DISSERTATION

EXTRINSIC AND INTRINSIC DRIVERS OF FELINE IMMUNODEFICIENCY VIRUS EVOLUTION IN THE MOUNTAIN LION

Submitted by

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ABSTRACT

EXTRINSIC AND INTRINSIC DRIVERS OF FELINE IMMUNODEFICIENCY VIRUS EVOLUTION IN THE MOUNTAIN LION

Viruses are among the most rapidly evolving entities in biology and are so intricately associated with their obligate hosts that the boundary between host and pathogen, and thus the study of one versus the other, is blurred by intimate interactions at scales ranging from proteins to populations. Viral genetic variation is both ecologically and molecularly determined, and thus viruses serve as measurably evolving populations that provide a window into adaptations and behaviors of their vertebrate hosts. Of all viral families, the biology of retroviruses is coupled especially tightly to that of the host due to permanent integration of viral DNA into eukaryotic chromosomes, producing an inherently dynamic infection that persists for life. Feline immunodeficiency virus (FIV) is among the oldest of viruses in the Lentivirus genus and the mountain lion, also known as the puma *(Puma concolor*), is the most extensively ranging New World terrestrial mammal. We used molecular analyses to investigate the host-pathogen interactions between pumas and FIV across geographic and temporal space, within and across populations, and among FIV subtypes.

In Chapter One, we investigate cross-species transmission of FIV from bobcats to pumas and compare the outcome of spillover infections in two populations separated by vast geographic space. Our findings reveal that the puma is typically a dead-end host of bobcat FIV infection, although altered population dynamics can promote stuttering chains of infection following spillover events. In Chapter Two, we employed a novel next generation sequencing technique to

investigate the impact of management interactions such as population supplementation on FIV dynamics in the endangered Florida panther. Results from this chapter show evidence for cointroduction of one subtype of FIV with translocated pumas from Texas, followed by local extinction of the previously circulating, 'less fit' subtype in the puma host. Chapter Three describes an important intrinsic driver of viral evolution through characterization of the APOBEC3 protein A3Z3 in the puma, a primary cellular restriction factor against FIV. We show evidence that at least one geographically associated genotype of puma FIV is able to evade lethal hypermutation typical of A3Z3 activity despite a deficiency in the viral counter protein Vif.

The collective findings of this work explore the ancient relationship between a vastly ranging apex predator and a chronic lentiviral infection by applying both novel and conventional methodologies to a unique, naturally occurring host-pathogen system. Although our questions were specific to FIV in pumas, the methodologies described here can be applied to other systems and models to address inherent limitations of opportunistic field studies including DNA degradation and sequencing of low copy number templates from archival biological samples. Ancient viral infections have the potential to elucidate the life history of mammalian hosts, which is particularly useful in the study of elusive and broadly ranging carnivores threatened by urbanization and habitat fragmentation. Future objectives of this work will expand analyses to incorporate additional populations, such as the modern Texas puma, and more thoroughly investigate genotype variation in Vif-A3Z3 interactions. Collectively, our results will inform additional studies that seek to elucidate determinants of host-pathogen interactions in naturally-occurring systems across diverse ecosystems and broad spatiotemporal scales.

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DEDICATION

To Sue, for being the exceptional mentor you are, and the strong female scientist I aspire to be.

TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS iv
DEDICATION vi
TABLE OF CONTENTS vii
INTRODUCTION 1
CHAPTER ONE: Feline immunodeficiency virus cross-species transmission: Implications for emergence of new lentiviral infections 14
Synopsis 14
Importance 15
Introduction 15
Methods 18
Results 25
Discussion 27
Figures 34
Tables 39
CHAPTER TWO: Genetic rescue of the Florida panther (*Puma concolor coryi*) correlates with exponential spread of extraordinary stable feline immunodeficiency virus. 42
Synopsis 42
Importance 43
Introduction 44
Results 47
Discussion 50
Methods 54
Figures 59
Tables 65
CHAPTER THREE: Differential degradation of puma APOBEC3 by geographically distinct variants of feline immunodeficiency virus 67
Introduction 67
Methods 74
Results 78
Discussion 82
Figures 87
Tables 92
CONCLUSIONS 94
LITERATURE CITED 96

INTRODUCTION

Host-pathogen interactions

The relationship between a pathogen and its host is among the most intimate, complex, and venerable associations occurring in the natural world. Although microbes were once defined by the ability to cause an injurious response or disease, current knowledge distinguishes pathogens as infectious organisms imparting highly variable outcomes dependent on myriad interacting factors across all biological scales, ranging from the level of the organelle to that of the ecosystem or biosphere. While many minute details and intricate associations ultimately determine a pathogen's capacity for infection, there are three distinct, critical determinants: (1) the host must be adequately exposed to the pathogen, (2) the host must be susceptible to infection, that is the pathogen must be able to enter, survive and replicate in the environment of the host, and (3) the host must shed the pathogen if onward transmission is to occur. In naturally occurring systems, parasite infectivity and host resistance are both ecologically and genetically determined, evolving under a cyclical adaptation and counter adaptation succession fueled by natural selection. This genetic conflict generates and maintains diversity both within parasites and mammals through a molecular 'arms race' driven by antagonistic infection and defense strategies.

Many ancient infections are characterized by chronic, asymptomatic states that reflect steady-state homeostasis of host and pathogen following many cycles of adaptation and counter-adaption. Novel infections, such as those emerging or re-emerging in a new host population or species, range from abortive or nonpathogenic to highly virulent with epidemic or even pandemic potential. In any case, the outcome of infection is determined by a multitude

of complex interactions between the inextricably linked biology of the parasite and the host. Of all categories of macro- and microparasites, retroviruses are especially revelatory of coevolutionary forces due to the established persistence that follows productive infection.

Retroviruses as evolutionary forces

Retroviruses comprise a diverse viral family found in all vertebrates and distinguished by a unique life cycle that involves reverse flow of genetic information from RNA to DNA, followed by integration of proviral DNA into the host genome (1). Integration of a provirus into the chromosome of an infected cell permits persistence in the host and enables remarkable and relatively rare events such as viral invasion of the germ line, establishment of latency in cellular reservoirs, oncogenic transformation and tumorigenesis (2, 3). Each such event drives host-pathogen coevolution in sophisticated and extraordinary ways, dependent on the molecular interplay between the immune system and the virus (4, 5). Scientists have long postulated that infectious agents have pervasively shaped the human genome by generating and maintaining genetic diversity (6-8). Recent studies provide evidence for this assumption, revealing that pathogens account for more selective pressure on humans and other mammals than any other environmental driver of genetic variation (9). Much of the evidence for pathogens as key drivers of diversity derives from studies of retroviruses and retroelements, particularly those of humans and other primates (10, 11). Nearly 8% of the human genome is retroviral in origin, consequent of ancient germ line invasion and Mendelian propagation, a process known as endogenization (12-14). The impact of retroviruses and endogenous retroelements on mammalian genomes is remarkably diverse, ranging from evolution of essential biological functions to adaptation of disease defense mechanisms, such as innate immunological restriction of infection (3, 15-17).

Diversity of the Lentivirus genus

Until recently, lentiviruses were considered a relatively modern genus of retrovirus, with origin estimates falling within the range of the past several hundred to several thousand years for the heavily studied primate subgroups based on application of the molecular clock (18). Recent advances in sequencing technology, especially within the fields of ancient DNA and paleovirology, however, have revealed ancient origins of the Lentivirus genus through discovery of ‘fossilized’ endogenous viral elements (EVEs) in rabbit, hare, and lemur genomes that confirm existence of retroviruses at least ~12-14 million years ago (Ma) (19-21). Deep evolutionary processes, such as ancient endogenization events, anchor viruses in the evolutionary context of their hosts and provide an ecological framework for studying present day virus-host interactions (22, 23).

Exogenous lentiviruses comprise a unique and divergent genus subdivided into 5 discrete evolutionary groupings (24). These include the widely recognized and heavily studied primate lentiviruses SIV and HIV, as well as feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), equine infectious anemia virus (EIAV) and the small ruminant lentiviruses (SRLVs), that is caprine arthritis and encephalitis virus (CAEV) and maedi-visna virus (MVV), the first of the lentiviruses to be discovered (25). Named for the ‘slow’ nature of infection, lentiviruses characteristically produce chronic-progressive infections and possess a unique capacity for replication within nondividing cells such as macrophages (26). Lentiviral genomes, like all retroviral genomes, are arranged in homodimers consisting of two identical, positive-sense, single-stranded RNA (ssRNA) monomers comprising functionally diploid virions (27). Genomes are ~7-13 kb in size (1).

Lentiviruses express structural genes *gag*, *pol* and *env* in addition to multiple accessory genes functioning in complex aspects of the lentiviral life cycle including persistent replication and evasion of immunological control (28, 29). The lentiviral genome is flanked by long terminal repeats (LTRs) comprised of sequence blocks known as U3, R and U5, of which U3 and U5 are duplicated during reverse transcription. Lentiviral LTRs interact with accessory proteins to function as promotors of viral transcription (30). Accessory genes vary in number and structure across evolutionary subgroups. All lentiviruses express *rev* and a downstream Rev-Response Element (RRE), which together control the relative contributions of full-length transcripts versus multiply spliced mRNAs (31-33). Most lentiviruses also express *tat,* which is a potent transactivator of transcription that binds to the TAR element through recruitment of cofactors cyclinT and Cdk9, initiating transcription at the promoter within the viral (LTR) (34). Excluding EIAV, all lentiviruses express viral infectivity factor (*vif*), which functions to counteract cytosine deamination of the viral genome by host-encoded restriction factors (35). Lentiviral *nef* is present at the 3' end of primate lentiviruses only, functioning in the downregulation of CD4 receptor expression by infected cells (36) . FIV encodes a small protein (OrfA) in a similar region that has recently been shown to decrease CD134 receptor expression and thus may be analogous to Nef of the primate lentiviruses (37). Transactivation functions similar to those of Tat have also been described for FIV OrfA (38, 39). In the intergenic region between *vif* and *env*, primate lentiviruses express several other accessory genes with varied structure and function (40-42).

Cellular tropism varies among lentiviruses; while all have the capacity to replicate in macrophages, some subgroups, such as the primate lentiviruses, preferentially replicate in CD4+ lymphocytes (43). The cellular tropism of FIV is relatively broad—the virus is

consistently found in a variety of lymphocyte subtypes, macrophages, and other cell types including those of the bone marrow and central nervous system (CNS) (44-46). Entry into target cells is accomplished via sequential coreceptor binding by primate lentiviruses and FIV. Both groups utilize T-cell activation markers as primary receptors; binding induces a conformation change, in turn permitting interaction with a secondary chemokine receptor followed by cellular entry (47). Primate lentiviruses specifically utilize CD4 as a primary receptor and CXCR4, CCR5, or other chemokine receptors secondarily (47, 48). In the domestic cat, FIV utilizes CD134 and CXCR4 (49), although other putative mechanisms permit viral entry in some nondomestic felids, such as the puma (50). The conformational change required by the sequential, dual receptor system partially shields the envelope of the virus, minimizing exposure of critical antigenic information to the humoral immune system of the host (39). Among other lentiviral subgroups, only EIAV receptor usage has been elucidated. EIAV enters macrophages via a member of the tumor necrosis factor (TNF) receptor superfamily known as equine lentivirus receptor 1 (ELR1) (51). It is unknown whether ELR1 comprises one unit of a dual receptor system, and whether all lentiviruses require two receptors remains to be determined. Natural modes of lentiviral transmission collectively include horizontal through semen and blood, and vertical through parturition and postnatally via milk (25). The nonprimate lentiviruses can sometimes be transmitted by saliva (especially FIV) (52), aerosols (especially SRLVs) (53, 54), and/or insect vectors (EIAV) (55). Within subgroups, some lentiviral infections cross into closely related hosts with relative ease and frequency (56-61). Transmission between distantly related hosts, however, is intrinsically restricted by cellular anti-viral proteins (62, 63).

Lentiviral restriction factors

Restriction factors are cellular proteins that inhibit viral infections and comprise an important arm of the innate immune system, serving as a first line of anti-viral defense. Diverse in both structure and function, restriction factors target a vast majority of viral pathogens at nearly every phase of the viral life cycle (64). Locked in an antagonistic battle with the viruses they restrict, restriction factors tend to evolve under positive selection (65). Gene duplications, losses, rearrangements, and length polymorphisms are thus a common phenomenon in comparative studies of restriction factors across species (16). Genes encoding restriction factors are often induced by interferon (IFN) signaling and are largely dedicated to antiviral activity, while essential cellular functions outside of those relating to the innate immune system are unidentified (64). Ancient gene duplications, however, perhaps facilitate subfunctionalization of paralogues, expanding the role of these proteins in the innate immune response (16). On the viral side of the genetic conflict, evidence of positive selection is reflected in auxiliary genes that have evolved in a gain of function fashion to combat the restrictive activity of the host (66). The most thoroughly characterized restriction factors to date are those that antagonize HIV, a virus which has indeed evolved a number of accessory genes in response to intrinsic cellular restriction (40).

Thoroughly characterized retroviral restriction factors include proteins of the apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3; A3) family, tripartitie motif-containing protein 5α (TRIM5α), cyclophilin A (CypA), tetherin (also known as bone marrow stromal antigen 2; BST-2 or CD317), sterile α motif domain and HD domain-containing protein (SAMHD1), and serine incorporators 3 and 5 (SERINC3 and 5). A comprehensive review of viral restriction mechanisms is beyond the scope of this work and can

be found elsewhere (41, 64, 67-69). A brief review of these factors follows.

(1) TRIM5α interacts with the retroviral capsid (CA) through its carboxyl-terminal B30.2 (SPRY) domain to interfere with viral uncoating in a species-specific fashion (70). In addition to this primary restriction function, a broader role for TRIM5α in innate immunity has recently been revealed, including function as a pattern-recognition receptor (PRR) (71) and a constitutive signaling intermediate in the nuclear factor kappaB (NF-κB) cascade (72). Interactions between the viral capsid protein and CypA are also important for infectivity of some lentiviruses (73-75). In a remarkable display of convergent evolution, in two genera of primates, macaques (Macaca spp.) and owl monkeys (Aotus spp.), retrotranspositions have independently produced TRIM5α-CypA (TRIMcyp) fusion proteins for efficient recognition and restriction of post-entry lentiviral uncoating (76).

(2) Tetherin, also known as BST2 or CD317, possesses relatively broad antiviral activity, restricting diverse infections of many viral families by preventing budding of progeny virions through 'tethering' of nascent particles to the plasma membrane of the newly infected cell (77). Several viral proteins have known anti-tetherin activity, including but not limited to Env, Vpu, and Nef of the primate lentiviruses (78, 79). Similar to TRIM5α, tetherin has also recently been shown to activate the NF-κB signaling in the innate immune response (80).

(3) SAMHD1 is a more recently identified retroviral restriction factor that hydrolyzes cellular dNTP and degrades viral RNA (81). The specifics of restriction by SAMHD1 have been the subject of intense scientific scrutiny because HIV-2 and some SIVs encode accessory proteins Vpx and/or Vpu that abrogate SAMHD1 activity, while

HIV-1 lacks a known counter protein (82). SAMHD1 additionally plays a role in the innate immune response to non-viral infections, and mutations in SAMHD1 are associated with autoimmunity (83).

(4) SERINC3 and 5 are among the most recently identified retroviral restriction factors, acting through a reduction in membrane fusion and antagonized by viral proteins including HIV and SIV Nef, Glyco-Gag of murine leukemia virus (MLV) and S2 of EIAV (84, 85).

