGGTGAC-3') as external primers, and U3-F(2) 5'-GCTCCCAGTTGACCAGAGT-3' and reverse G-R (2): 5'-GCTTCGGTACCAAACCGAAA-3', previously described (Miyazawa and Jarrett 1997). The PCR was performed using One*Taq*® Quick-Load® (New England Biolabs) and each reaction consisted of: 12.5µl of One*Taq* Quick-load Master mix with Standard Buffer (2x); 0.5µl (10µM) Forward primer; 0.5µl (10µM) Reverse primer; 9.5µl of Nuclease-Free water; 2µl of template DNA to reach a final volume of 25µl per reaction. Thermo-cycler conditions used a Gene-Touch Thermal-cycler, from Bioer Technology® following the (Miyazawa and Jarrett 1997) protocol with some modifications according to the polymerase requirements. The protocol was: initial denaturation at 94ºC for 30 s followed by 45 cycles of denaturation at 95ºC for 30 s; annealing at 60 ºC for 30 s; extension at 68ºC for 1 min and a final extension at 68ºC at 5 mins. The same positive control and water as negative control were used. The PCR products were run by electrophoresis on a 1% agarose TAE (Fisher scientific®) gel at 80V, 400 mA for 30 minutes. DNA purification used the Nucleospin® extract II kit (Macherey-Nagel®), according to the manufacturer's instructions.

5.3.2 Illumina Sequencing

Amplicons from *env* genes were amplified in 9 captive non-domestic felids (Table 14). These were sequenced by the Illumina platform (HiSeq4000) with 300-bp paired-end distances using a coverage of 30x by Novogene Europe, Cambridge, United Kingdom.

Table 15. Non-domestic felid DNA quantification measured in Qubit 4 Fluorometer. Quantification was done individually for FeLV-A and FeLV-B amplicons. These samples were sent for Illumina short read sequencing by Novogene Europe (Cambridge, The United Kingdom).

Species	Amplicon sequenced	DNA Concentration (ng/µl)	Total Amount (µg)
Tiger	FeLV-A	166.8	0.13440
Jaguar	FeLV-A	138.4	0.77500
Caracal	FeLV-A	138	1.07200
Ocelot	FeLV-A	99.2	0.17928
Snow Leopard	FeLV-A	192	0.78000
Guigna	FeLV-A	164	2.29600
Snow Leopard	FeLV-B	70.8	1.13280
Caracal	FeLV-B	99.6	1.49400
Jaguar	FeLV-B	98.8	1.77840

Illumina reads were trimmed, and adaptors removed using FastP v0.23.1 (Chen et al. 2018). De Novo Assembly from Illumina reads was performed using MEGAHIT (Li et al. 2015). The contigs generated by MEGAHIT were mapped to FeLV-A FAIDS (M18247) using Bowtie2 (Langmead and Salzberg 2012). All contigs and consensus sequence were extracted and imported to Geneious v2023.0.4 (Biomatters Inc., Newark, NJ) to annotate and continue phylogenetic analysis.

All contigs and consensus sequence were extracted and imported to Geneious v2023.0.4 (Biomatters Inc., Newark, NJ) to annotate and continue phylogenetic analysis.

The dataset was constructed in Geneious (Biomatters Inc., Newark, NJ) using all available complete *env* gene FeLV related sequences and were annotated and aligned with MAFFT (Katoh and Standley 2013). This was exported and used to construct phylogenetic trees and evaluate recombination between FeLV-A and enFeLV sequences.

The alignment was sent to the IQTREE web server (Trifinopoulos et al. 2016), where a maximum likelihood tree was inferred using the ModelFinder (Kalyaanamoorthy et al. 2017) to select the best-fit model nucleotide substitution. The model chosen was general time reversible (TIM2+F+G4), branch support analysis was ultrafast with 1,000 bootstrap (Hoang et al. 2018). EnFeLV was used as an outgroup to root the tree, and the phylogenetic tree was visualized in FigTree v1.4.4 (Rambaut 2018).

5.3.3 Intra-Host Variation Analysis

The intra-host single nucleotide variation (iSNV) analysis was done using BWA-MEM (Li 2013) and iVar (Grubaugh et al. 2019). All libraries were mapped to the FeLV-A reference sequence (M18247) using BWA-MEM and consensus sequences were generated using the iVar consensus command for each library. All reference sequences generated by iVar were cut at nucleotide 66 (M18247), to initiate at the same nucleotide position. The output was used to call single nucleotide variants and indels in iVar. The minimum quality parameter was <Q10 and the minimum frequency selected was 0.01 and only statistically significant ($p < 0.05$) results were considered as true.

5.4 Results

All amplicons obtained from captive non-domestic felids amplified random sections of the felid genome and tested negative in the LTR-gag PCR diagnostic PCR. As a result, these samples were excluded from further analyses.

The *env* gene was successfully sequenced from 5 free-range guignas, corresponding to 5 FeLV-A amplicons and 2 FeLV-B amplicons. All resulting contigs were approximately ~1.9kb in length. The FeLV-A PCR amplicons matched the expected amplicon size (~1.9kb) and did not show the insertions or deletions commonly found in other variants of FeLV.

Although FeLV-B specific primers yielded amplicons, sequences revealed only FeLV-A sequences with random nucleotides attached to the 5'-end. The FeLV-A contigs shared 97.2% to 100% nucleotide identity among themselves and 98.8-100% identity at amino acid level.

When compared to FeLV sequences from Chilean domestic cats (Chapter 3), the guigna sequences showed 97.2-99% nucleotide identity and 95.6%-99.7% amino acid identity.

BLASTn analysis revealed that the guigna sequences shared 98.86% identity with FeLV-FAIDS strain (GenBank accession M18247.1), and 98.44% identity with FeLV-A_Fca2018 from the US (ON995432.1). The FeLV-B amplicons demonstrated a similar identity percentage, although with a reduced query coverage due to random nucleotides attached to the 5' end.

Contigs assembled using MEGAHIT were aligned with FeLV-A sequences from domestic cats in Europe, the US, Japan, Brazil, Chilean sequences from previous chapters, as well as with sequences from non-domestic felids such as pumas and Iberian Lynx, where available. Guigna samples clustered together, without distinction between FeLV-A and FeLV-B amplicons (**Figure 38**). This clade grouped with the cluster of Chilean domestic cats and was positioned near a puma sequence from the US. All were located inside a bigger cluster that includes the FeLV-FAIDS (M18147.1), and Rickard (AF052723) reference strains.