(5) APOBEC3 (A3) proteins comprise a diverse family of cytosine deaminases best known for the ability to hypermutate lentiviral genomes (86). A3 proteins are countered by lentiviral Vif, an interaction that is reviewed more extensively in Chapter Three. The discovery of A3 protein presence and function transpired through the study of HIV-1 emergence and evolution (86).

Emergence and evolution of HIV

Of all examples of the arms race between host and pathogen, none is more thoroughly investigated than that comprising the emergence and evolution of human immunodeficiency virus (HIV). The inception of HIV was facilitated by a ‘perfect storm’ that permitted each of the three critical determinants of infection previously described, giving rise to the pandemic that persists today. Much scientific effort has been dedicated to unearthing the origin of HIV, and it is now known that four phylogenetic lineages of HIV-1 (M, N, O and P) arose from independent cross-species transmission events, with chimpanzees (*Pan troglodytes*) and gorillas (*Gorilla beringei*) serving as reservoirs of lentiviruses capable of crossing host barriers into humans (87-91). Additional cross-species transmissions led to the emergence of

nonpandemic HIV-2, which originated from sooty mangabey (*Cercocebus atys*) SIV in West Africa (92-94).

Given that lentiviruses have species-specific tropisms and are restricted by host barriers of the intrinsic immune system, the circumstances giving rise to HIV have been the subject of much scrutiny (90, 95). It is widely accepted that the SIVs that gave rise to HIV circulated within natural hosts for many years before spillover and adaptation to humans occurred (90, 96). Extrinsic drivers of HIV emergence are known to include socioecological circumstances that permitted the exposure of humans through hunting and consumption of infected bush meat (89, 90). The intrinsic factors permitting replication and productive infection in the novel (human) host have also been elucidated by recent studies (97) and provide a critical base for additional discoveries such as those relating to functions of viral accessory genes, which have evolved the critical capacity to overcome host barriers and infect new hosts.

Feline immunodeficiency virus

Feline immunodeficiency virus (FIV) of domestic cats (*Felis catus*) is recognized as a corollary to HIV due to clinical similarities such as robust depletion of T cells and a chronic, progressive decline in immune function (98). FIV is in fact the only nonprimate lentivirus known to cause an acquired immunodeficiency syndrome (AIDS), and produces a wide range of clinical outcomes including neurologic disease, renal disease, and tumorigenesis (99). For a complete review of FIV immunopathogenesis, see Miller et al. 2018 (99). Importantly, FIV, like SIV, circulates naturally in different host species in a subtype-specific pattern (100, 101), producing no detectable disease in most free-ranging feline hosts (102). It has been hypothesized that like HIV, FIV in the domestic cat represents a more recent viral infection and a departure from the apparent subclinical homeostasis reached by the ancient FIV

infections in members of the Felidae family and most SIV infections in nonhuman primates (103). Because FIV is both similar and different from HIV in distinct ways, it has served as an informative model for lentiviral studies and HIV investigations (99).

The genome of FIV, like all lentiviruses, is structured around three primary open reading frames (ORFs), *gag*, *pol*, and *env*, which encode the structural components and critical enzymes for production of nascent virions (see Figure 1.5) (25). Structural proteins matrix (MA), capsid (CA) and nucleocapsid (NC) are formed from cleavage of the Gag polyprotein by the viral protease (PR), one of several critical enzymes encoded by the *pol* gene (39, 104). Additional enzymes encoded by *pol* include reverse transcriptase (RT), an RNA-dependent DNA polymerase that produces a DNA intermediate from the viral RNA genome during the early phase of the viral life cycle, and integrase (IN) which permits stable integration of the proviral DNA into the host chromosome (39). FIV *pol* also encodes dUTPase (DU), which functions to prevent misincorporation of uracil during viral replication, thereby minimizing mutagenesis (105). FIV *env* encodes glycosylated surface unit (SU) and transmembrane (TM) proteins that function in attachment and entry of the virion into target cells (104). Along with these primary polyproteins, the aforementioned accessory proteins Vif, OrfA, and Rev complete the coding regions of the FIV genome, which is flanked by long terminal repeat (LTR) elements containing promotors for viral transcription (1).

The prevailing antagonists of feline retroviral infection are the A3 proteins capable of lethal hypermutation of viral genomes (67, 106). Interactions between feline A3 proteins and retroviral infections are reviewed here in Chapter Three. As has been demonstrated for other restriction factors, feline A3 proteins comprise a robust, but permeable barrier to cross-species infections (67, 106, 107). Interestingly, feline tetherin shows restrictive activity against the

release of nascent viral particles but is not capable of restricting direct cell-to-cell spread of FIV (108). A virus-encoded antagonist against feline tetherin has not been identified. Felids express a truncated TRIM5 gene due to a premature stop codon also seen in some primate species (109). Unlike in primates, however, the missing domain of feline TRIM5 is not replaced by CypA, and TRIM5 has no known antiviral activity in the Felidae family. Synthetic TRIMcyp chimeras, however, produce potent anti-FIV and anti-HIV activity (110, 111). It is also possible that feline TRIM5 participates in LPS-mediated signaling during pathogen recognition and response as has been described in humans and other primates (112, 113).

Puma FIV

The North American puma (*Puma concolor*) is host to at least two subtypes of FIV—the host-adapted, naturally occurring subtype FIVpco, and the bobcat-adapted subtype FIVlru, which predominantly infects pumas through cross-species transmission (114). Although the clinical impacts of FIV in pumas are difficult to assess given the species' solitary and elusive nature, FIV has not been associated with morbidity in pumas and is not known to produce an immunodeficiency syndrome (63, 115). Subtype FIVlru has poor replicative capacity (fitness) in the puma with viremia typically below the lower limit of detection (114). In contrast, FIVpco shares some aspects of host-adapted SIVs, such as SIVagm in the African green monkey and SIVsm in the sooty mangabey, which have relatively high fitness but do not produce clinical disease or measurable immunodeficiency in their respective hosts (114, 116, 117). This suggests that primates have evolved distinct mechanisms that protect against the pathogenicity of their own species adapted SIV subtypes (118), and similar adaptations might therefore be expected in the puma.

Previous studies have reported a correlation between the age of a host-lentivirus relationship and the level of pathogenicity. It has been hypothesized that prosimian immunodeficiency virus (PSIV), an endogenous lentivirus in the contemporary genomes of divergent genera of lemurs (23), originated from an ancestral FIV that crossed species from a felid carnivore to a prosimian host in ancient times (22). This hypothesis suggests that FIV is among the oldest of all members of the Lentivirus genus. An ancient history of FIV in pumas and a long period of host-pathogen coevolution could thus explain many patterns seen in FIVpco infections. Interestingly, the life history of the North America puma is marked by a regional extinction during the Pleistocene era approximately 10,000 years ago, followed by recolonization by a founder population from eastern South America (119). Previous studies suggest that these life history events are reflected in ancient and present-day FIV dynamics (120), providing an exciting opportunity to unify the study of host and pathogen in a unique, naturally occurring system that parallels that of primates but with informative differences.

Deep investigation of FIV in the puma has elucidated numerous exceptions to typical patterns and assumptions of lentiviral infections. Such findings include low viral fitness, but high prevalence in some populations, remarkable variation in evolutionary rates across populations, and rare stuttering chains of infection of a ‘poorly fit’ virus with minimal active replication. These findings are described in detail in the following chapters and highlight the unique relationship between pumas and FIV, which varies across populations. Lessons gleaned from FIV in pumas, also referred to in this work as mountain lions and panthers, are of interest to conservation biologists, disease ecologists, and HIV researchers alike, and offer rare opportunities to employ lentiviruses as strategic tools of investigation in other systems. Each

chapter comprising this work uniquely exemplifies the complexities of host-pathogen interactions and underscores the many ecological and molecular determinants of lentiviral infections.

CHAPTER ONE

Feline immunodeficiency virus cross-species transmission: Implications for emergence of new lentiviral infections

Synopsis

Owing to a complex history of host-parasite coevolution, lentiviruses exhibit a high degree of species specificity. Given the well-documented viral archeology of HIV emergence following human exposures to SIV, understanding processes that promote successful cross-species lentiviral transmissions is highly relevant. We have previously reported natural cross-species transmission of a subtype of feline immunodeficiency virus, puma lentivirus A (PLVA), between bobcats (*Lynx rufus*) and pumas (*Puma concolor*) in a small number of animals in California and Florida. In this study we investigate host-specific selection pressures, within-host viral fitness, and inter- vs. intra-species transmission patterns among a larger collection of PLV isolates from free-ranging bobcats and pumas. Analysis of proviral and viral RNA levels demonstrates that PLVA fitness is severely restricted in pumas compared to bobcats. We document evidence of diversifying selection in three of six PLVA genomes from pumas but did not detect selection among twenty PLVA isolates from bobcats. These findings support that PLVA is a bobcat-adapted virus, which is less fit in pumas and under intense selection pressure in the novel host. Ancestral reconstruction of transmission events reveals intraspecific PLVA transmission has occurred among panthers (*Puma concolor coryi*) in Florida following initial cross-species infection from bobcats. In contrast, interspecific transmission from bobcats to

pumas predominates in California. These findings document outcomes of cross-species lentiviral transmission events among felids that compare to emergence of HIV from nonhuman primates.

Importance

Cross-species transmission episodes can be singular, dead-end events or can result in viral replication and spread in the new species. The factors that determine which outcome will occur are complex, and the risk of new virus emergence is therefore difficult to predict. Here we use molecular techniques to evaluate transmission, fitness, and adaptation of puma lentivirus A (PLVA) between bobcats and pumas in two geographic regions. Our findings illustrate that puma exposure to PLVA is relatively common but does not routinely result in infections communicable in the new host. This is attributed to efficient species barriers that largely prevent lentiviral adaptation. However, the evolutionary capacity for lentiviruses to adapt to novel environments may ultimately overcome host restriction mechanisms over time and under certain ecological circumstances. This phenomenon provides a unique opportunity to examine cross-species transmission events leading to new lentiviral emergence.

Introduction

The *Lentivirus* genus comprises complex retroviruses with a propensity for rapid mutation and recombination, resulting in a high rate of viral evolution. Lentiviruses typically infect hosts in a species-specific manner, and distinct viral subtypes or clades are characteristically associated with a single host species. Transmission of these host-adapted viruses to new species is uncommon (121, 122). Host restriction is attributed to several factors, including lower viral fitness in the novel host, intrinsic anti-viral defense mechanisms, and/or

limited contact sufficient for transmission between different host species (63, 67, 123-125). Notable examples of successful cross-species lentiviral infection include multiple transmissions of simian immunodeficiency viruses (SIVs) from non-human primates to humans, which gave rise to the various circulating subtypes of human immunodeficiency virus (HIV) (reviewed in (89)). It is thought that a convergence of social, cultural, and behavioral factors resulted in viral transmission and subsequent adaptation, culminating in a devastating pandemic infecting an estimated 35 million people worldwide (126).

At least eleven felid species have been diagnosed with lentiviruses known as feline immunodeficiency viruses (FIVs), which represent the most well-defined lentiviral group outside of the SIVs (115, 127). As with other lentiviruses, FIV phylogenetic relationships support a pattern of species-specific viral evolution (102, 128). In domestic cats (*Felis catus*), reported morbidity and mortality vary widely from mild or inapparent infection to a terminal AIDS-like syndrome (129-132). In non-domestic felids, infections are apparently subclinical, though reduced CD4 T-lymphocyte counts and increased prevalence of opportunistic pathogens have been documented in some feline hosts (133-135). Experimental transmission of FIV isolated from puma (*Puma concolor* – also referred to as mountain lion, cougar, and panther) to domestic cats resulted in productive, yet avirulent infections (136). Host-mediated cytidine deamination ultimately produced defective viral genomes (136, 137), suggesting that adaptation of FIVs to new host species does not readily occur.

An exception to the pattern of lentiviral host-specificity, puma lentivirus A (PLVA, also referred to as FIVpcoA), is an FIV subtype documented to infect two different species in the wild – bobcats (*Lynx rufus*) and pumas (56). Pumas inhabit a geographic range from western Canada to southern Chile, while bobcats are sympatric mesopredators throughout much of North

America (Figure 1.1). Both species are habitat generalists but are sensitive to anthropogenic influences and have experienced regional extinctions and population subdivision due to overhunting and habitat degradation (138-141). PLVA is the only FIV that has been isolated from bobcats and is endemic in California and Florida but has not been identified in other geographic regions (56, 120, 142-144). PLVA has only been identified in pumas that are sympatric with PLVA infected bobcats (Figure 1.1), is absent throughout most of the puma geographic range, and is much less common in pumas as compared to a second FIV subtype, puma lentivirus B (PLVB, also referred to as FIVpcoB), in regions where the two viruses co-circulate (56, 101, 102, 122, 139, 142). In contrast to PLVA, PLVB has been shown to infect pumas throughout their entire geographic range and thus has likely co-evolved with pumas since prior to their proposed recolonization of North America after the last Ice Age (10,000 – 15,000 years ago) (142, 145). The genetic distance between PLVA and PLVB is similar to that separating the other species-specific strains of FIV and is suggestive of a divergent evolutionary history in separate host species (101, 102).

We previously described several aspects of PLV evolution including the role of mutation, recombination, and natural selection in generating genetic diversity over time and space (120). Specifically, both PLVA and B evolve under predominantly purifying selection, with high rates of synonymous mutations occurring at the nucleotide level but relatively infrequent non-synonymous substitutions resulting in diversity in the corresponding proteins. Multiple recombination break points were detected across the PLV genomes, indicating this is an important mechanism for generating genetic diversity, which can lead to fit viral variants that differ from both parental isolates. Finally, we were able to document through viral phylogenies

that PLV diversity reflects the population structure of its hosts, with large genetic distances separating geographically distinct host populations, but apparent admixture within populations.

In this study, we present new findings following examination of an expanded set of samples to specifically characterize the dual-host tropism of PLVA. This report represents the first analysis of cross-species transmission, viral fitness, and viral adaptation in relation to the evolution of PLVA in the native host (bobcat) and secondary host (puma). Our results indicate that PLVA viral fitness is severely reduced in the puma compared to the bobcat, and that adaptation (episodic diversifying selection) has occurred in puma PLVA isolates. In California, most puma isolates have arisen from cross-species transmission from bobcats. In contrast, PLVA phylogeny in Florida panthers (a regional subspecies of puma, *Puma concolor coryi*) is consistent with primarily intraspecific transmission events, suggesting possible PLVA adaptation in this population. The dual-host tropism of PLVA provides a unique opportunity to understand the ecological and evolutionary factors involved in lentivirus host-range expansion, analogous to the transmission of SIVs to humans leading to the emergence of HIV.

Methods

Sample Collection and Nucleic Acid Extraction

Puma and bobcat samples were collected from natural populations in three locations over the following time spans: California (1996-2010), Colorado (2009-2013), and Florida (1983-2010). Isolates yielding PLVA sequences from bobcats or pumas are illustrated in Figure 1.1. Samples were collected from live, free-ranging animals captured using baited cage traps or scent-trained tracking hounds, as previously described (146). Animals were chemically sedated for blood collection. All animal capture and handling protocols followed approved Animal Care and

Use Committee guidelines, and where applicable, local government regulations. Additional samples were opportunistically collected during routine postmortem examinations by local government authorities. Aliquots of blood and tissue samples were sent to Colorado State University for characterization as described below. Tissue samples consisted of lymphoid organs, including lymph node, spleen, and thymus, as well as one sample each of liver, kidney, and muscle. Table 1.1 provides the sex, age, location, and collection date for the samples included in this study.