Figure 38 Phylogenetic tree of the FeLV *env* gene (nucleotide sequence) was constructed using 1,000 bootstrap approximations and rooted against enFeLV (M25425). The analysis includes only FeLV-A sequences from domestic and non-domestic felids, including all previously reported Chilean sequences. Guigna sequences are shown in red and form a distinct cluster adjacent to the Chilean domestic cat clade.

The iVar analysis identified a total of 96 intra-host single nucleotide variants (iSNVs), corresponding to 45 unique nucleotide positions across the 7 amplicons obtained from 5 guigna individuals. Among them, Lpgui_B7 and Lpgui_B123 showed the highest number of iSNVs with substantial variation also observed in sample Lgui_A169. In contrast, Lpgui_A128 presented only two iSNVs, while Lpgui_152 showed 8 iSNVs but with comparatively higher variant frequencies compared with the other animals (**Figure 39**).

In terms of genomic distribution, the majority of iSNVs (50) were in the in SU region, followed by 34 positions within TM region. Only 7 iSNVs were identified in the LTR region. Within the SU domain, 31 iSNVs were identified in the RBD, 3 in the PRR, and 16 in the C-domain. As expected, the RBD was the most variable segment of the *env* gene.

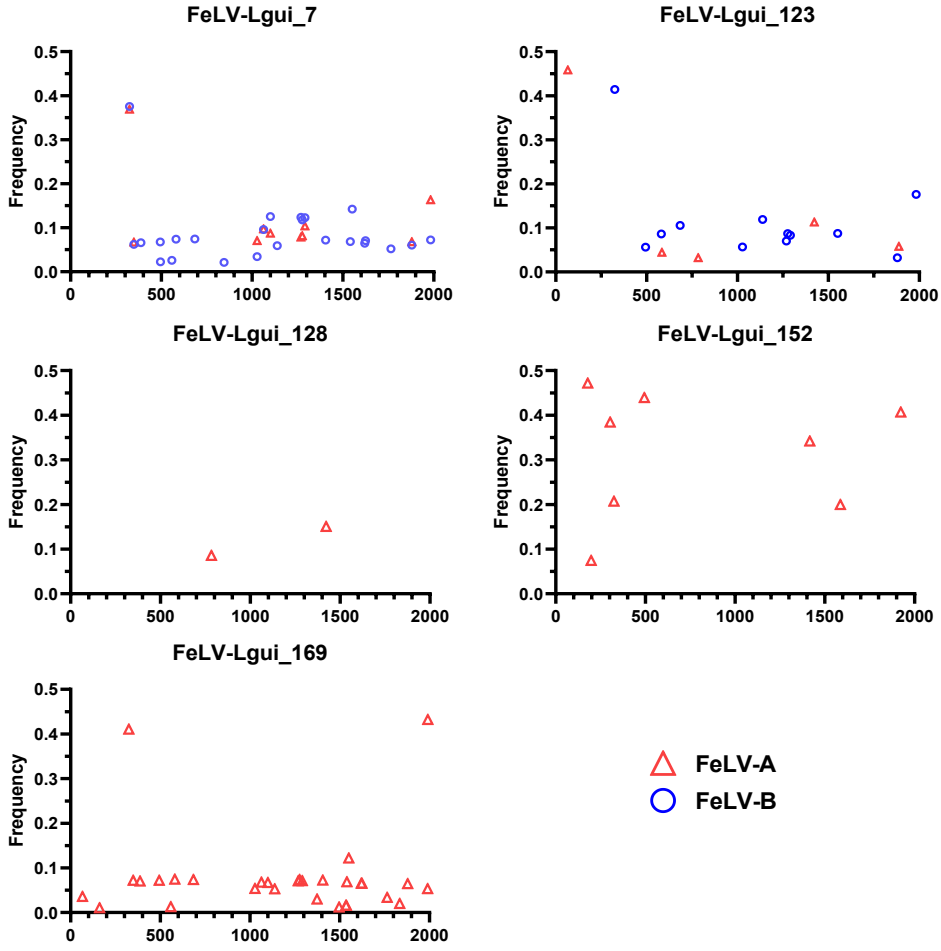

Figure 39 iSNV sites detected in *env* gene amplicons from Illumina sequencing. iVar software was used to generate a consensus sequences per library and to be used as reference sequence (~1.8kb). The sequence. Blue circles represent iSNV obtained using primers to amplify FeLV-B and red triangles represent iSNV obtained with FeLV-A primers. Each graph represents a different animal.

Analysis of the type of iSNVs showed that G→A transitions were the most common, with a total of 57 events (62.6%). Other types of substitutions included 16 A→G (17.6%), 3 A→T and 3 T→A (3.3%), 2 C→G (2.2%), C→A and C→T had 1 event each (1.1%), 6 events of T→C 6 (6.6%), and 2 T→G (2.2%).

Out of the total 96 iSNVs, 84 were located within coding regions, with 20 synonymous and 64 non-synonymous mutations. These non-synonymous variants were detected at different frequencies across samples, involving 26 distinct amino acid positions. Of these, 15 were G→A transitions, with 13 of them strongly suggestive of APOBEC-mediation, based on typical APOBEC motifs (AGG or GGG). Only one was less likely to be an APOBEC substitution (GAA to AAA).

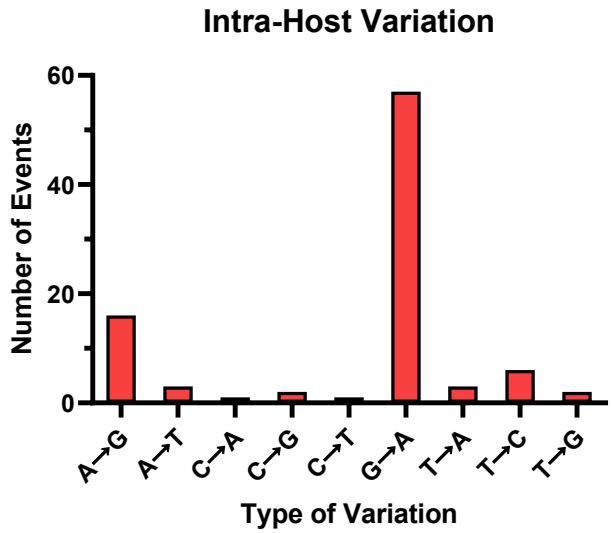

Figure 40 Bar graph of nucleotide variation in sequences obtained from Illumina sequencing for guigna samples.