DNA was extracted from tissue, whole blood or peripheral blood mononuclear cells (PBMCs) using the DNeasy Blood and Tissue protocol (Qiagen Inc., Valencia, CA). Approximately 130 samples from archival collections were screened to identify the maximal number of positive samples that could be further sequenced or subjected to quantitative PCR. Plasma or serum was available for a subset of positive samples (n=7 bobcat and n=24 puma). RNA was extracted from these samples using the QIAmp viral RNA mini kit (Qiagen Inc., Valencia, CA) according to manufacturer recommendations.

Phylogenetic Relationships

Proviral DNA sequences of the *pol* gene (474 bp) from 59 PLV isolates (28 PLVA isolates and 31 PLVB isolates) were obtained from GenBank (Table 2.1) (147). Previously described nested PCR protocols (101) were used to amplify PLVA proviral DNA from additional bobcats (n=12) and pumas (n=6). This represented approximately 20% of bobcat samples tested and 10% of available puma samples, consistent with previously reported prevalence in these regions (56, 120, 144). Reactions contained 100–1000 ng of genomic DNA. PCR products were sequenced on an ABI 3130xL Genetic Analyzer 136 (Applied Biosystems Inc., Foster City, CA) and aligned using default parameters in MEGA6 (148). A maximum likelihood phylogenetic tree

was constructed using PhyML (149) parameters in Seaview (150) based on the GTR+i model of nucleotide substitution. Cluster support was estimated with 1000 bootstrap replicates. An HIV-1 sequence and three domestic cat FIV sequences (subtypes A, B and C) were included as outgroups.

Two maximum likelihood phylogenetic subtrees were constructed using the above methods and including PLVA sequences from either Florida or California. A PLVB sequence was included in each subtree as an outgroup, and cluster support was estimated with 1000 bootstrap replicates. We further investigated relationships between bobcat and puma PLVA by generating heat maps based on pairwise distance matrices using Sequence Demarcation Tool (151).

Within-Host Viral Fitness

We developed PLVA- and PLVB-specific quantitative polymerase chain reaction (qPCR) assays to quantify proviral load and viremia from natural infections. Thirty-four full-length PLVA genomes and 33 full-length PLVB genomes were aligned for primer design. Despite high intra-subtype genetic diversity, we identified primer-binding sites in PLVA *env* and PLVB *gag* that were 100% conserved. PLVA primers were 8083F, GCA GCC CTG ACG GTA TCC, and 8165R, GCA GTC TCC TCT GAA CAA TCC; and PLVB primers were 643F, CTG TCT GTC ATG GGG AAT GAG T, and 773R, GTC CTG TAG CTA CCA AGG CAA.

Provirus: qPCR reactions for PLVA (n = 30 bobcats; n = 10 pumas) and PLVB (n = 18 pumas) (Table 1.1) were conducted under identical conditions using 50-100 ng of genomic target DNA. All samples were run in triplicate while standards and controls were run in duplicate. Reactions were conducted using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) according to manufacturer recommendations with 20 µM primer concentrations and cycling

conditions of 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 62°C for 10 s. Reactions were analyzed by melt-curve with the temperature varying from 65°C to 95°C (increasing 0.5°C every 10 seconds) in order to confirm PCR product size and uniformity. All melt-curves generated from PLV infected feline DNA matched melt-curves generated from plasmid standards, indicating consistent detection of virus-specific DNA. Plasmid standards of known copy number were prepared by cloning PLVA and PLVB target sequences into pCR4-TOPO using a TOPO TA cloning kit (Life Technologies, Carlsbad, CA). Plasmid standards from 10^6 to 10^2 PLV copies per reaction were prepared using TE buffer (10 mM Tris and 1mM EDTA) containing 10 ng/µl DNA from FIV-negative bobcats. All qPCR reactions had amplification efficiencies within the acceptable range of 90-110% (152).

Negative control DNA samples from specific pathogen-free domestic cats and PLV-negative bobcats yielded negative results with both PLVA and PLVB qPCR assays, demonstrating that the assay primers do not cross-react with feline genomic DNA. PLV proviral copies per 10^6 cells were calculated for each sample based on the number of cell equivalents of input DNA as described by (153). The lower limit of detection of this assay is approximately 100 proviral copies per reaction.

Plasma viremia: cDNA synthesis was performed using 10 ul of purified RNA, and the following reagents: 4 µl of 5x First Strand Buffer (Life Technologies, Grand Island, NY), 1 µl of 10 mM dNTP (Bio-Rad), 1 µl of 0.1 M DTT (Life Technologies), 0.25 µl of 40 U/µl RNase Out™ (Life Technologies), 0.25 µl of 200 U/µl SuperScript® II Reverse Transcriptase (Life Technologies), 2 µl of 300 ng/µl Random Primers (Life Technologies), and 1.5 µl of nuclease-free water. Samples were incubated at 42°C for 50 min, followed by 95°C for 5 min. cDNA was stored at -20°C until testing in the qPCR assays. qPCR was performed as described above for

provirus quantification. PLV RNA copies per ml of plasma were calculated as follows:

(SQ) (1/C) (D) (E) (1/P) = RNA copies per ml of plasma

SQ: Mean starting quantity per sample

C: cDNA volume (µl) added per well of the qPCR assay (5 µl)

D: Inverse of the dilution used for cDNA synthesis (e.g., for 1-in-2 dilution D equals 2)

E: Elution volume (µl) used for viral RNA extraction (60 µl)

P: Plasma volume (ml) used for viral RNA extraction (generally, 0.14 ml).

All calculated values of RNA copies/ml of plasma were rounded to two significant digits based on the precision of the method. To determine the Lower Limit of Quantitation (LLOQ), PLV standards (diluted in TE buffer) were tested on multiple days to determine the lowest concentration that could be consistently quantified. Standard concentrations tested were 10^2 to 10^7 plasmid copies/well (10-fold dilution), as well as values below this range (10, 20, 40, 50, 60, and 80 copies/well). The LLOQ was determined to be 10^2 copies/well, equivalent to 17,000 RNA copies/ml plasma.

For each virus-host relationship, mean copies of integrated provirus (proviral load) and circulating viral RNA (plasma viremia) were statistically compared by one-way analysis of variance (ANOVA), followed by Tukey's multiple means comparison test using Prism v. 5.0 (Graph Pad Software, La Jolla, CA).

Host-Specific Selection

Previous analysis of PLVA and PLVB genomic characterization has been performed using Data Monkey tools MEME and FEL analysis and results have been reported on a gene-by-gene basis independent of host of origin (30). To compare selection pressures across host species, separate PLVA (n=26) and PLVB (n=33) translation alignments were generated using

the Muscle plugin with default parameters in Geneious (154, 155). Each alignment was split into multiple non-recombinant sections based on the recombination breakpoints identified in (120), and screened using branch-site random effects likelihood analysis (branch-site REL) in Datamonkey (156). Branch-site REL does not use *a priori* assumptions about which viruses may be diversifying, but rather selection is allowed to vary among all lineages and across all sites. Viral lineages identified as evolving under episodic diversifying selection have a non-synonymous substitution rate (dN) significantly greater than the synonymous substitution rate (dS) at a proportion of sites within any region of the genome (dN>dS; corrected p-value < 0.05). All full-length PLV sequences were included in these analyses (Table 1.1).

Host-Specific Substitution Bias

To further examine host-pathogen interactions, we quantified each type of nucleotide substitution within a non-recombinant segment of the *pol* gene for all available PLVA isolates. Consensus sequences were generated for each host in each region (California and Florida) based on strict (50%) majority. Mutations at polymorphic sites were summed in each isolate and the mean number of each type of base change (i.e. cytosine to thymine) within each group was compared. Additionally, we screened for evidence of host-mediated cytidine-deamination under the working hypothesis that a bias for guanine to adenine (G-to-A) transitions could be detected in isolates of non-adapted virus in a novel host. Using the same region of *pol*, the mean number of G and A nucleotides was compared between host species in Florida.

Transmission Dynamics

Discrete trait mapping in BEAST v1.8.3 (157) was used to infer ancestral host states across the PLV *pol* phylogeny. Ancestral states were reconstructed at nodes and along branches, and state change counts were estimated across the entire tree (158). Cross-species transmission

events were evaluated with an asymmetric Bayesian stochastic search variable selection model (159). Model parameters for the phylogeny included an HKY substitution model with estimated base frequencies. Substitution rates were estimated separately for two codon partitions (1+2 and 3) with a gamma distribution and four rate categories. The relative rate of mutation for both codon partitions was modeled using a lognormal prior with standard deviation and initial value set equal to one. The tree prior included a coalescent model with a constant population size and a random starting tree (157). Evolution over time was modeled using an uncorrelated relaxed molecular clock with a lognormal distribution of rates, an initial value of 0.1, and standard deviation equal to 0.5. Two separate Markov chain Monte Carlo (MCMC) runs were performed of $1x10^8$ generations each sampled every $1x10^4$ generation.

The log files were viewed in Tracer (160) to confirm the models reached convergence (as indicated by effective sample sizes greater than 200), and ensure posterior parameter estimates were similar between the two independent MCMC chains. The posterior distributions were down-sampled to every $2x10^4$ generation and combined using Log Combiner (161) after removing the first 10% of sampled states from each file as burn-in. The posterior tree files were similarly combined and then the maximum clade credibility tree was identified and annotated with median node heights using Tree Annotator (159). Discrete trait transitions were categorized as intra- vs. inter-specific transmission events based on posterior probability support greater than 0.80 that a node ancestral to a given branch was of the same or different host species respectively.

Results

Host-virus phylogeny

We previously evaluated PLV phylogenetic relationships in the context of broad-scale geographic patterns and gene-by-gene evolution (30). In this analysis we report on yet uncharacterized traits of PLV diversity that provide insight into the evolutionary dynamics of this two-host, two-virus system. The PLVB clade contains only puma isolates, and ancestral host-state reconstruction of the basal node of this clade resulted in a 0.99 posterior probability of puma ancestry (Figure 1.2A and Figure 1.3). In contrast, PLVA comprises 15 puma isolates and 32 bobcat isolates, and the basal node of PLVA was assigned a probability of 0.84 of bobcat ancestry.

PLVA isolates form two distinct groups of viral sequences exclusively from California or Florida (Figure 1.2). Samples from Florida cluster by host species - 14 of 14 bobcat and 7 of 8 panther isolates have a most recent common ancestor predicted to be a bobcat and panther, respectively (Figure 1.2B and Figure 1.3). In California, 18 of 18 bobcat PLVA isolates arise from predicted bobcat ancestors; however, in contrast to Florida, 5 of 7 California puma isolates were predicted to arise from most recent bobcat ancestors (Figure 1.2C and Figure 1.3). No puma-to-bobcat transmission was inferred within either population. In support of these results from the host-state ancestral reconstruction analysis, pairwise identity matrices demonstrate different patterns of host-virus relationships in California and Florida (Figure 1.2B and Figure 1.2C). In Florida, the majority of panther isolates share higher pairwise identity with other panther isolates than with bobcat isolates, while in California, the most closely related isolate to most puma isolates is a bobcat isolate. One viral isolate from a Florida panther (Pco87.FL1984) is paraphyletic to all PLV isolates with high bootstrap support of its exclusion from PLVA and

PLVB (Figure 1.2A). This isolate clusters with the domestic cat FIV isolates and is most similar to FIV_{Fca} subtype B (92% pairwise identity) (data not shown).

Within-Host Fitness

PLVA proviral loads in bobcats (mean = $10^{3.8}$; sd = 0.49) and PLVB proviral loads in pumas (mean = $10^{4.7}$; sd = 0.50) were 1 to 2 orders of magnitude greater than PLVA proviral loads in pumas (mean = $10^{3.0}$; sd = 0.93) (ANOVA; $p < 0.0001$, Figure 1.4A). This result was consistent for proviral loads quantified from both blood and tissue samples. The qPCR assay did not detect PLVA provirus in 6 PLVA infected pumas, despite amplification of integrated proviral DNA by nested PCR assays.

Analysis of the raw data documented significantly higher viremia for PLVA in bobcats and PLVB in pumas than for PLVA in pumas (Figure 1.4B), and no significant difference between viremia values of each virus in its apparent primary host. The majority of bobcat and puma plasma viral loads for both PLVA and PLVB were below LLOQ, and 10 of 10 pumas with detectable PLVA provirus had no detectable viremia.

Host-Specific Selection

Full genome sequences of PLVA (n=26) and PLVB (n=33) were analyzed to detect individual isolates subject to episodic bursts of diversifying selection. Episodic diversifying selection (dN>dS) was detected in at least one genomic region in 50% (n=3/6) of the PLVA sequences isolated from pumas (Figure 1.5). Selective pressure was detected within *vif* and several regions of *env*, including the leader domain, the transmembrane domain, and the region spanning the surface and transmembrane domains. In contrast, no evidence of diversifying

selection was detected in any segments of 20 PLVA genome sequences from bobcats. One of 33 PLVB genome sequences had evidence of selection in a single region in *env (*data not shown*).*

Host-Specific Substitution Bias

The most common type of nucleotide substitution in 3 of 4 groups of hosts (California bobcats, California pumas, and Florida panthers) was A-to-G (Table 1.3A). G-to-A transitions were slightly more common within bobcat isolates from Florida but we did not identify a significant bias for G-to-A substitutions in any group (Table 1.3B).

Discussion

Investigations of cross-species viral transmission events and "host jumps" have improved our understanding of the factors that can lead to virus emergence in new hosts (162, 163). While the specific ecological and adaptive drivers of emergence are case- and virus-specific, some general patterns and processes have been elucidated. Independent cross-species transmissions of host-adapted viruses often occur with little to no subsequent transmission among the new host (spillover events – i.e. transmission of avian influenza A viruses to humans) (164). This is because non-host adapted viruses may have low fitness, low transmission efficiency, or both in the novel host (165). However, with low fitness in a novel environment, selective pressures acting on existing and *de novo* genetic diversity can increase the frequency of beneficial mutations, leading to viral adaptation. This process may be observed through the accumulation of genetic changes that differ from those present in the reservoir population (166). This process of viral adaptation leading to increased fitness and transmission efficiency may be necessary for the virus to persist in the new host (host jump – i.e. the emergence of canine parvovirus from felids) (167).

SIV spillover into humans has occurred many times but only a small number of these resulted in sufficient viral adaptation to allow widespread human-to-human transmission in the worldwide HIV epidemic (89, 90, 168). We thus hypothesized that the jump of PLVA from bobcats into pumas would similarly represent a poorly adapted viral infection in a new host species with relatively low rate of transmission within the novel host. To test this hypothesis, we reconstructed ancestral viral-host phylogenetic relationships, measured within-host viral fitness, and analyzed patterns of viral adaptation for PLVA and PLVB isolates.

We inferred intra- and inter-species transmission dynamics using a phylogenetic analysis with ancestral reconstruction of each host species along the phylogeny (Figure 1.3). The results support our hypothesis that PLVA and PLVB have evolved with different primary hosts, as the ancestral host at the basal node of each clade was inferred with high posterior support to be the bobcat and puma, respectively. Further, bobcats were predicted to be the ancestral host-state at over 85% of nodes across the PLVA phylogeny, and all cross-species transmissions occurred in a unidirectional pattern from bobcats to pumas.

Although a similar number of bobcat and puma PLVA isolates were evaluated from California and Florida, the phylogenetic relationships and predicted patterns of cross-species transmission differ greatly between the two sample sites. Most PLVA isolates sampled from pumas in California arose via cross-species transmission, while the majority of panther isolates sampled in Florida resulted from intra-species transmission. The median posterior date estimates of the cross-species transmission events in Florida pre-date those in California, suggesting that PLVA has circulated in Florida panthers since prior to any detected cross-species transmission events in California (original tree annotated with dates and 95% highest posterior density intervals available upon request). Additionally, the branch lengths and pairwise identity values

between PLVA isolates from Florida panthers demonstrate that lineages in Florida have diverged since their shared ancestry with bobcat PLVAs, a pattern that differs from the California population (Figure 1.2). These findings are consistent with PLVA cross-species transmission from bobcats to panthers followed by viral divergence through drift or adaptation during subsequent intra-specific spread in Florida.

This contrast in transmission patterns between California and Florida provides a unique opportunity to consider ecological and host factors that may contribute to viral adaptation in a new host. The Florida panther is historically endangered, reduced to a dwindling population of 20-25 individuals by the early 1990s (169). Remaining panthers were highly inbred with significant deleterious impacts on the population, including congenital defects and an array of infections unlikely to occur to a similar degree in immunocompetent hosts (170). The low population size, small patches of suitable habitat, and isolation from other populations significantly restricted interactions among conspecifics for decades (169, 170). This ecological situation increased contact rates among remnant panthers concentrated within small habitat patches(170). Such drastic shifts in density and distribution, paired with changes in social structure and decreased host genetic diversity, may underlie our observation that PLVA transmission occurred within the Florida panther cohort and was followed by ongoing genetic divergence in the new host.

Interestingly, many of the California pumas with PLVA also derive from small isolated populations with similar genetic characteristics as those documented in the historic Florida panther population. Pumas from the Santa Ana Mountains (south of Los Angeles) and Santa Monica Mountains (north of Los Angeles) have high average pairwise relatedness, low estimated effective population sizes, and strong evidence of past genetic bottlenecks (171, 172). While our

phylogenetic data suggests that PLVA in California pumas is primarily acquired from bobcats versus intra-specific contacts, the condition of these small, isolated populations may similarly predispose them to chains of intraspecific infection and emergence of a puma-adapted PLVA infection over time. Observations gleaned from these results support the notion that the outcome of lentiviral infection in a new host is highly circumstantial, typically restricted from emerging in the new host, and dependent on complex interplay between ecological and evolutionary forces to adapt to a new species.

We detected both host-specific and clade-specific differences in viral fitness as measured by proviral loads and plasma viremia (Figures 1.4A & 1.4B). PLVA proviral loads in bobcats and PLVB proviral loads in pumas were significantly higher than PLVA loads in pumas. PLVB viral loads in pumas were significantly higher than PLVA proviral loads in bobcats, though viral copy numbers in both species were within a range similar to those reported for host-adapted subtypes of FIV in domestic cats (~10^3 – 10^4 proviral copies/10^6 cells) (153, 173, 174). Our inability to quantitate PLVA provirus from a number of animals that were positive by conventional PCR is likely a reflection of low proviral load, and potentially reflects partial degradation of some archival samples. Alternatively, the possibility of mutations in the primer/probe region of the *env* gene resulting from selection and/or genetic drift cannot be excluded as a plausible cause for inconsistencies in the assay.

A second measure of fitness that may relate more directly to transmission efficiency is plasma viremia. Similar to the proviral load data, the level of viremia in bobcat PLVA and puma PLVB were higher than in puma PLVA. While many of the viremia values were below LLOQ precluding a robust quantitative comparison among groups, the fact that PLVA was not detectable in any of the pumas analyzed is interesting and suggests that in most instances, PLVA

is not highly replication competent in pumas, despite the fact that intraspecific transmission of PLVA in Florida panthers is strongly supported by viral phylogenies.

Given that adaptive viral evolution is known to occur in episodic bursts driven by the host immune response, we predicted that the low fitness of PLVA in pumas would result in detectable genetic signatures of adaptation. As a measure of adaptation, we therefore estimated which viral lineages may be evolving under episodic diversifying selection, identified as an increase in non-synonymous substitutions relative to synonymous substitutions within short segments of viral proteins. In line with our prediction, diversifying selection was detected in segments from half of the puma PLVA isolates analyzed (3 of 6), but was rare among PLVB isolates (1 of 33) and not detected among bobcat PLVA isolates (0 of 20) (Figure 1.5). All diversifying lineages were evolving under episodic selection in *env*, an antigenic protein involved in the binding and entry of viral particles into host cells, and a target for neutralizing antibodies. Env variation has important fitness implications for other lentiviruses (175-180), and our results suggest Env may be important for the adaptation of PLVA in pumas.

In two of the three PLVA puma isolates under selection, viral adaptation was also detected in *vif*, the gene encoding the accessory protein that counteracts the innate antiviral activity of apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 (APOBEC3). In the absence of Vif, APOBEC3 enzymes are packaged into virions to restrict replication via deamination of cytidines during reverse transcription (181). APOBEC3 activity is detectable in proviral DNA as increased G-to-A mutation rates, which can result in eventual viral degradation. The complex interplay between Vif and APOBEC3 has been identified as an important driver of host-pathogen coevolution in a number of lentiviruses (64, 182), and G-to-A alterations have been documented in the infection of domestic cats with PLV (137). Thus adaptation in the Vif

protein of PLVA in pumas is therefore not unexpected and could represent a critical evolutionary mechanism shaped by host immune system and intrinsic restriction pressures.

Given the well-documented importance of Vif adaptations to host-range expansion of other lentiviruses, we surmised that APOBEC3 antiviral activity could be a factor limiting PLVA fitness in the Florida panther. However, we did not detect a bias of G-to-A substitutions in puma PLVA sequences. While this limited analysis of a small segment of PLVA genomes did not provide evidence of APOBEC3-mediated cytidine deamination, it is possible that highly mutated genomes may have been eliminated from circulation.

Our findings support growing evidence that pumas, the apex feline carnivore in North America, are regularly exposed to a diverse array of pathogens (183). Recent reports have documented puma predation on bobcats and domestic cats (184, 185), a likely mechanism by which pumas are exposed to pathogens of sympatric felids. The relationship between predator-prey interactions and viral transmission is not well studied, and may represent a process by which predators accumulate pathogens by a mechanism that is similar to the process of bioaccumulation of environmental toxins (186). For example, feline leukemia virus has spilled over from domestic cats and caused outbreaks with high morbidity in Florida panthers on multiple occasions (187). Another example is the recent discovery of a novel feline gammaherpesvirus that appears to be transmitted from bobcats to pumas (188). This pattern is further documented in this study by the identification of frequent transmission of PLV from bobcats to pumas, and by the first recorded account of domestic cat FIV in a free-ranging Florida panther (Figure 1.3 and Figure 1.2A). This phenomenon of predator-driven pathogen exposure warrants further study as it may represent a yet unappreciated ecological driver of emerging diseases.

With the notable exception of SIVs, this study represents the first robust analysis documenting contemporary, naturally occurring lentiviral cross-species transmission. Our findings confirm that PLVA and PLVB are different viral species, deriving from bobcats and pumas, respectively. We therefore propose that PLVA be reclassified as a distinct species of FIV, designated as FIV_{Lru} to indicate that the bobcat is the primary host. We further suggest that reference to 'puma lentivirus' be reserved for PLVB, more accurately referred to as FIV_{Pco}.

Our findings demonstrate that viral transmission from a reservoir host into a sympatric relative can occur frequently. Further, we found evidence of PLVA adaptation to pumas, an evolutionary process that may be influenced by both host and ecological conditions. Given the prevalence of PLVA in pumas that are sympatric with infected bobcats and the evidence for sustained intraspecies transmission of PLVA in relic Florida panthers, the emergence of a puma adapted PLVA with a broader geographic distribution is clearly possible.

This study provides further evidence that viral transmission from one species to another does not routinely result in intraspecific spread within the new host, but also suggests that viral evolution may ultimately overcome host restriction mechanisms. This contribution to the current understanding of pathogen-host dynamics also highlights the susceptibility of small, stressed populations to novel viruses and sets the stage for further study of risk factors for viral emergence in apex predators.

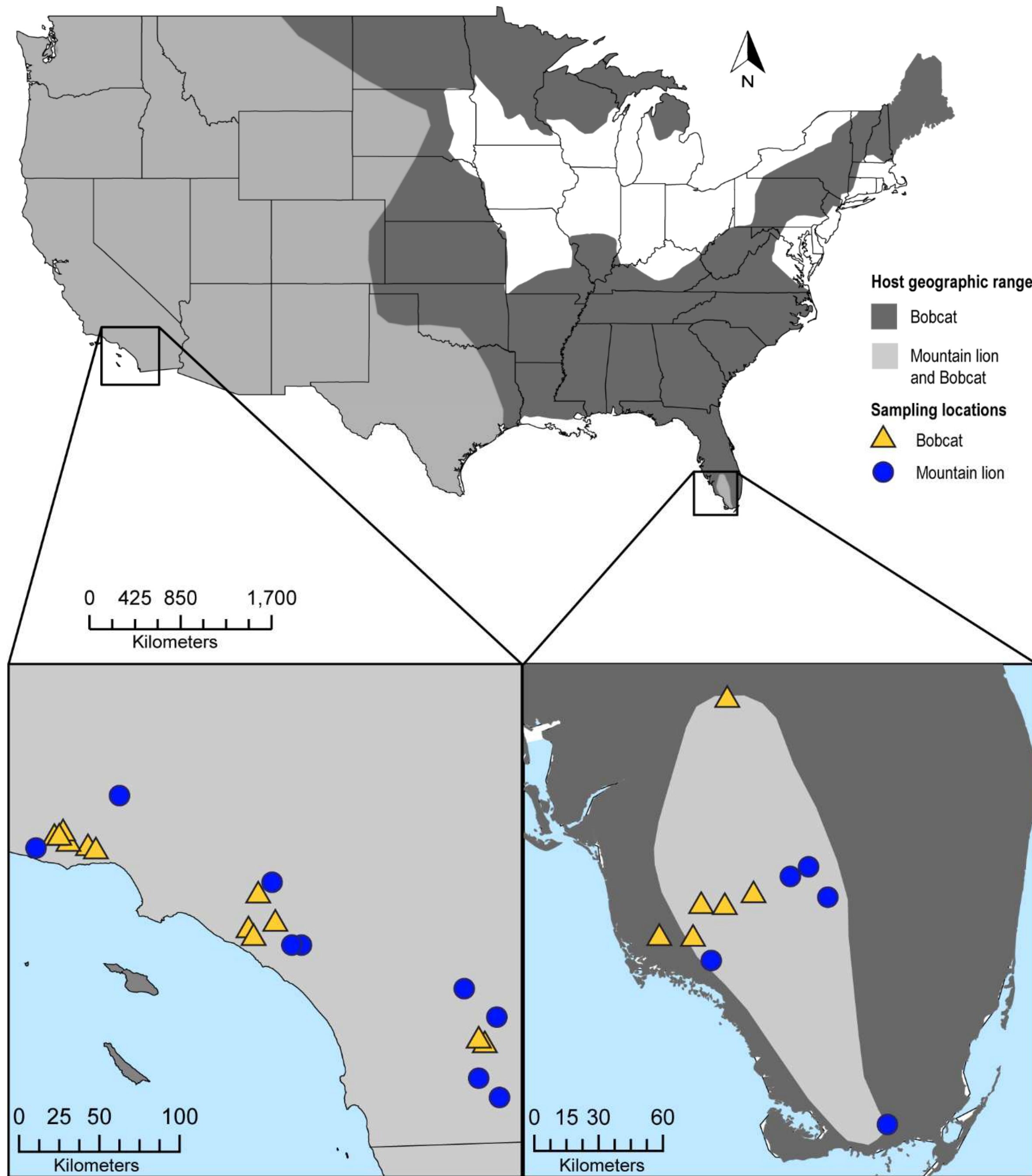


Figure 1.1: Host species geographic ranges and sample collection sites for PLVA isolates from bobcats and pumas in California and Florida. Light grey shading represents regional sympatry; the geographic range of bobcats overlaps that of pumas throughout the United States. (http://www.icunredlist.org/)

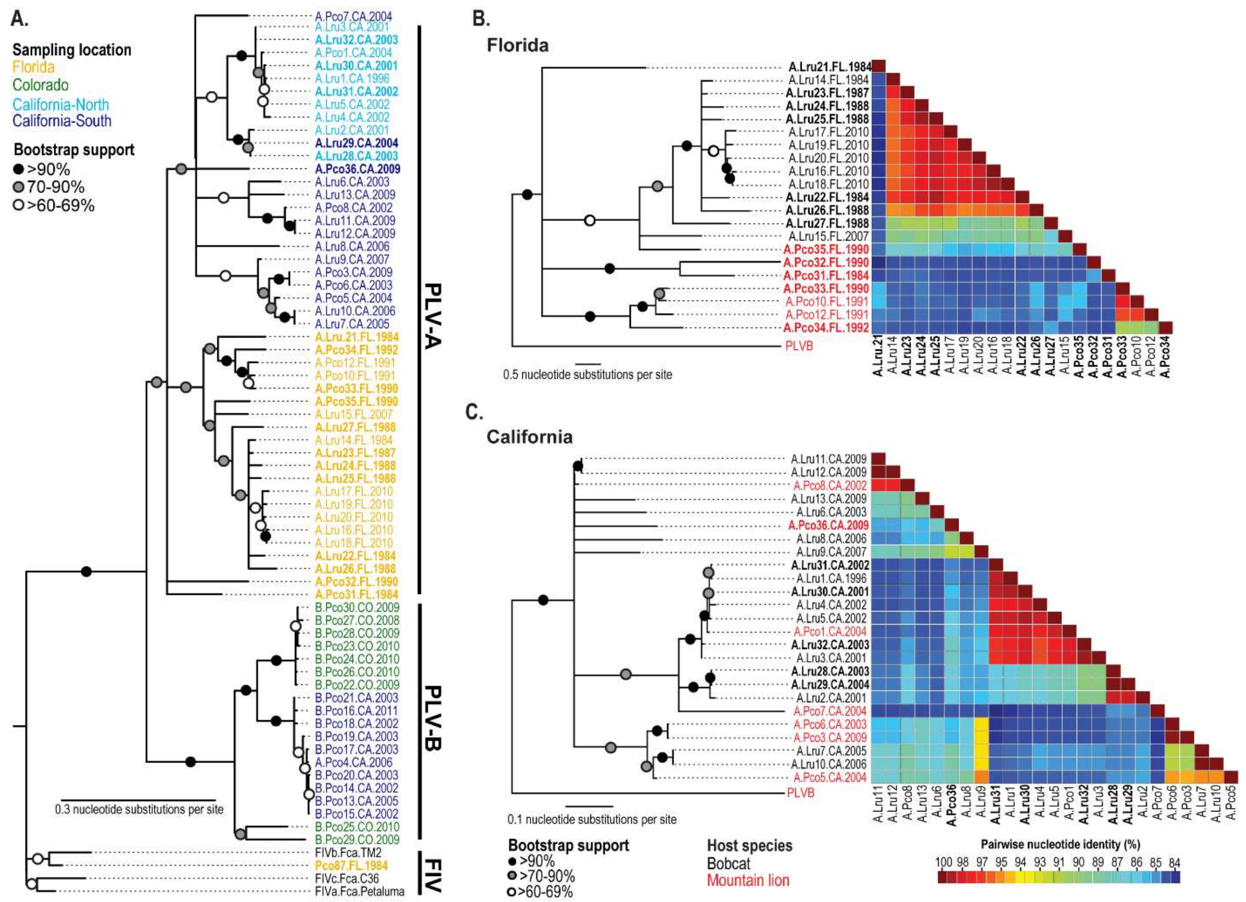


Figure 1.2: PLVA and PLVB are separated by large genetic distances and each comprises geographically associated subgroups. A. Maximum-likelihood phylogenetic tree constructed from a 474 bp region of *pol*. Isolates original to this study are highlighted by bold text. Nodes with <60% bootstrap support have been collapsed. Isolate names provide the following information: (i) PLV clade (clade A or B), (ii) host species (Lru, bobcat; Pco, puma), (iii) animal identification number (see Table 1.1), (iv) sampling location, and (v) sample year (1984 to 2011). Regional PLVA subtrees and pairwise identity matrices demonstrate different patterns of host-virus relationships. Isolates in Florida (B) tend to form well supported clusters by host species, while most California pumas isolates (C) are more closely related to sympatric bobcat isolates than to viruses from other pumas.