Non-synonymous iSNVs were found in both the SU and TM regions, affecting 8 positions in SU and 7 in TM. The most frequently substituted amino acid replaced was glycine replaced in 8 positions by Glutamic acid, Arginine or Serine. One notable iSNV at position 1620nt introduced a premature stop codon, potentially truncating the envelope protein and impacting viral function.

Based on the de novo assembled contigs, guigna samples were more conserved at the amino acid level compared to Chilean domestic cat sequences. Four notable amino acid variations were identified differing between the Chilean cats and other reference sequences (Table 16). At position 59 of the *env* gene, within the VRA, guigna sequences showed a serine (Ser), whereas

Chilean cats presented either aspartic acid (Asp) or asparagine (Asn) at the same position. At position 60, three guigna contigs exhibited Ser, while 4 contigs showed proline (Pro), in contrast to cats which consistently had Pro at this position. Afterwards, at position 71 all Chilean cats and guignas, showed aspartic acid (Asp) compared with reference sequences. Position 165, however showed only variation in guignas, carrying either arginine (Arg) or lysine (Lys). Notably, position 249 consistently showed leucine (Leu) across all Chilean sequences, unique to the Chilean cluster and not identified in other sequences from GenBank.

Table 16 Amino acid variation identified across guigna and domestic cats.

Amino Acid position	59	60	71	165	249
DC_Chile	D	P	D	R/K	L
Lpgui_A7	S	P	D	R	L
Lpgui_B7	S	P	D	R	L
Lpgui_B123	S	P	D	R	L
Lpgui_123	S	S	D	K	L
Lpgui_169	S	S	D	K	L
Lpgui_152	S	S	D	K	L
Lpgui_128	**S**	S	D	K	L
FAIDS	S	P	S	K	P
Rickard	D	P	S	R	P
Glasgow	N	P	S	R	P
Iberian Lynx (EU293175-94)	D	P	S	R	P
Puma US	D/S	P	S	R/K	P

***DC_Chile:** Chilean domestic cats from previous chapters; **FAIDS**: FeLV-A strain (M18247); **US**: United States; **Rickard** strain (NC_001940.1); **Glasgow** FeLV-A (M12500)

Several positions were recurrent across individuals: positions 325 and 1269 were present in 5 samples, while positions 494, 1027, 1269, 1276, 1291, and 1879 were found in 4 samples. Notably, the nucleotide common variants identified in the de novo assembly were also detected in other samples at lower frequencies, confirming common variant sites within the population.

For example, the amino acid substitution at position 61 was observed in sample A152 at a frequency of 47%, and in other samples at lower frequences (<10%). Similarly, that at position 169 was found at 43% in A152, and at 7% in both B123 and A169. Overall, the frequency variant was between 47% to 1% of total reads.

5.4.1 Nested PCR U3-LTR to *gag* fragment

A confirmatory nested PCR for samples for which env gene sequence failed was used. Two domestic cats were used as controls to implement the nested PCR. The PCR products were ~601bp in length, consistent with the expected amplicon size, these were also Sanger sequenced to verify amplicon identity. No non-domestic felid samples were positive using this PCR approach.

BLASTn analysis of the Sanger sequences against the NCBI database showed a 98.45% identity with previously reported sequences from Colombia (GenBank accession: MT229941).

For the phylogenetic analysis of this gene region, all available sequences of the same size deposited in GenBank were considered for the analysis. This phylogenetic tree was rooted using an enFeLV (GenBank number: M25425). The resulting U3'LTR-gag phylogeny showed a major clade encompassing sequences from the US, Brazil, Colombia, and the UK. The Chilean sequences clustered together, forming a distinct sub-clade near to several Colombian sequences, indicating a close genetic relationship. (**Figure 41**).

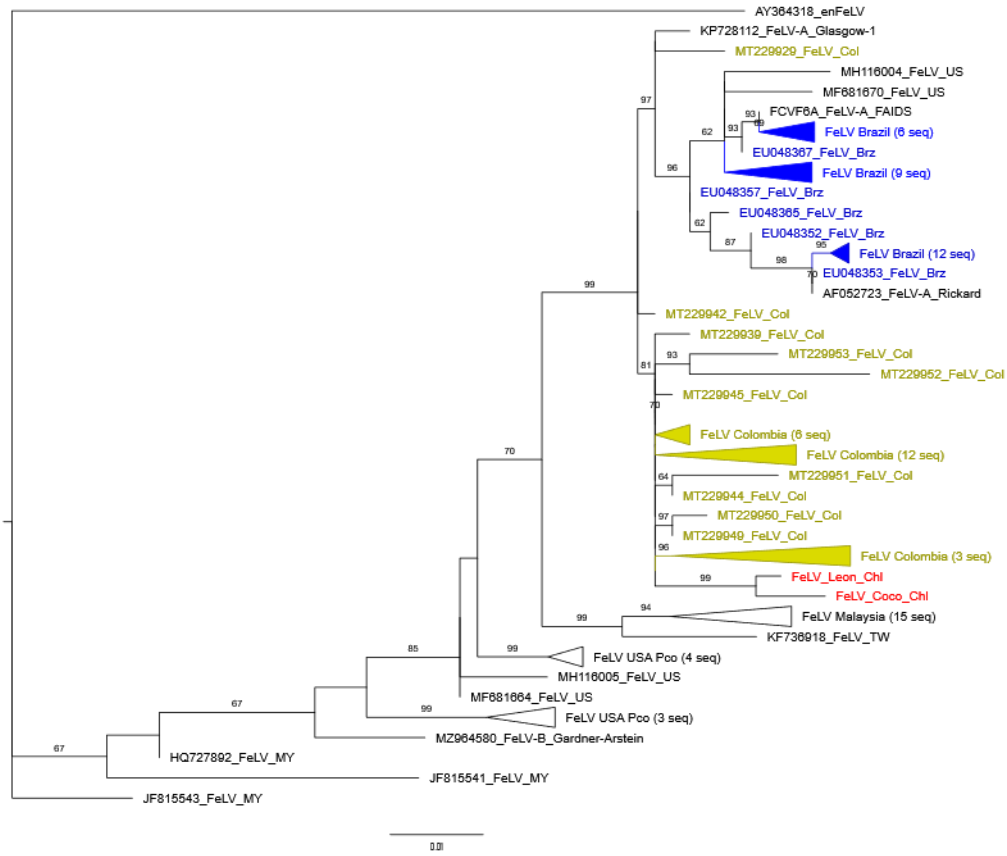

Figure 41 Maximum Likelihood phylogenetic tree of nucleotide 3'-LTR to *gag* constructed with 1,000 bootstrap approximation and rooted in enFeLV (M25425). Ninety-seven FeLV sequences from GenBank were considered. Large clades from the same location have been collapsed for clarity (horizontal triangles). The coloured sequences belong to South American countries: Blue for Brazil and yellow for Colombia. Red colour indicates Chilean sequences obtained in this study.