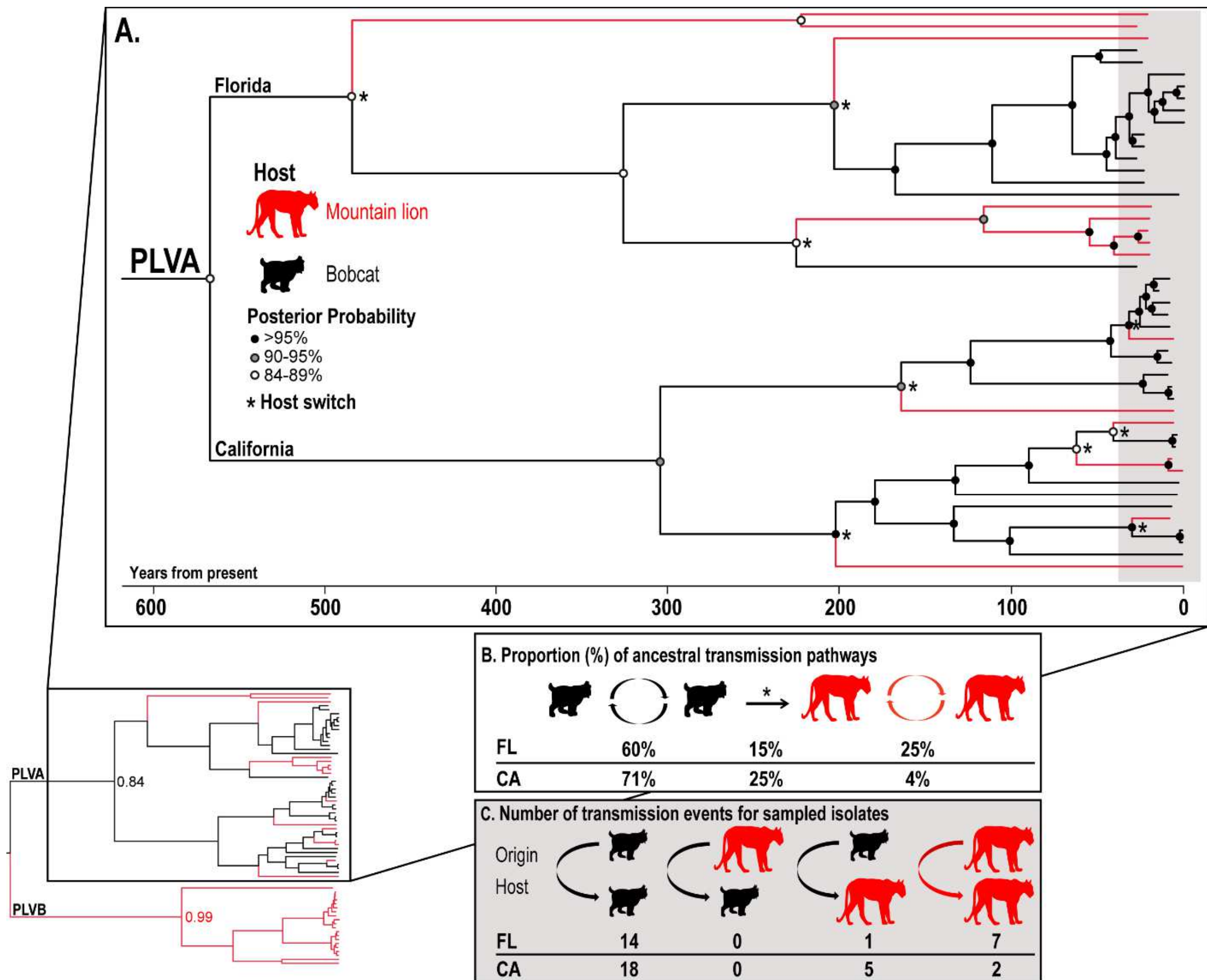


Figure 1.3: Ancestral reconstruction of host-state across the PLVA phylogeny depicts different patterns of intra- and inter-species transmission in California and Florida. A. Maximum-clade credibility tree constructed from the *pol* sequences used in Figure 1.2 depicting historic and contemporary transmission dynamics. Host-state posterior probability values relevant to transmission directionality are indicated by shaded circles at nodes. *Indicates predicted cross-species transmission events (3 in Florida, 6 in California). B. The proportion of inferred host-state transitions across the PLVA phylogeny depicts substantial bobcat to puma transmission rates at each site (15% of Florida and 25% of California transmissions). Predicted puma-to-puma transmissions occur with far greater frequency in Florida (25%) than California (4%). C. The gray shaded region of A corresponds to host states for sampled isolates depicted here in C. More sampled puma isolates were predicted to arise from intra-host transmission events in Florida (7 of 8) than California (2 of 7).

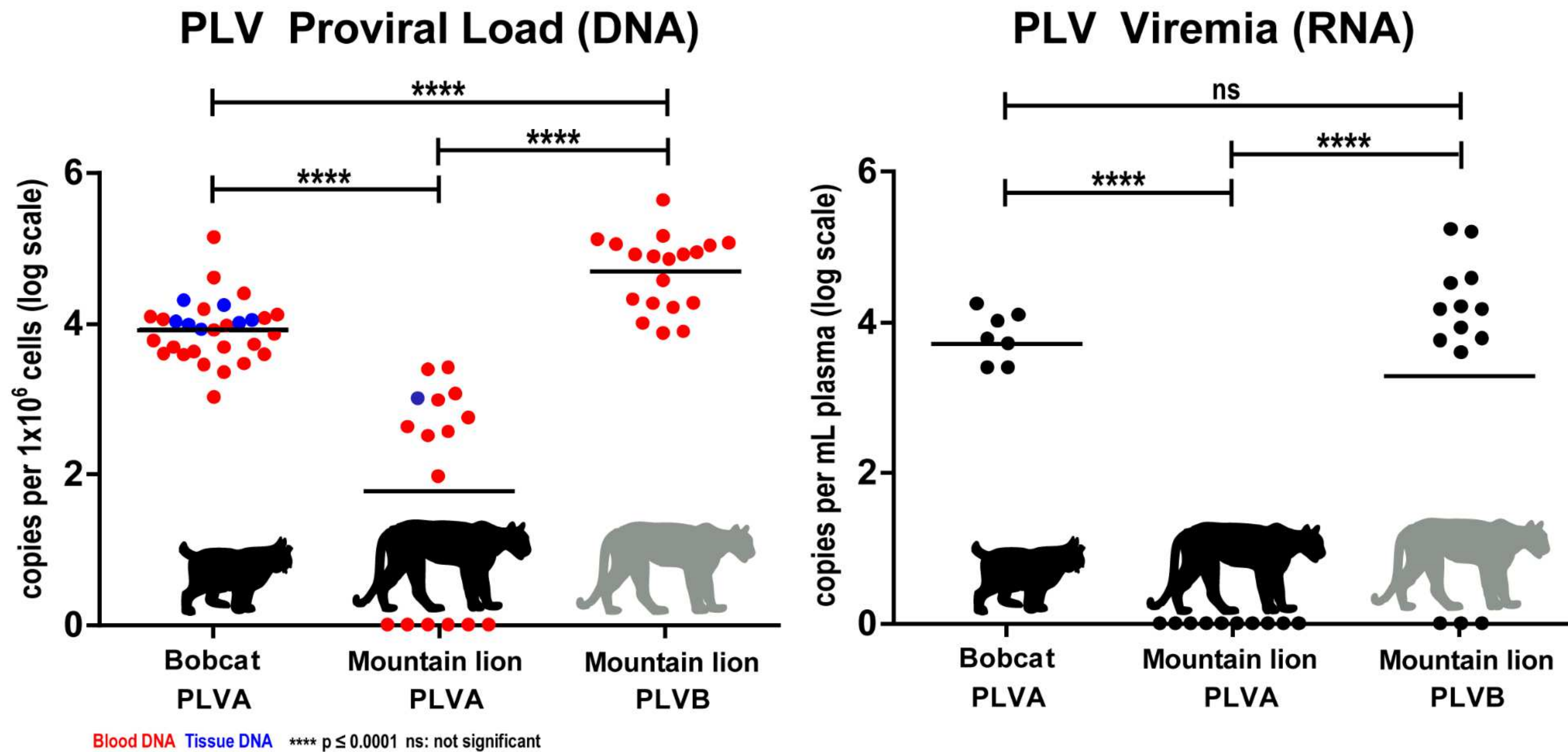


Figure 1.4: Within-host viral fitness differs significantly among hosts and among viral clades. A: Individual sample proviral loads (triplicate mean) are depicted for PLVA in bobcats (n = 30), PLVA in pumas (n = 10), and PLVB in pumas (n = 18). Lines represent mean values for each host-virus relationship. Mountaion lion PLVA proviral copy number is significantly lower than PLVA in bobcats and PLVB in pumas (both $p < 0.0001$). Puma PLVB proviral load is significantly higher than bobcat PLVA proviral load ($p < 0.0001$). No significant difference was detected between proviral loads obtained from blood (represented in red) versus tissue (represented in blue). B: Individual sample viremia values (triplicate mean) are depicted for PLVA in bobcats (n = 7), PLVA in pumas (n = 10), and PLVB in pumas (n = 14). PLVA viremia was below the limit of quantification in all puma samples. PLVA viremia in bobcats was not significantly different (ns) than mean PLVB viremia in pumas.

Proportion of isolates under episodic diversifying selection

Mountain lion PLVA: 3/6 (50%)
Mountain lion PLVB: 1/33 (3%)
Bobcat PLVA: 0/20 (0%)

Isolate	Genome region (gene)	Protein domain	Proportion of sites with dN>dS	p-value
PLVA				
A.Pco7.CA.2004	5419-6249 (vif)	-	0.03	0.020
	6551-6758 (env)	L	0.04	0.019
	8643-8772 (env)	TM	0.24	0.014
A.Pco4.CA.2006	6250-6550 (env/vif)	L	0.02	0.048
A.Pco6.CA.2003	6759-8337 (env)	L, SU, TM	0.03	0.035
PLVB				
B.Pco9.CO.2009	7122-7632 (env)	SU	0.01	0.02

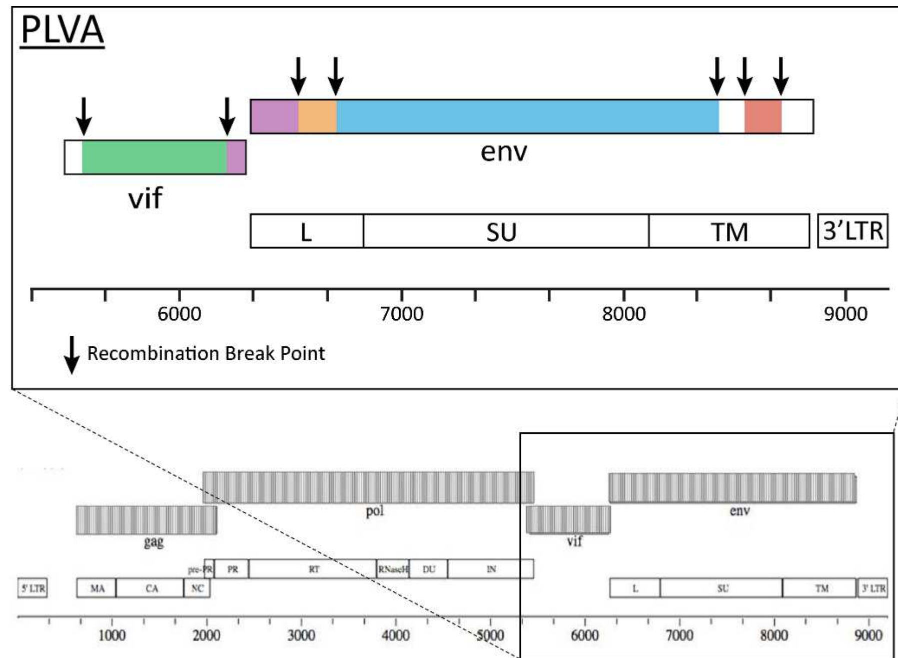


Figure 1.5: Selection results are consistent with the hypothesis that PLVA is not adapted to the puma and is therefore under strong pressure to evolve in this host. All non-recombinant regions of PLVA and PLVB genomes were analyzed by branch-site REL without *a priori* designation of isolates as originating from a bobcat or a puma. Diversifying selection was detected in 3 of 6 PLVA isolates from pumas, 0 of 20 bobcat PLVA isolates, and 1 of 33 PLVB isolates from pumas. The table on the left lists the isolates evolving under diversifying selection, the corresponding genomic regions under selection, and the proportion of sites (codons) diversifying in each region (L=leader, SU=surface, TM=transmembrane). P-values shown were derived via ANOVA and corrected for multiple tests using the Holm-Bonferroni method. The right side of the figure depicts the locations of the PLVA regions identified as evolving under episodic diversifying selection. All segments under selection were at the 3' end of the genome and included multiple loci within *env* and *vif*. Previously characterized recombination breakpoints are indicated by arrows. The full PLVA genome is outlined below for reference.

Table 1.1: Demographic information for all samples included in analyses.

PLVA - Mountain Lions			
Animal ID	Sex	Age	Location
A.Pco1.CA.2004[f,p]	F	A	CA[1]
A.Pco3.CA.2009[p]	M	A	CA[1]
A.Pco4.CA.2006[f]	M	A	CA[1]
A.Pco5.CA.2004[f,p]	F	A	CA[2]
A.Pco6.CA.2003[p]	M	A	CA[3]
A.Pco7.CA.2004[p]	F	A	CA[3]
A.Pco8.CA.2002[f,p]	F	A	CA[3]
A.Pco9.CA.2010[f]	M	A	CA[2]
A.Pco31.FL.1984[p]	F	A	FL
A.Pco32.FL.1990[f,p]	M	A	FL
A.Pco33.FL.1990[p]	F	A	FL
A.Pco34.FL.1992[p]	F	Y	FL
A.Pco35.FL.1990[p]	F	A	FL
A.Pco36.CA.2009[f,p]	M	Y	CA[3]
A.x988.CA.2009[f]	F	A	CA[3]
A.VCPF22.CA[f]	F	-	CA[1]
A.x121.CA.2002[f]	F	A	CA[3]

PLVA - Bobcats			
Animal ID	Sex	Age	Location
A.Lru1.CA.1996[f,s]	M	A	CA
A.Lru2.CA.2001[f,s,p]	M	A	CA
A.Lru3.CA.2001[s,p]	M	A	CA
A.Lru4.CA.2002[s,p]	M	A	CA
A.Lru5.CA.2002[f,s,p]	F	Y	CA
A.Lru6.CA.2003[f,s,p]	M	A	CA
A.Lru7.CA.2005[s,p]	M	A	CA
A.Lru8.CA.2006[s,p]	M	A	CA
A.Lru9CA.2007[s,p]	M	A	CA
A.Lru10.CA.2006[f,s,p]	M	A	CA
A.Lru11CA.2009[f,s,p]	M	A	CA
A.Lru12.CA.2009[s,p]	F	A	CA
A.Lru13.CA.2009[s,p]	M	A	CA
A.Lru14.FL.1984[f,s,p]	M	-	FL
A.Lru15.FL.2007[f,s,p]	F	A	FL
A.Lru16.FL.2010[s,p]	M	A	FL
A.Lru17.FL.2010[f,s,p]	M	A	FL
A.Lru18.FL.2010f[f,s,p]	M	A	FL
A.Lru19.FL.2010[f,s,p]	M	A	FL
A.Lru20.FL.2010[f,s,p]	M	A	FL
A.Lru21.FL.1984[f,p]	M	A	FL
A.Lru22.FL.1984[f,p]	F	-	FL
A.Lru23.FL.1987[f,p]	M	-	FL
A.Lru24.FL.1988[f,p]	M	-	FL
A.Lru25.FL.1988[f,p]	M	-	FL
A.Lru26.FL.1988[f,p]	F	-	FL
A.Lru27.FL.1988[f,p]	M	-	FL
A.Lru28.CA.2003[f,p]	M	A	CA
A.Lru29.CA.2004[f,p]	M	A	CA
A.Lru30.CA.2001[f,p]	M	A	CA
A.Lru31.CA.2002[f,p]	F	A	CA
A.Lru32.CA.2003[f,p]	F	Y	CA
A.x202.CA.2001[f]	M	A	CA
A.x152.CA.2003[f]	F	Y	CA
A.x154.CA.2003[f]	M	A	CA
A.x165.CA.2004[f]	M	A	CA
A.x195.CA.2001[f]	M	A	CA
A.x202.CA.2001[f]	M	A	CA
A.x215.CA.2002[f]	M	A	CA

PLVB - Mountain Lions			
Animal ID	Sex	Age	Location
B.x369r1.C0[f]	F	A	CO
B.x427r1.CO.2010[f]	F	A	CO
B.x1120.CO.2010[f]	F	A	CO
B.x1131.CO.2010[f]	M	A	CO
B.x1342r1.CO.2010[f]	F	A	CO
B.x1372r1.CO.2011[f]	F	A	CO
B.x1389.CO.2011[f]	F	A	CO
B.x1402r1.CO.2012[f]	M	A	CO
B.x1403.CO.2011[f]	F	A	CO
B.x1406.CO.2011[f]	M	A	CO
B.x1407.CO.2011[f]	F	A	CO
B.x1555r1.CO.2011[f]	M	A	CO
B.x1643.CO.2011[f]	F	A	CO
B.x1647.CO.2012[f]	M	Y	CO
B.Pco2.CA.2002[s]	M	A	CA[1]
B.Pco4.CA.2006[s,p]	M	A	CA[3]
B.Pco13.CA.2005[s,p]	F	A	CA[3]
B.Pco14.CA.2002[f,s,p]	F	A	CA[3]
B.Pco15.CA.2002[s,p]	F	A	CA[3]
B.Pco16.CA.2011[s,p]	M	-	CA[3]
B.Pco17.CA.2003[s,p]	M	A	CA[3]
B.Pco18.CA.2002[f,s,p]	F	A	CA[3]
B.Pco19.CA.2003[s,p]	F	A	CA[3]
B.Pco20.CA.2003[s,p]	F	A	CA[3]
B.Pco21.CA.2003[f,s,p]	F	A	CA[3]
B.Pco22.CO.2009[s,p]	M	-	CO
B.Pco23.CO.2010[s,p]	F	A	CO
B.Pco24.CO.2010[s,p]	F	Y	CO
B.Pco25.CO.2010[s,p]	F	A	CO
B.Pco26.CO.2010[f,s,p]	F	A	CO
B.Pco27.CO.2008[s,p]	F	A	CO
B.Pco28.CO.2009[s,p]	M	A	CO
B.Pco29.CO.2009[s,p]	F	A	CO
B.Pco30.CO.2009[s,p]	M	A	CO
B.Pco603.MT.2001[s]	-	-	MT
B.Pco604.MT.2002[s]	-	-	MT
B.Pco605.MT.2001[s]	-	-	MT
B.Pco606.MT.2001[s]	-	-	MT
B.Pco607.Yel.1992[s]	-	-	WY
B.Pco608.Yel.1992[s]	-	-	WY
B.Pco609.WY.2004[s]	-	-	WY
B.Pco610.WY.2003[s]	-	-	WY
B.Pco611.Yel.2001[s]	-	-	WY
B.Pco612.Yel.2001[s]	-	-	WY
B.Pco613.WY.2001[s]	-	-	WY
B.Pco.615.BC[s]	-	-	BC
B.PLV1695.BC.1995[s]	-	-	BC

FIV - Mountain Lions			
Animal ID	Sex	Age	Location
Pco87.FL.1984*	M	A	FL

[f] Fitness analysis
[s] Selection analysis
[p] Phylogenetic analysis
M=male; F=female
A=adult; Y=yearling

CA populations:
[1] Santa Monica
[2] Santa Ana
[3] Peninsular

Table 1.2: GenBank accession numbers for published PLV sequences. New isolates from this study are indicated by bold text.

PLVA Isolate	GenBank Accession #	PLVB Isolate	GenBank Accession #
A.Lru1.CA.1996	KF906143	B.Pco4.CA.2006	KF906182
A.Lru2.CA.2001	KF906144	B.Pco13.CA.2005	KF906180
A.Lru3.CA.2001	KF906145	B.Pco14.CA.2002	KF906182
A.Lru4.CA.2002	KF906146	B.Pco15.CA.2002	KF906183
A.Lru5.CA.2002	KF906147	B.Pco16.CA.2011	KF906193
A.Lru6.CA.2003	KF906148	B.Pco17.CA.2003	KF906175
A.Lru7.CA.2005	KF906149	B.Pco18.CA.2002	KF906176
A.Lru8.CA.2006	KF906150	B.Pco19.CA.2003	KF906177
A.Lru9.CA.2007	KF906151	B.Pco20.CA.2003	KF906178
A.Lru10.CA.2006	KF906152	B.Pco21.CA.2003	KF906179
A.Lru11CA.2009	KF906153	B.Pco22.CO.2000	KF906188
A.Lru12.CA.2009	KF906154	B.Pco23.CO.2010	KF906189
A.Lru13.CA.2009	KF906155	B.Pco24.CO.2010	KF906190
A.Lru14.FL.1984	KF906156	B.Pco25.CO.2010	KF906191
A.Lru15.FL.2007	KF906157	B.Pco26.CO.2010	KF906192
A.Lru16.FL.2010	KF906158	B.Pco27.CO.2008	KF906194
A.Lru17.FL.2010	KF906159	B.Pco28.CO.2009	KF906184
A.Lru18.FL.2010	KF906160	B.Pco29.CO.2009	KF906186
A.Lru19.FL.2010	KF906161	B.Pco30.CO.2009	KF906187
A.Lru20.FL.2010	KF906162	B.Pco605.MT.2001	EF455605
A.Lru21.FL.1984	**KX899911**	B.Pco604.MT.2002	EF455604
A.Lru22.FL.1984	**KX899912**	B.Pco606.MT.2001	EF455606
A.Lru23.FL.1987	**KX899913**	B.Pco607.Yel.1992	EF455607
A.Lru24.FL.1988	**KX899914**	B.Pco608.Yel.1992	EF455608
A.Lru25.FL.1988	**KX899915**	B.Pco611.Yel.2001	EF455611
A.Lru26.FL.1988	**KX899916**	B.Pco612.Yel.2001	EF455612
A.Lru27.FL.1988	**KX899917**	B.Pco603.MT.2001	EF455603
A.Lru28.CA.2003	**KX899905**	B.Pco609.WY.2004	EF455609
A.Lru29.CA.2004	**KX899906**	B.Pco610.WY.2003	EF455610
A.Lru30.CA.2001	**KX899907**	B.Pco613.WY.2001	EF455613
A.Lru31.CA.2002	**KX899908**	B.PLV1695.BC.1995	PLV-1695
A.Lru32.CA.2003	**KX899909**		
A.Pco1.CA.2004	KF906163	FIV Isolate	GenBank Accession #
A.Pco3.CA.2009	KF906165	**Pco87.FL.1984**	**KX899923**
A.Pco5.CA.2004	KF906167		
A.Pco6.CA.2003	KF906168		
A.Pco7.CA.2004	KF906169		
A.Pco8.CA.2002	KF906170		
A.Pco10.FL.1991	KF906172		
A.Pco12.FL.1991	KF906174		
A.Pco31.FL.1984	**KX899918**		
A.Pco32.FL.1990	**KX899919**		
A.Pco33.FL.1990	**KX899920**		
A.Pco34.FL.1992	**KX899921**		
A.Pco35.FL.1990	**KX899922**		
A.Pco36.CA.2009	**KX899910**		

Table 1.3A: G to A substitution rates across a 474 bp segment of PLVA *pol* do not vary by host species. FL=Florida; CA=California; Pco=*Puma concolor*; Lru=*Lynx rufus*.

PLVA Subgroup	Original base	Mean substitutions per animal			
		G	A	T	C
FL Pco	**G**		3.0	0.3	0.0
	A	5.5		5.1	1.5
	T	1.0	3.8		3.4
	C	0.4	1.5	1.3	
FL Lru	**G**		3.2	0.1	0.0
	A	3.1		1.2	0.3
	T	0.3	2.1		2.6
	C	0.1	0.4	2.2	
CA Pco	**G**		5.1	0.3	0.0
	A	10.9		3.9	1.1
	T	0.3	3.7		6.9
	C	0.1	0.1	1.6	
CA Lru	**G**		5.1	0.1	0.1
	A	10.0		5.7	2.8
	T	1.1	2.1		6.6
	C	0.1	0.9	2.4	

Table 1.3B: G/A variability was identified at18%of sites within this *pol* segment of Florida isolates but both host species had the same proportions of G and A nucleotides.

	Host (n)	
	Bobcat (14)	Panther (9)
Total no. of G (%)	297 (26.5)	192 (26.2)
Total no. of A (%)	820 (73.4)	540 (73.7)
% other	0.1	0.1
Mean G/animal	21.2	21.3
Mean A/animal	58.6	60

CHAPTER TWO

Genetic rescue of the Florida panther (*Puma concolor coryi*) correlates with exponential spread of extraordinary stable feline immunodeficiency virus

Synopsis

Wildlife translocations are an important conservation tool and a commonly used strategy in endangered species recovery programs. Although translocation efforts require detailed assessment of risk, the impact on parasite distribution has remained a low priority. This is despite the observation that actions that alter host-parasite distributions can drive evolution, or introduce new parasites to previously sequestered populations, resulting in a heightened threat of infectious disease as a cause of species' extinction. Here we use a contemporary approach to amplify viral sequences from remnant biological samples and characterize a previously undocumented impact of successful supplementation efforts to rescue the endangered Florida panther (*Puma concolor coryi*). Our efforts reveal transmission and evolution of feline immunodeficiency virus (FIV) during translocation of pumas from Texas to Florida, resulting in a shift in the predominant circulating subtype from FIVlru to FIVpco. We used coalescent theory to estimate viral demography across time and show an exponential increase in the effective population size of FIVpco coincident with expansion of the panther population. Additionally, we show that FIVpco isolates from Texas are basal to all isolates from Florida. Interestingly, FIVpco infections of Florida panthers and Texas pumas demonstrate exceptionally low interhost divergence relative to any other lentiviruses evaluated. Low host genomic diversity and lack of continued introgressions of FIVpco infected individuals into the population may underlie the

surprising lack of apparent FIVpco evolution over two decades. We conclude that the dominant FIV lineage in the contemporary Florida panther originated from Texas and disseminated following translocations of infected pumas, and that infectious disease risks should be carefully considered during conservation efforts involving translocations. Further, viral evolutionary dynamics may be significantly altered by ecological niche, host diversity, and connectivity between host populations.

Importance

Wildlife translocations have become a common tool for conservation biologists in response to anthropogenic threats to biodiversity. Although translocations involve detailed health assessments, few empirical studies have thoroughly investigated the impacts of such efforts on the persistence and evolution of subclinical microparasites such as rapidly evolving RNA viruses. We investigated the consequences of genetic rescue of the iconic Florida panther on lentiviral infection dynamics. Our findings support a founder population of translocated Texas pumas as the source of modern feline immunodeficiency virus (FIV) in the panther. We show that modern panther FIV originating from Texas replaced a poorly adapted historic FIV subtype, and further report on low viral genetic variation in the isolated panther population. These findings provide empirical evidence that translocations impact subclinical infections in unpredictable ways that could promote viral evolution and disease emergence in threatened wildlife populations.

Introduction

Anthropogenic introduction of a parasite to a geographical region or host species outside of its natural range has been termed 'pathogen pollution' and is increasingly recognized as an important driver of disease emergence (189-191). Multiple mechanisms underlie pathogen pollution, chief among which are wildlife conservation efforts involving translocation (192, 193). In the face of shrinking habitat and expansive urbanization, active management strategies such as translocation have become pervasive tools to prevent extinction of threatened species (194, 195). Common goals of translocations include establishment of new populations, reintroduction of a species to historic range, movement of species from regions of human development or conflict, and supplementation of existing populations to enhance population size, genetic diversity, or both (196). Translocations have historic precedent, and infectious disease has only recently been recognized as an important associated threat (192). Important examples of host-parasite cointroductions include the translocation of rabid raccoons from Florida to Virginia in the late 1970s and early 1980s (197), the release of captive-bred plains bison harboring bovine tuberculosis and brucellosis into Wood Bison National Park (198), and human-mediated introduction and dissemination of the causative agent of salmonid whirling disease across the United States (199). While these instances exemplify the magnitude of potential risk, indirect impacts of translocated parasites on extant species can be subtle and may become evident only with time and concurrent ecological change (193). Apart from examples with obvious impacts on disease emergence or reemergence, broad and long-term consequences of host-parasite cointroductions are rarely studied in detail. The evolutionary response of a parasite to selection pressures imposed by a naïve host in a new geographic region is a particularly important, yet understudied determinant of community dynamics and ecosystem health.

The Florida panther represents an apparent and widely documented translocation success story (200). Once widely ranging throughout the southeastern United States, this iconic subspecies of puma (*Puma concolor coryi*) was isolated to a rapidly urbanizing region of southern Florida following decades of persecution and habitat destruction (201, 202). After nearly a century of bounty hunting, the state of Florida declared the panther a protected species in 1958 (203). Despite this designation, the population continued to decline, and the Florida panther was listed as federally endangered in 1967 (204, 205). In the 1980s, in effort to assess northern Florida habitat suitability for recolonization of historic range, conservation biologists translocated several pumas from Texas to northern Florida (202). Pumas released preliminarily as a trial were later removed from northern Florida. Meanwhile, numbers and genetics of the remaining panthers in the shrinking primary range to the south continued to diminish.