5.5 Discussion

The findings described in this chapter extends previous knowledge regarding the FeLV situation in guignas. Our results confirm that domestic cats are the primary source of FeLV infection for guignas with very close clustering of Chilean domestic cat and guigna sequences indicating region specific circulation. We also identified a distinct guigna-specific FeLV cluster, characterized by unique mutations not observed in domestic cats. It is possible that very rapid

adaption of virus to guigna (as occurs for avian flu isolates in mammals) is occurring but the complete separation of the guigna and cat sequences in our study suggests that, while the virus originally arose from Chilean domestic cats, it is now being transmitted guigna-to-guigna.

Previous studies assessing infection prevalence across large regions of Chile have relied on the 3'LTR diagnosis (Mora et al. 2015, Sacristán et al. 2021a). This method is more sensitive considering that it is smaller amplicon (211bp), and it is also highly conserved, decreasing false negative results (Miyazawa and Jarrett 1997). These characteristics make it suitable for prevalence calculations. However, analysis of the envelope gene, which is a hypervariable gene, provides additional information about the genetic dynamics of the virus (Watanabe et al. 2013, Petch et al. 2022, Chiu et al. 2019), including receptor usage for cell entry and potential clinical progression (Rohn, J L et al. 1994, Cano-Ortiz et al. 2022). Sacristan et al (2021a) previously reported difficulties amplifying longer fragments due to limited sample quality. A similar situation was described in a lynx in Germany, after an attempt to sequence the whole genome of FeLV using NGS yielded only partial sequences, requiring re-amplification with the Sanger method to complete the genome sequencing. This sequence showed notable differences regarding published reports, but there is no evidence of FeLV in lynxes from that area (Gregor et al. 2024).

In the US, FeLV in pumas has demonstrated multiple introduction events from domestic cat to pumas, followed by puma-to-puma transmission (Chiu et al. 2019, Petch et al. 2022). Two distinct FeLV-A clades were identified, resembling the situation in Japan where multiple FeLV-A clusters coexist (Petch et al. 2022, Watanabe et al. 2013). The epidemiological situation in pumas and Iberian lynx differs markedly from the guigna context. Both Iberian lynx and North American pumas are apex predators, frequently preying on domestic cats (Lee et al. 2017, Nájera et al. 2019, Brown et al. 2008). In contrast, the guigna is one of the smallest wild felids, similar in size to small domestic cats, with less frequent direct interactions. Contact between domestic cats and guigna happen less frequently with cats, mainly occurring at forest edges near to human settlements (López-Jara et al. 2021), where guigna may enter human areas hunting poultry (Schüttler et al. 2017, Zorondo-Rodríguez, Reyes-García, and Simonetti 2014), and potentially leading to aggressive encounters with cats (Mora et al. 2015). This behavioural context likely also explains the higher prevalence in male guigna and Iberian Lynx (Meli et al.

2010, Sacristán et al. 2021, Nájera et al. 2024). Following infection, males can transmit the virus to females during mating, as guigna, like most felids, are solitary outside of mating periods (Sanderson, Sunquist, and W. Iriarte 2002).

The phylogenetic tree presented in this chapter incorporated sequences previously described in prior chapters, including samples from animals from diverse geographic origins, two species, and using three different sequencing methods. A distinct Chilean cluster was clearly identified when compared to reference sequences from Europe and US (Cano-Ortiz et al., 2022; Gallina et al., 2024; Petch et al., 2022). Within this cluster, all Chilean sequences form a terminal cluster with a specific subcluster for guigna sequences. Interestingly, none of the Chilean sequences were related to the Brazilian strains, consistent with observations from the LTR-*gag* PCR analysis, demonstrating the circulation of distinct FeLV-A strains across the continent.

At a first overview, the absence of large deletions or insertions in *env* suggest these viruses may not be highly pathogenic variants such as FeLV-C, T, B strains (Rohn et al. 1994, Stewart et al. 2012). Among the Chilean sequences, two amino acid changes were uniquely identified in guignas at positions 60 and 61, within the VRA region, which is critical for receptor interaction (Rohn et al. 1994, Stewart et al. 2012, Cano-Ortiz et al. 2022). The presence of Ser at position 60 has been described previously in Iberian lynx (Geret et al. 2011), some pumas (Petch et al. 2022), and domestic cats with tumours (Rohn, J L et al. 1994). However, the Ser at position 61 has not been previously described. While presence of amino acid combinations of Asp52 and Asn60Asp mutations are known to enhance receptor binding, viral entry and viral replication (Stewart et al. 2012, Miyake et al. 2016), the significance of Ser at this position is unknown. These unique variations may represent specific viral adaptations to guigna receptors, and comparative studies on the sequence and structure of guigna FeLV receptors could elucidate the virus infectivity and replication efficiency in this wild felid. Another notable amino acid substitution, Pro249Leu, was observed exclusively in all Chilean FeLV strains, whether from wild or domestic felids and appears to be a feature of the currently circulating Chilean strains.

Evidence from Iberian lynx suggest that FeLV infection in this species is typically in a regressive state, with viral replication largely controlled except in a few progressively infected individuals

(Nájera et al. 2024). This may partly explain the challenges in amplifying longer viral fragments observed here and in previous studies (Sacristán et al. 2021b, Gregor et al. 2024). Although we cannot definitively conclude that guigna experience similar regressive infections, the low diversity of FeLV strains in our samples and the low variation within each host suggest limited active replication and few new introductions of virus into the guigna population.