By the early 1990s, panthers were reduced to a dwindling population of less than 25 adults with a projected time to extinction of less than two decades (169, 206). This prompted the translocation of 8 female pumas from Texas to Florida in effort to increase genetic diversity and stabilize the population. Subsequently, survival of kittens and females increased, heterozygosity doubled, fitness parameters improved, and the population has since expanded to an estimated 120-230 individuals (207-209). This outcome has provided fundamental support for intensive management as a useful and necessary conservation tool (210, 211). The initial decision to pursue genetic introgression through translocation, however, was highly controversial, with compelling arguments on both sides (202, 209, 212). Many arguments against translocation, such as those citing an inability of hybrid panthers to recolonize and survive in historic range (212), have since been laid to rest by the geographic and demographic expansion that has followed

(169). While genetic rescue efforts have not guaranteed the persistence of panther, they have undoubtedly prolonged its presence (200, 209).

Puma are host to several putatively nonpathogenic retroviruses, including the lentivirus feline immunodeficiency virus (FIV). Two subtypes of FIV have been reported in the puma; the host-adapted FIVpco (also known as puma lentivirus B (PLVB)), and the bobcat-adapted spillover virus, known as FIVlru (PLVA) (56, 114). Previous studies showed a predominance of FIVlru in the Florida panther prior to translocation of Texas pumas (114, 120, 142). In contrast, FIVpco is more prevalent in all other sampled puma populations (120, 134). The impact of the translocated Texas pumas on circulating FIV subtypes has not been previously described. We therefore initiated a study to investigate the dynamics of FIV infection in the Florida panther pre- and post-Texas puma translocation. Here we document in detail: (1) translocation of multiple Texas pumas infected with FIVpco; (2) a shift from predominant subtype FIVlru to subtype FIVpco following translocation; (3) an exponential increase in FIVpco infections concurrent with population expansion; and (4) low genetic variation of FIVpco among and between Florida panthers and Texas pumas across a 23-year sampling period. The low genetic diversity of this viral lineage is highly unusual and may relate to the unique demographics and isolated nature of this population. This work provides rare empirical evidence of altered viral ecology as a direct effect of wildlife translocation. It is the first to thoroughly characterize viral evolutionary processes concurrent with genetic introgression of the host and offers an unusual opportunity to investigate the impacts of human-mediated dissemination of a subclinical parasite in a naïve host population.

Results

Historic translocations impacted FIV subtype dynamics in the Florida panther.

To investigate FIV in the Florida panther pre- and post-Texas puma translocation, we adapted a tiled amplicon approach to whole genome sequencing from Quick et al. 2017 using multiplex PCR and subtype specific primers for targeted enrichment of low copy number viral DNA (213). Our analysis revealed a remarkable shift in occurrence of circulating FIV subtypes that coincided temporally with the translocation of Texas pumas to Florida (Figure 2.1). Previously published FIV isolates from Florida panthers were exclusively of FIVlru subtype recovered from samples collected in the late 1980s and early 1990s (142). We sequenced the first and the two earliest FIVpco isolates recovered from Florida to date (May and June 1988), which originated from Texas pumas released in northern Florida in the 1980s in effort to assess habitat suitability for potential recolonization beyond the modern southern Florida range. An additional isolate of FIVpco was recovered from a relic Florida panther sample collected in August of 1988. Of the 8 females from Texas that comprised the well-documented translocation in 1995, we show that at least 2 were positive for FIVpco, and a 3rd had a detectable FIVpco infection by 1997 (Figure 2.1). We calculated the relative risk of a sample testing positive for FIVpco after 1995 and found that Florida panthers sampled after the Texas introductions were 3.8 times more likely to test positive for FIVpco as compared to those sampled prior to 1995 (95% CI 1.8-8.0, $p=0.0004$). We then used coalescent theory to estimate the effective number of FIVpco infections across time based on whole genome sequence data (214) which revealed an exponential increase in infections between ~2000 and 2002, corresponding to exponential expansion of the Florida panther population (Figure 2.2A and 2.2B). Interestingly, FIVlru infections fell below the level of detection after 1992 (Figure 2.1), despite previous findings that

intraspecific panther transmission was strongly supported by phylogenetic analysis (114). Collectively, these results demonstrate a sharp increase in FIVpco infections concurrent with the apparent disappearance of FIVlru in the Florida panther, and provide evidence for Texas origin of FIVpco in Florida.

Genetic variation of FIVpco in the Florida panther is remarkably low.

To investigate the evolution of FIVpco in the Florida panther in the context of Texas translocations, we constructed FIVpco consensus sequences (n=64, Table 2.1) from overlapping fragments within a tiled amplicon framework (213). We report strikingly low interhost diversity of FIVpco in the panther population, demonstrated by a mean pairwise nucleotide identity of ~99% for nearly whole genome sequences spanning over two decades of sample collection (Table 2.2). Across the 23-year sampling period (1983-2011), divergence between isolates from translocated Texas pumas and Florida panthers is extraordinarily low (pairwise identity 98.8-100% excluding missing data sites) (Table 2.2). Similarly, low divergence is reported across isolates from historic Florida panthers and those from modern Florida-Texas hybrid animals, also included in Table 2.2. The genetic stability of FIVpco and previously reported relative fitness advantage over FIVlru (114) likely contributed to an increase in highly homologous FIVpco infections and a circulating FIV subtype shift mediated by multiple ecological and molecular determinants of infection dynamics through time (Figure 2.3).

FIVpco isolates from Texas and Florida comprise a single lineage.

To further investigate relationships between and among FIVpco isolates from Texas and Florida, consensus sequences were subjected to phylogenetic analyses. Due to short branch lengths derived from highly homologous sequences, a Bayesian maximum clade credibility tree was converted to a cladogram for improved visualization (Figure 2.4). Two isolates from

translocated Texas pumas (TX104 and TX106) are basal to all other sequences from Florida. TX104 and TX106 were translocated as a pair to Fakahatchee Strand State Preserve in 1995 (Figure 2.5). TX106 was positive for FIVpco in 1995, while TX104 samples from 1995 consistently tested negative. A sample collected during recapture of TX104 in 1997, however, yielded a positive result with a consensus sequence highly homologous (99% pairwise identity) to that from TX106. FIVpco was additionally recovered from a third breeding female translocated to Everglades National Park (TX105) in 1995 (Figure 2.5). Early isolates (1988-1994) recovered from 'trial' release Texas pumas in the northern historic range share high sequence homology with those of Florida origin and do not comprise a separate lineage (Figure 2.4).

The genetics of the Florida panther population have been shaped by numerous anthropogenic introductions in addition to those from Texas, including escape and/or release of captive animals from Big Cypress Seminole Indian Reservation and a privately-owned collection housed at Piper's Everglades Wonder Garden (169). We therefore sought to identify unique FIVpco lineages corresponding to the genetic ancestry of the host. Somewhat surprisingly, distinct clades of FIVpco corresponding to panther ancestry were not identified (Figure 2.4). Rather all FIVpco sequences were highly homologous, providing further evidence of a point source introduction of FIVpco from Texas. To investigate the frequency of inferred vertical transmission, we sought to identify phylogenetic relatedness of viruses isolated from dam-offspring pairs. Putative maternal transmission was identified in two cases (FP124 to FP126 and FP161 to K27); transmission divergence was greater for dam-offspring pair FP113 and FP171, suggesting intermediate transmission(s) rather than direct dam to offspring infection (Figure 2.4). While many of the infected panthers had morphologic defects including tail kinks, thoracic

cowlicks, atrial septal defects, and cryptorchidism, we did not find phylogenetic evidence for a correlation between host phenotype and FIVpco genotype (Figure 2.4).

FIVpco from Florida and Texas is genetically distinct from all other puma FIVs.

Following phylogenetic analysis of FIVpco isolates from Florida, we sought to compare this unique FIV lineage to other puma FIVs across North America. Using sequences obtained from GenBank and a representative subset of the sequences from this study, we constructed a Bayesian maximum clade credibility tree from ~480 bp within the reverse transcriptase (RT) region of *pol*, a highly conserved region that has classically been used to characterize FIV and other lentiviruses (Figure 2.6). The RT-pol phylogeny illustrates that all previously published sequences of FIV from Florida panthers prior to the 1995 translocation are of subtype FIVlru. Sequences from the current study share high homology with two isolates derived from zoos in Texas (Pco-28 and Pco-733), comprising a single lineage distinct from others in the Americas. Branch lengths are notably short within the Florida-Texas clade, as well as within the clade comprising southern California, highlighting the low genetic variation of FIV in Florida and similarly high sequence homology in other isolated populations with restricted gene flow, as has been reported in southern California (171).

Discussion

Few empirical studies provide managers with detailed findings regarding the complexities of translocation (210). The Florida panther represents a rare opportunity to document the role of translocations in dissemination, persistence and evolution of an apparently nonpathogenic virus. Based on our findings, three pumas released in northern Florida between 1988 and 1994 were carriers of FIVpco, in addition to at least two that were translocated to the

primary southern range in 1995. FIVpco sequences from Texas pumas released in 1988 represent the oldest isolates recovered from this region and share high sequence homology with isolates subsequently recovered from Florida panthers. These findings, combined with an apparent absence of FIVpco in Florida prior to 1988, strongly suggest translocated Texas pumas as the introduction source of FIVpco in the Florida panther. While it is notable that pumas released in northern Florida in the 1980s are not known to have been in contact with panthers inhabiting peninsular Florida, the probability of contact, and therefore the risk of FIVpco transmission, increases with the tendency for long-distance dispersal by translocated subadult males looking to breed, return to native territory, or establish a new home range (215). Further, the genomes of all FIVpco isolates from Florida are highly homologous to those from Texas, providing strong evidence for a single lineage and supporting the Texas translocations as a point source origin.

We further document the replacement of subtype FIVlru by FIVpco following translocation of infected Texas pumas, a remarkable and unequivocal finding. Previous analyses revealed that FIVlru is a primary bobcat virus, with most puma infections resulting from cross-species transmission (56, 114). As an exception to this norm, however, we previously reported strong evidence for a chain of FIVlru infections in the Florida panther in the late 1980s and early 1990s (114). It is therefore interesting that FIVlru could not be detected in 264 samples collected from Florida panthers after 1992. The apparent disappearance of FIVlru from the panther population was followed by an exponential increase in the effective number of FIVpco infections after translocation of infected Texas pumas. We previously reported low fitness of FIVlru in the puma as compared to FIVpco (114), and therefore propose a competitive fitness advantage of FIVpco as a theoretical explanation for the observed shift in FIV subtype.

Whole genome sequencing of FIVpco isolates revealed exceptionally low diversity across Florida and Texas, a surprising finding given that a previous study reported diversification of eight FIVpco lineages in the northern Rocky Mountains occurring within the last 20 to 80 years (216). Moreover, diversity among other FIVpco lineages is similar to that reported for other FIVs, and evidence for ongoing positive selection and adaptation was reported in a recent genome-wide analysis of FIVpco in the puma (120). The limited genetic variation of FIVpco in Florida is thus unusual and could be related in part to the isolation of the panther and the 'closed' status of the population. Among 'open' populations with contiguous habitat, FIVpco genetic variation can be partially attributed to recombination events arising from coinfections following dispersal of infected animals (217). Because new members do not naturally enter and disperse from Florida, viral recombination as a source of genetic variation is limited by the absence of divergent FIV lineages from other geographic regions. This premise is supported by a study of FIVpco in the Snowy Range of Wyoming, which concluded that endemic FIV in pumas evolves slowly in absence of coinfections and recombination (218). A similar pattern of relatively low FIVpco genetic variation is seen in southern California, where urbanization is comparable to Florida and gene flow of the puma is restricted (171).

The genetic stability of FIVpco in Florida could additionally be promoted by intrinsic interactions between the virus and the innate immune system of the host. It is notable that copy number of FIVpco in the panther, while significantly high compared to that of FIVlru, is inconsistent with reported high replication rates of other nonpathogenic host-adapted lentiviruses, such as SIV (219). Furthermore, we have reported viremia levels below the lower limit of detection in the face of consistent detection of FIVpco proviral DNA. Low level replication combined with the apparent absence of robust immunological activation suggests

latent reservoirs of stably integrated viral DNA (220). Alternate replication mechanisms such as clonal expansion of infected cells or cell-to-cell spread could further contribute to mutational robustness by limiting error-prone reverse transcription of the viral genome (220, 221). The relatively large proportion of infected panthers suggests frequent vertical transmission, which could in part explain the observed exponential increase in infections in absence of evidence for epidemic parameters such as an enhanced replication rate. High rates of vertical transmission have indeed been reported for endemic FIVpco; one study reported infection of more than 50% of cubs born to infected dams (218). Vertical transmission provides a simple putative mechanism underlying the observed increase in FIVpco infections concurrent with demographic recovery of the panther, a pattern that has also been reported for pumas in the Rocky Mountains (216). While the mechanistic details remain theoretical, the relative risk associated with translocation is striking and strongly supports a role for infected Texas pumas as primary drivers of FIVpco spread through Florida.

Outside of the sequences derived from Texas founders in this study, only two additional isolates from Texas have been partially sequenced: Pco-28 from a zoo in San Antonio and Pco-733 from a zoo in Houston (142). These isolates share high sequence homology with those from this study, a finding that provides additional evidence for Texas origin. We additionally screened 12 tissue samples (skeletal muscle and heart) from contemporary Texas pumas but did not detect FIV in any available tissues. Additional analysis of Texas FIVpco is warranted to determine the phylogenetic relationships between contemporary Texas isolates, those from Florida, and those from other regions with contiguous habitat to other circulating FIVpco lineages.

Collectively, our findings document a shift in circulating FIV subtype following translocation of Texas pumas infected with FIVpco. This work represents rare documentation of

human-mediated alterations in viral ecology and is the first study to thoroughly investigate the evolution of a virus concurrent with genetic introgression of the host. Further, we report on evolutionary patterns that diverge from the expected adaptive processes typical of lentiviruses. This contribution provides a distinct example of the potential impacts of translocation on extant parasite communities and highlights the deterministic influence of intensive management on species interactions and persistence. We show that management actions drive changes in parasite occurrence and distribution and emphasize the difficulty in accurate prediction of the long-term consequences of anthropogenic movement of parasites with their hosts. We conclude that while the benefits of genetic rescue clearly outweigh the costs in the case of the Florida panther, dynamic alterations in host-pathogen relationships should be expected in response to translocation, as illustrated by the cautionary tale of Florida panther FIV.

Methods

Sample collection and nuclei acid extraction

Blood and tissue samples were collected from Florida panthers and translocated Texas pumas between 1988 and 2011. Capture of free-ranging animals involved the use of baited cage traps or trained tracking hounds, as previously described (146). Capture and handling protocols followed approved Institutional Animal Care and Use Committee guidelines, as well as applicable local government regulations. Additional samples were opportunistically collected during routine postmortem examinations by local government agencies. Aliquots of blood and tissue samples were sent to Colorado State University for analyses described below. Multiple tissue types were analyzed as available with priority assigned to lymphoid organs (i.e. spleen, followed by lymph node). DNA was extracted from tissue, whole blood, or peripheral blood

mononuclear cells (PBMCs) using an adapted version of the DNeasy Blood and Tissue protocol (Qiagen Inc., Valencia, CA.) Tissues were homogenized using the benchtop FastPrep-24 cell and tissue homogenizer (MP Biomedicals, LLC., Santa Ana, CA.). Blood samples were incubated in lysis buffer at 56℃ overnight. 264 archival samples were screened for FIV using a multiplex PCR protocol as described below. Collection date, location, and host demographic information for each sample selected for viral genotyping (Table 2.1). Capture locations for FIV-infected panthers and translocated Texas pumas are displayed in Figure 2.5.