A notable finding was the unusually high proportion of iSNVs in guignas that occurred in the TM region of the *env* gene, resulting in amino acid variations and even premature stop codons. In domestic cats, in contrast, variation is typically concentrated in the SU region, especially the RBD area, while the TM domain remains highly conserved even between FeLV-A, FeLV-B and other recombinants (Cano-Ortiz et al. 2022a).

A plausible explanation for the unexpected higher ratio of TM iSNVs is pressure from the guigna APOBEC antiviral system. APOBEC3 enzymes preferentially deaminate cytosines in AGG and GGG motifs (Stavrou and Ross 2015), generating higher numbers of G→A hypermutations that produce non-infective virions (Terry et al. 2017). Although APOBEC has a stronger impact on FIV and FFV than on FeLV in domestic cats (Münk et al. 2008), its activity may still drive the unique variation patterns we observe in guignas. A previous study using the Roche 454 sequencing platform to sequence the SU gene in Iberian lynx infections, reported some effects of the APOBEC system. They identified 25 sites of variations in SU, nearly half of which (48%) led to nonsynonymous changes, being predominantly Trp→Stop-Codon and Gly→Arg. (Geret et al. 2011). By contrast, our analysis additionally included TM protein and also indicated Gly as a frequently replaced amino acid. We identified a higher level of nonsynonymous variations (62.6%) and a sole premature stop codon was identified within the TM protein. The Iberian lynx study sampled fewer animals and detected fewer iSNVs per animal, while in our dataset one guigna harboured only two iSNVs and another showed over 50 different positions. Therefore, individual and species effects may also influence the efficacy of the APOBEC3 system in FeLV infections.

Comparative phylogenetic analyses of felid APOBEC systems indicates that the domestic cat and puma systems are closely related, whereas the lynx APOBEC diverged slightly earlier

(Münk et al. 2008). Specifically, puma and domestic cat lineages diverged ~6.7Mya, followed by lynx lineage divergence ~7.2Mya (Werdelin et al. 2010). The *Leopardus* lineage (including guigna) diverged over 8 Mya (Werdelin et al. 2010, Lescroart et al. 2023). This evolutionary distance could indicate that guigna APOBEC3 may interact differently with FeLV genomes compared to domestic cat APOBEC3. Although it cannot be directly compared, FIV from pumas was less effective at infecting domestic cats, because the virus was less adapted to a different species and in consequence was more susceptible to domestic cat APOBEC (Münk et al. 2008). These findings support the idea that FeLV from domestic cats may likewise be poorly adapted to guigna APOBEC3, resulting in increased viral hypermutation in guignas.

When our iSNVs were mapped against published FeLV sequences, most appeared to be random, but several matched mutations documented in other hosts. For instance, the G109R substitution was originally described in domestic cats with tumours (Rohn, J L et al. 1994); the V262I variation had been described in cats suffering tumours and in Brazilian cats (Rohn, J L et al. 1994, Cano-Ortiz et al. 2022). Lys343Glu has been described across a wide range of hosts, including cats from US, an Iberian Lynx, European cats, the Rickard reference strain, Pumas, Brazilian cats (Chandhasin, Coan, and Levy 2005, Geret et al. 2011, Chen et al. 1998, Petch et al. 2022, Cano-Ortiz et al. 2022); Ala469Thr and Ala513Thr substitutions have been previously observed in US pumas (Chiu et al. 2019); and the Glu542 Lys mutation has reported in a US domestic cat (Erbeck et al. 2021).

Only two other studies have used NGS to characterize FeLV in non-domestic felids, a case report and using one of the first NGS methods, which limits direct comparisons with our data set (Geret et al. 2011, Gregor et al. 2024). Nevertheless, we have successfully described *env* variation in guignas using Illumina sequencing. A key advantage was the absence of enFeLV in guigna genomes, which reduced the interference of incorrect amplification and bioinformatic artefacts that were observed in our domestic cat analysis reported in Chapter 3. The Illumina method proved highly effective for variant detection and produced results broadly consistent with those from domestic cats using the same method.

Rapid human migration from urban areas into Chilean native forest is driving significant landscape changes (Gálvez et al. 2018). As contact between domestic cats and other non-domestic felids increases, pathogen spillover risk is continuously increasing. It is therefore critical to maintain ongoing surveillance of FeLV both in domestic cats and in the guigna population. This study provides evidence that there is likely guigna to guigna transmission occurring and highlights the need for monitoring for clinical impacts in this vulnerable species. The effectiveness of the commercial domestic cat FeLV vaccines in controlling diseases has been widely demonstrated in the Iberian Lynx and North American Puma species recovery programmes and there is clearly an urgent need to establish such a programme with guigna.

Chapter 6: General Discussion

This study presents a comprehensive overview of the FeLV situation in Chile, employing modern molecular approaches and comparing these with more traditional diagnostic methods. This dual strategy offers valuable insights into both the molecular and epidemiological context of FeLV in the country.

FeLV remains one of the most significant infectious threats to felid species worldwide. It is responsible for a high burden of clinical disease and can lead to severe clinical outcomes, including fatal outbreaks in non-domestic felids. In several countries, FeLV prevalence has been effectively reduced through vaccination programs (Studer et al. 2019). However, in certain circumstances, the prevalence may still rise. In regions where a low prevalence has been historically described, vaccination has been underused or discontinued, leading to vaccine shortages which can disrupt or increase the problem in areas when the infection is highly endemic (Luckman and Gates 2017). This study has described a wide perspective of the FeLV situation in Chile, using modern methods and comparing them with traditional methods used, providing an extra point of view about the FeLV molecular and epidemiological situation in Chile.

The aim of this thesis was to provide a comprehensive overview of the FeLV situation in Chile and assess the risk of transmission from domestic cats to non-domestic felids. Traditionally, FeLV detection has focused on highly conserved viral regions (such as *pol* or LTR), which offer reliable diagnostic targets but limited insight into the molecular diversity and epidemiology of the virus. Otherwise, analysis of longer genomic fragments and hypervariable regions can yield more detailed information, although these are more difficult to amplify. Successful amplification requires high viral loads and high-quality samples, which often limits the success rate of sequencing efforts in these areas.

Additional challenges arise when diagnostic strategies target the envelope gene, which is prone to recombination between ERVs and exogenous FeLV elements. Recombination events can interfere with PCR amplification and sequence interpretation. Moreover, the high sequence

identity between endogenous and exogenous forms often results in non-specific amplification, making it difficult to distinguish between them in molecular analyses.

Traditionally, analysis of the *env* gene has relied on PCR amplifications followed by cloning of amplicons into plasmids and sequencing via the Sanger method. While effective, this approach is labour intensive, time consuming and costly when compared with LTR or *gag* region targets. In contrast, NGS methods began emerging approximately 15 years ago and have since evolved rapidly. These advancements have led to increased accuracy, higher throughput, and the development of tailored sequencing platforms for specific purposes. Although initially, these were prohibitively expensive, NGS technologies have reduced in cost, becoming more affordable and accessible at a wide level with platforms like Oxford Nanopore enabling field based genomic analysis.

The declining costs and reduced laboratory infrastructure requirements have made NGS increasingly feasible for applications in veterinary medicine and wildlife conservation. In parallel, the recognition of companion animals as integral family members and the growing adoption of the One Health concept have significantly influenced public health priorities over the past two decades.

As sequencing technologies have advanced, the resulting increase in data output has required the development of more sophisticated software analysis and computational tools. Previously, informatic sciences was a specialized field limited to researchers with computational backgrounds and access to costly proprietary software. Nowadays the accessibility of open-source platforms and improved user-friendly tools have expanded bioinformatics capacity globally. These advances are helping to bridge knowledge gaps in pathogen dynamics, allowing researchers to better understand how infectious agents behave under diverse ecological and epidemiological conditions.

To understand the FeLV dynamics in wild species it is essential to assess the FeLV situation in domestic cats as the primary source of infection. Consequently, each chapter of this thesis contributes a different perspective, spanning molecular characterization, epidemiology and host-

pathogen interaction to build a comprehensive understanding of FeLV impact in Chilean non-domestic species.

6.1 Modelling Survival Analysis and Prevalence with Risk Factors of FeLV in Domestic cats.

Chapter 2 included two modelling analyses, a lifetime analysis comparing FeLV, FIV and uninfected cats and a risk factor analysis for FeLV. The Kaplan Meier Lifetime analysis was conducted using a 20-year record of domestic cats from a shelter of domestic cats in the UK. This analysis highlighted the severe impact of FeLV on feline longevity. FeLV infected cats had a markedly reduced life expectancy, averaging less than four years. In contrast, FIV infected cats exhibited lifetime patterns comparable to the general domestic cat population in the UK, with significant differences observed only at advanced ages (over 14 years). Additionally, FeLV infection was strongly correlated with the development of lymphomas, further shortening life expectancy by approximately one year (reducing it to an average of ~3 years), whereas FIV cats showed no greater predisposition to lymphomas than uninfected cats, suggesting a more stable clinical progression.

FeLV prevalence is shaped by multiple factors, each reflecting a distinct epidemiological context. In Chile, two reports investigated FeLV prevalence in rural domestic cats to assess the risk of transmission to guigna's (Mora et al. 2015, Sacristán et al. 2021). However, these rural-focused studies may not accurately represent the broader domestic cat population in Chile, particularly in urban areas. Another study assessed FeLV prevalence from Santiago (Choi et al. 2024), which is the capital and wealthiest city in Chile. FeLV prevalence was determined in a specific area of the capital of Chile (Santiago), based on data from only three veterinary clinics. This narrow focus is not representative, likely reflects localized socioeconomic conditions rather than the national scenario.

The FeLV prevalence in Chile in our study was found to be substantially higher than in countries such as the UK, the USA, Canada, Germany, or even countries with moderate prevalence like Spain, and Italy (Studer et al. 2019). Instead, the Chilean context more closely resembles that of

the other Latin American countries or certain regions of Asia, where FeLV remains more widespread.

This study identified three major contributing factors to FeLV dynamics. First, vaccination: although vaccination is highly effective against FeLV, vaccination rates remain low when compared with countries where the disease has been controlled (Studer et al. 2019). The second factor was socioeconomic disparities, leading to different FeLV prevalence across regions, particularly in underserved areas with lower access to veterinary care. The last factor was a potential stochastic factor that may have played a role in the COVID-19 pandemic. The highest number of cases in this study were detected during 2021, a period marked by strict movement restrictions in Chile. Reduced veterinary access and changes in owner behaviour during this time may have increased the number of positive tests taken.

6.2 Molecular analysis of *env* gene of Feline Leukaemia Virus between domestic cats and RNA folding.

Chapter 3 explores the application of NGS to the *env* gene of FeLV, highlighting the benefits and limitations of various sequencing strategies. The advancements in NGS have led to new virological approaches that offer insights from multiple perspectives. These different methodologies can yield varying results; thus, careful consideration must be given to the sequencing approach and its impact on the conclusions drawn. Additionally, modern bioinformatics tools now enable re-analysis of previously published datasets, providing opportunities to reinterpret the existing knowledge with improved computational power.

The *env* gene of FeLV is highly variable, and prone to insertions, deletions, and recombinant events with endogenous retroviruses. The most well-known recombination occurs between FeLV-A and enFeLV, producing the FeLV-B variant. Nonetheless, other recombination events can be produced by interaction between the exogenous FeLV and ERV-DC to produce FeLV-D. Besides, FeLV-A by itself, can generate mutations that give rise to other subtypes such as FeLV-C, FeLV-E or FeLV-T (Miyake et al. 2019, Overbaugh 2000, Riedel et al. 1986). The *env*

gene is critical for determining receptor usage and has been linked to clinical progression (Faix et al. 2002, Cano-Ortiz et al. 2022).

Sequencing this gene presents multiple challenges. The high sequence similarity between FeLV-A and enFeLV often results in amplification artefacts during PCR, especially when using Sanger sequencing directly on amplicons. This frequently leads to mixed sequences that are difficult to interpret. One solution is the plasmid cloning of PCR products before Sanger sequencing, allowing for the isolation and accurate sequencing of individual variants. However, this approach is labour-intensive and more costly compared to sequencing conserved regions like *gag* or LTR.

In Chapter 3, the *env* gene was molecularly analysed in 8 domestic cats using 4 sequencing approaches: Sanger sequencing directly from PCR amplicon, Sanger sequencing following plasmid cloning, amplicon-based Illumina sequencing and Nanopore sequencing. All methods successfully identified FeLV-A and FeLV-B sequences. Among these, direct Sanger sequencing produced the most divergent results, while sequences obtained through other methods tended to cluster based on the sequencing platform used. Although minor differences were observed between direct amplicon and plasmid cloning sequences, no substantial differences were detected. Plasmid cloning provided cleaner sequences, but deletions and insertions were observed across all methods.

At a recombination level, RDP4 software identified four distinct recombination breakpoint patterns. Interestingly, the longest recombination was detected only through Sanger sequencing, while Nanopore sequencing was uniquely able to detect small recombination events and large indels. However, Nanopore has a high per base error rate which limits its reliability in identifying SNPs, resulting in the detection of over 200 variants, many of which may be sequencing artefacts.

Most of the genetic variation occurred in RBD, while the C-Domain was the least variable. These findings align with previous studies (Rey, Prasad, and Tailor 2008, Cano-Ortiz et al. 2022). In addition, G→A transitions were the most common single nucleotide variation observed, consistent with the activity of feline APOBEC 3 mutations.

To further validate variation patterns, Synplot2 analysis was performed, incorporating additional sequences from GenBank to increase its statistical significance. This analysis confirmed strong synonymous site conservation in the C-Domain of FeLV-A and revealed a smaller conserved region in the PRR, previously associated with β-turn helix motifs (Fontenot et al. 1994, Lavillette et al. 1998). In contrast, FeLV-B showed a broader peak of variation from RBD to the PRR, consistent with its recombinant nature. Unfortunately, a lack of available enFeLV *env* sequences meant that this analysis was unable to be performed at this time with the *env* gene from enFeLV.

RNA secondary structure analysis also provided insights into envelope gene complexity. FeLV-A displayed more structurally diverse and complex RNA folding patterns than FeLV-B. Due to the unavailability of sufficient enFeLV *env* sequences, RNA structure predictions for endogenous variants could not be performed. Such analysis would be valuable in understanding how recombination breakpoints and length of enFeLV insertion affect the RNA stability and potentially influence viral replication efficiency.

One limitation of this chapter is that not all cats were sequenced using every method. The NGS method requires higher sample concentrations and better sample conditions than Sanger sequencing. Therefore, a comparative analysis of all cats across all methods was not possible. In conclusion, the sequencing method of choice should be chosen based on what the objective of the study is, Nanopore for recombination events and large indels, and Illumina for SNP evaluation. Which platform sequencing has been performed on should also be considered when different comparisons are evaluated, because bias in the sequencing method can lead to artificial clustering, complicating the construction of large-scale viral phylogenies.

6.3 Endogenous Gamma retroviruses in Chilean Domestic Cats.

Several gamma retroviruses have been described in domestic cats. ERVs are known to influence host genomes through multiple mechanisms, including truncation of host gene transcripts, enhancements of gene expression, and the formation of chimeric transcripts. (Uren et al. 2005). The genomic insertion sites of ERVs can vary depending on the timing of endogenization, which influences their distribution and potential biological effects.

Traditionally, the analysis of ERV integration sites has been conducted using PCR-based methods and FISH (Uren et al. 2005, Fujino 2003). However, these approaches are often laborious and limited by issues specific to each technique (Fujino, Ohno, and Tsujimoto 2008). More recently, NGS technologies have been employed to enable faster and less labour-intensive detection of ERVs and their integration sites (McEwen et al. 2021, Chiu et al. 2024).

In this study, ONT together with ReadFish software were employed to run NAS, to enrich for both exogenous FeLV and enFeLV. Eight samples were successfully sequenced. Taxonomic classification using Kraken2 enrichment was performed, with robust sequences of enFeLV, Fc-gamma4, exFeLV, RDRS, and ERV-DC identified. These ERVs have been mainly described in domestic cat populations from Spain, Japan, or the USA. Although the structural features were consistent with previous works, notable differences were identified in Chilean cat populations, such as the number of insertion sites, sequence variations and sub-groups. More domestic cat populations across genetically and geographically diverse should be considered to understand the full biological implications of these insertions for domestic felids.

The enFeLV insertions were randomly distributed across the chromosomes, with the majority occurring as solo-LTRs. The number of insertions varied randomly among the individual cats. The observed insertion patterns were consistent with those previously reported by Chiu et al. (2024), with most insertions being unique to individual cats, although some were shared with integration sites identified in earlier studies. Notably, 15 IS were located within protein coding genes, suggesting a potential impact on gene expression. One such gene, KIAA1211, known to have cancer-related effects (lung cell cancer, colorectal cancer, etc) was disrupted in five of the sequenced cats. Furthermore, pathway analysis indicated possible associations with the FBXW7 and NOTCH1 pathways. However, further research is necessary to validate these findings and to elucidate the functional consequences of these insertion events.

Regarding ERV-DC, the number of insertions varied among individual cats and did not follow the same pattern observed for enFeLV. Among the ERV-DC groups, GI was the most frequently represented, followed by GIII, while group GII was poorly represented. GII encodes the Refrex-1 protein, an antiviral molecule known to inhibit exFeLV infection by interfering with viral entry. In

contrast, GIII is considered the most prone to recombination with exFeLV to produce FeLV-D. These findings may change the composition of ERV-DC groups, influencing susceptibility to exogenous FeLV and shaping the virological situation in different feline populations.

Fc-gamma4 represents an older endogenous retrovirus, primarily identified in the form of solo-LTR rather than complete proviral sequences. The ratio between solo-LTRs and full viral elements showed a marked shift, with nearly two-thirds classified as CIS, supporting the hypothesis of earlier endogenization compared to more recently integrated retroviruses.

In contrast, RDRS are among the most recent endogenous viruses and were infrequently detected in the Chilean domestic cat population, appearing in only six of eight cats in small proportions. This limited distribution suggests a more recent or localized endogenization event.

All these data were generated from a single sequencing run using Nanopore Adaptive Sampling, which successfully enriched retroviral sequences. However, the resulting coverage per sequence was relatively low, limiting the depth of certain analyses. NAS is a continuously evolving tool with frequent updates and improvements to its functionality. As such, it represents a promising approach for simultaneously identifying a broad range of viral structures within a single sequencing run, offering the potential for more comprehensive virological analyses in future applications.

6.4 Cross-species transmission of feline leukaemia virus between domestic felids and guigna.

The virulence of FeLV in non-domestic felids has been well documented in outbreaks that began over a decade ago, particularly affecting Iberian lynxes and Florida panthers. These outbreaks were successfully mitigated through targeted management measures, significantly reducing both the risk of infection and mortality rates within these endangered populations.

In Chile, the guigna population faces similar risks due to pathogen transmission from domestic cats to guigna, including FeLV, canine parvovirus and morbilliviruses (Sieg et al. 2020, Sacristán et al. 2021). This threat is aggravated by habitat fragmentation, which has been associated with

reduced genetic diversity (Napolitano et al. 2015) and a geographically restricted distribution in central and south-central Chile, close to human populations (Iriarte et al. 2013).

These samples from guignas were previously analysed by Sacristan et al (2021a). This study revealed high 3'LTR sequence homology between domestic cats and guignas. The 3'LTR, conserved region capable of distinguishing between endogenous and exogenous FeLV, provides a short amplicon (~200bp) but a sensitive target for diagnosis. Their phylogenetic analysis clustered guigna samples closely with domestic cats.

The findings from this thesis, classified guigna sequences in their own cluster. These findings suggest that FeLV is either actively circulating among guigna populations or has undergone a very rapid host adaptation. Of particular interest are variations at positions 59 and 60, located within the VRA, which is a determinant of cell attachment and receptor usage, with potential implications for host specificity and pathogenicity. While mutations in these positions have previously been linked to changes in viral fitness, the specific roles of these amino acids remain undefined.

Moreover, the iSNVs in guignas displayed a distinct pattern from those seen in domestic cats. While the RBD remains the most variable region in both species, guigna sequences exhibited a higher number of iSNVs in the transmembrane domain, typically conserved in domestic cats. Notably, nearly two-thirds of guigna variants involved G→A transitions, consistent with APOBEC mediated hypermutation. Although APOBEC activity has shown limited impact on FeLV in domestic cats, these results suggest a stronger antiviral effect in guignas, potentially reflecting host-specific differences in innate immune pressure.

Interestingly, Illumina sequencing produced better-quality results for guigna samples than for domestic cat samples. This observation is likely due to the absence of enFeLV in guignas, which in domestic cats can cause sequencing artefacts because of the high similarity between exFeLV and enFeLV.

These findings not only advance our understanding of FeLV pathogenesis in non-domestic felids but also highlight key questions about how *env* gene variation affects cross-species infection and disease progression. From an epidemiological perspective, if FeLV is now primarily

circulating within guigna populations, future control efforts may need to shift focus, from managing disease in domestic cats to vaccination intervention directly within guigna populations.

6.5 Future work

The data generated in this study contribute significantly to the understanding of FeLV epidemiology in Chile, while also providing insights of broader relevance to understanding the FeLV biology. However, further research targeting deeper topics and understudied geographical areas could help to understand the behaviour of FeLV under different contexts.

From an epidemiological and survival modelling perspective, the consequences of high virus circulation, remain insufficiently evaluated. Future analysis should incorporate the distribution of infection outcomes (i.e. abortive, regressive, and progressive infections), as these infections significantly influence the clinical signs, clinical progression and survival rates. Thus, understanding the proportion of each infection type in highly endemic areas will support earlier and more informed decision-making in veterinary practice.

The increasing use of digital veterinary records presents an opportunity to compile more comprehensive and representative datasets, helping to demonstrate a broader and consistent context than specific situations described in local veterinary clinics.

At a molecular level, current knowledge of FeLV *env* gene variations remains limited to data from a few wealthy countries, generating substantial geographical gaps about the evolution of exogenous FeLV-A strains, especially in Latin American strains. Most published studies have described FeLV-A clusters from Japan and the USA, identifying different subgroups within FeLV-A. However, this genetic divergence has not been observed in the limited Latin American sequences currently available. This raises important questions about whether alternative FeLV-A variants are sampling underrepresented in this region, or whether a single dominant strain is circulating in the population. In terms of recombination, only recent studies in Japan have reported the emergence of recombinant strains beyond FeLV-B, underestimating the importance of characterizing alternative recombination events to understand their consequence on cats rather than relying on isolated case studies.

The broader topic of gamma retroviruses should be considered re-emergent. Although many ERVs were described over one or two decades ago, their biological significance is still being elucidated. Recent findings have challenged the paradigms about ERVs as “junk” DNA, revealing roles in complex diseases, especially in species such as humans or koalas. These discoveries have reshaped our understanding of ERV-host interactions and suggest that their impact is being underestimated (Tarlinton et al. 2022, McEwen et al. 2021, Tarlinton et al. 2020).

Domestic cats represent a particular species for ERV research. They exhibit higher cancer rates than many other species and harbour an unusually high number of gamma retroviruses compared to other carnivores. Our analysis of Chilean cats identified distinct ERV patterns, but deeper, more targeted investigations are needed to understand evolutionary, genetic events, and functional dynamics of each ERV. In this context, NAS offers a promising approach. As an evolving sequencing technology, NAS can increase both accuracy and resolution while enabling targeted enrichment of viral sequences in real time. This technique allows for rapid, high-throughput characterization of viral genomes and their integration sites, which could be extrapolated to other species, offering advantages over traditional methods that are slower and less flexible.

Regarding non-domestic felids infected by FeLV, the *env* gene has demonstrated valuable insights, therefore further non-domestic diagnosis should focus on this region. It will help to understand how FeLV adapts to different hosts and the implications for cross-species transmission. While our findings suggest the presence of host-specific amino acid changes, larger sample sizes and broader species comparison are needed to validate these observations. Therefore, the implementation of continuous surveillance of this infection across non-domestic species in Chile is necessary. Ideally the implementation of a vaccination campaign would start mitigation measures to avoid dissemination to species under severe genetic bottlenecks or habitat restriction, where viral adaptation could have profound conservation and health implications.

6.6 Conclusion

This thesis has provided important insights into the epidemiological and molecular situation of FeLV in Chile. From both perspectives, a high prevalence of FeLV was identified, aligning with findings from other Latin American countries. At a molecular level, NGS methods were successfully applied to characterize viral sequences; however, their application should be tailored to specific research objectives. In addition, NAS was implemented to detect gamma retroviruses with promising results to evaluate viral structures and insertion sites. Finally, FeLV strains from guigna showed species-specific variants, including nucleotide-level changes that suggest selective pressure in this host, potentially leading to unique viral adaptations.

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