Detection and sequencing of FIVpco

FIVpco was sequenced from 58 Florida panthers and 6 Texas pumas using a method adapted from Quick et al (213). Briefly, 62 primers (31 pairs) spanning the coding regions of the FIVpco genome were designed for amplification of ~400 bp amplicons using Primal Scheme (213). Primer sequences are available upon request. Extracted DNA was subjected to two multiplex PCR reactions using Q5 High-Fidelity DNA Polymerase Enzyme (New England Biolabs Inc., Ipswith, MA) and touch-down cycling conditions. Specifically, thermocycling was initiated at 68℃ and decreased by 0.5℃ for 6 cycles, followed by 34 additional cycles at 65℃ for a total of 40 cycles. Negative samples and no template reactions were included and were consistently negative. Amplicons were labeled using Nextflex Dual-Indexed Barcodes and a library was prepared using Nextflex Rapid DNA-Seq Library Prep Kit (Bioo Scientific Inc., Austin, TX). Products were then sequenced on an Illumina MiSeq using the MiSeq reagent kit v2 (500 cycles). Paired fastq reads of ~250bp with ~50bp overlap were analyzed as follows: (1) trimming of indexes, primers, low quality (phred <20), and short reads (<50 bp) using Cutadapt (222), (2) mapping of trimmed reads to a multiple reference index using Bowtie2 (223), (3) conversion of .sam files to .bam files using Samtools (224), and (4) viewing of sorted .bam files

in Geneious (155). The multiple reference index included all FIVpco whole genome sequences generated in our laboratory previously and/or all those available from GenBank (42 total sequences including 7 unpublished and GenBank accession numbers EF455603-EF455615, DQ192583, KF906185-KF906174). All reads were then mapped to the single 'best-fit' reference with the highest number of mapped reads to maximize genome-wide coverage. Reads with low mapping coverage (<75%) were additionally mapped to an FIVlru reference sequence derived from a Florida bobcat. In all cases, mapping coverage to the FIVlru index was poor (<20%). Consensus sequences were generated from mapped reads using the highest quality parameter in Geneious as a threshold. 'N' was assigned to sites with coverage <2 to represent missing data.

Detection of FIVlru

Primers spanning the coding regions of the FIVlru genome (58 total/29 pairs) were additionally generated using Primal Scheme with parameters as described above. 264 puma blood and tissue samples were subjected to at least one of two multiplex PCR reactions to screen for FIVlru. Previously sequenced positive control samples from Florida bobcats were used to confirm assay detection. Positive control samples consistently generated products of the expected size (~400 bp), as confirmed by gel electrophoresis. Reactions produced weak bands for 2 known positive, partially sequenced isolates from Florida panthers (GenBank accession numbers KX899918 and KX899922) (114). However, of 264 panther samples screened, no newly discovered FIVlru infections were detected. Reactions for the 4 partially sequenced isolates failed to meet minimum concentration requirements for library preparation despite efforts at increasing template DNA, doubling the amount of Q5 polymerase, increasing cycle number, and touch-down cycling at lower annealing temperatures. Negative samples and no template control reactions were consistently negative.

Phylogenetic analysis

Codon alignments of consensus sequences were constructed using the Clustal W algorithm and adjusted manually in MEGA (148). Multiple sequence alignments were partitioned into open reading frames and screened for recombination using GARD through the Datamonkey interface of the HyPhy package (225). Model selection was performed independently for each alignment subject to phylogenetic analysis using jModelTest (226). To examine ancestral relationships within Florida, a genome alignment of all newly sequenced Florida and Texas FIVpco isolates was subjected to Bayesian analysis performed using the MrBayes 3.2.6 Geneious plugin (227) with gamma-distributed rate variation and the HKY85+G substitution model. Missing data ('N') was included to maximize the number of sites sampled. Four heated chains of 1,100,000 chain length were run with a subsampling frequency of 200 with the initial 10% discarded as burn-in. An isolate recovered from Vancouver Island (PLV-1695) was used an outgroup (accession number DQ192583).

To estimate changes in effective number of infections through time as approximated by effective population size of the virus (216), we examined the distribution of coalescent events using a Bayesian skyline plot constructed in BEAST 2 (228). Effective population size was inferred for 5 intervals based on coalescence under the HKY+G model with 4 rate categories and an uncorrelated relaxed molecular clock with lognormal rate distribution. Two independent Markov chain Monte Carlo (MCMC) runs were performed for 10,000,000 generations, sampled every 500th generation. Examination of the MCMC samples revealed convergence and adequate mixing of the chain with estimated sample sizes >200.

Because most studies of FIV in pumas have been based on a conserved ~480bp region encoding the reverse transcriptase enzyme within *pol* (RT-pol), a representative subset of the

sequences derived from Florida and Texas in this study were subjected to further phylogenetic analysis, along with additional sequences obtained from GenBank (accession numbers KX899918-KX899922, KF906167-KF906170, KF906163-KF906174, EF455603-EF455615, U53718-U53766, U03982, DQ19258). Bayesian analysis was used to infer phylogenetic relationships for RT-pol under the GTR+G substitution model with 4 gamma-distributed rate categories and uncorrelated branch lengths, again using the MrBayes Geneious plug-in (227) with chain length and burn-in as described above. HIV-1 was used as an outgroup (accession number LT726763). All trees were annotated in iTOL v3 (229).

Assessment of translocation risk

To investigate Texas translocations as a risk factor for FIVpco infection, we calculated the relative risk (230) of a sample testing positive after 1995 as compared to previous years. The 1995 translocation was treated as the exposure and relative risk (RR) was calculated as follows:

$$R = \left(\frac{a}{a+b}\right) / \left(\frac{c}{c+d}\right)$$

where *a = # exposed testing positive, b = # exposed testing negative, c = # of controls (not exposed) testing positive,* and *d = # of controls testing negative*. The standard error of the log relative risk is:

$$SE\{\ln RR\} = \sqrt{\left(\frac{1}{a} + \frac{1}{c} - \frac{1}{a+b} - \frac{1}{c=d}\right)}$$

and the 95% confidence interval is:

$$95\%\ CI = \exp\ (\ln(RR) - 1.96\ \times\ SE\{\ln(RR)\})\ \ \text{to}\ \ \exp\ (\ln(RR) + 1.96\ \times SE\{\ln(RR)\}).$$

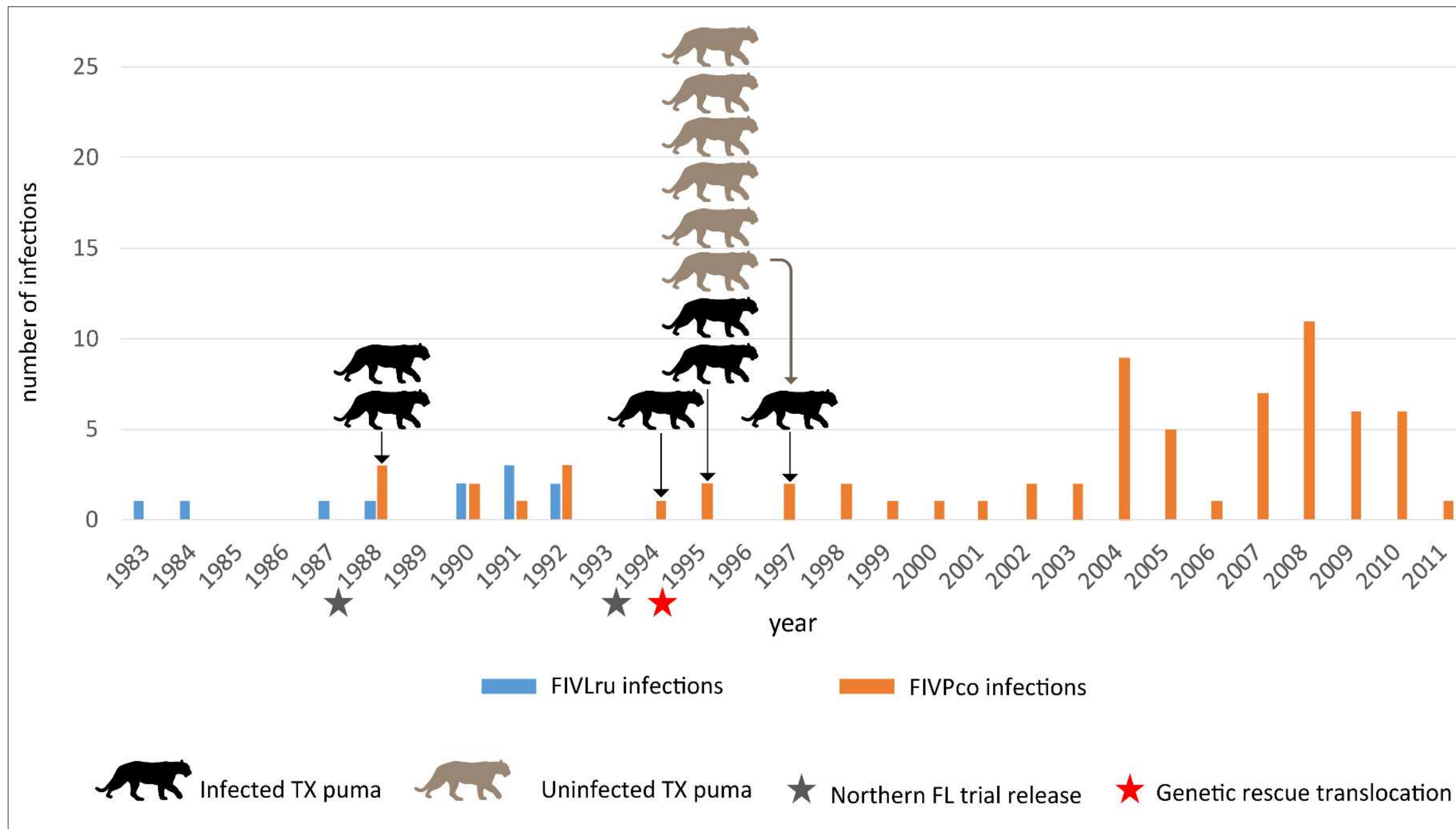
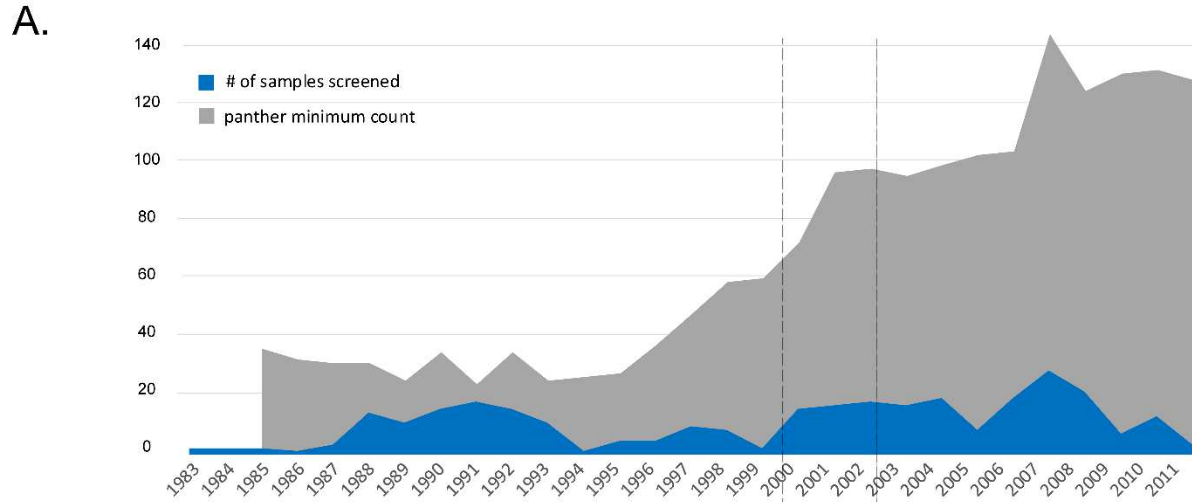


Figure 2.1: FIV dynamics in Florida are altered by introductions of infected Texas pumas. Following translocation of FIVpco infected Texas pumas in the 1980s and 1990s, the number of FIVpco infected Florida panthers increased dramatically while FIVlru infections fell below detectable levels. Texas puma 104 tested negative in 1995 but was positive on recapture in 1997 (grey arrow).

A.



B.

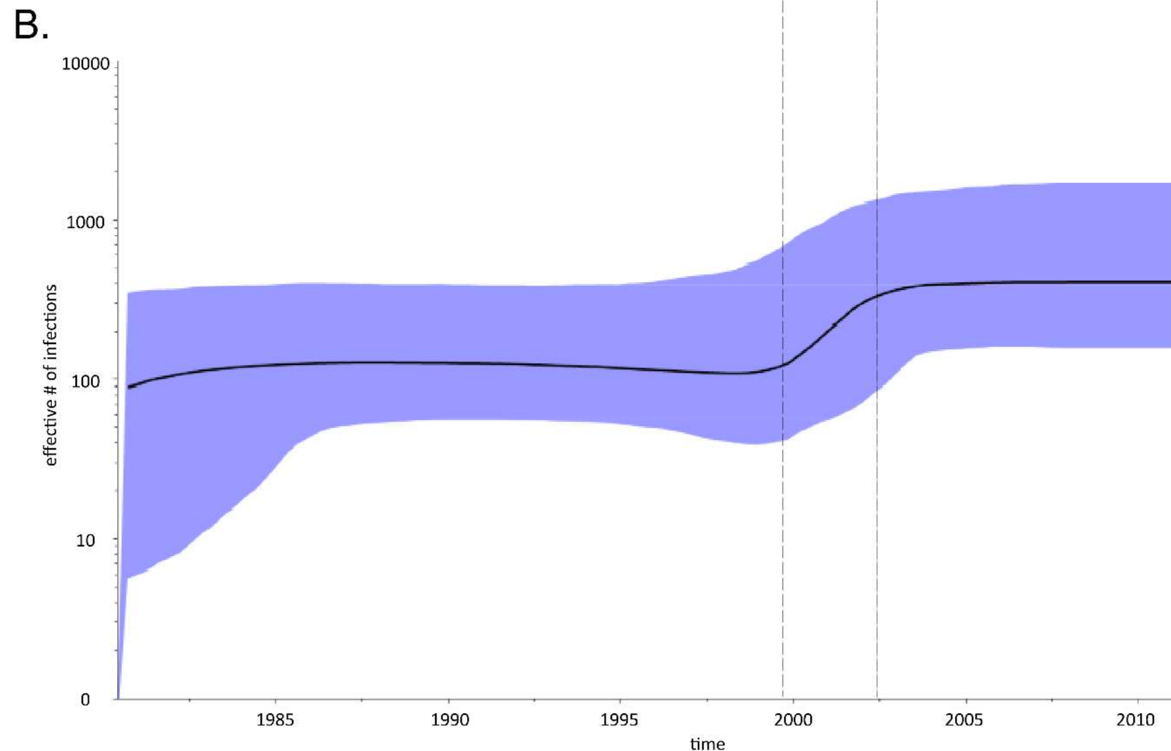


Figure 2.2A & 2.2B: An exponential increase in the effective number of FIVpco infections corresponds to a period of demographic panther recovery. A. The number of samples screened for FIVpco and FIVlru is plotted alongside the minimum panther count for each year. B. Bayesian skyline plot shows a sharp rise in the effective population size of FIVpco (effective number of infections) between ~2000 and 2002. The rise in effective number of infections coincides temporally with exponential expansion of the Florida panther population as estimated by minimum panther count. Dotted lines highlight the correlation.

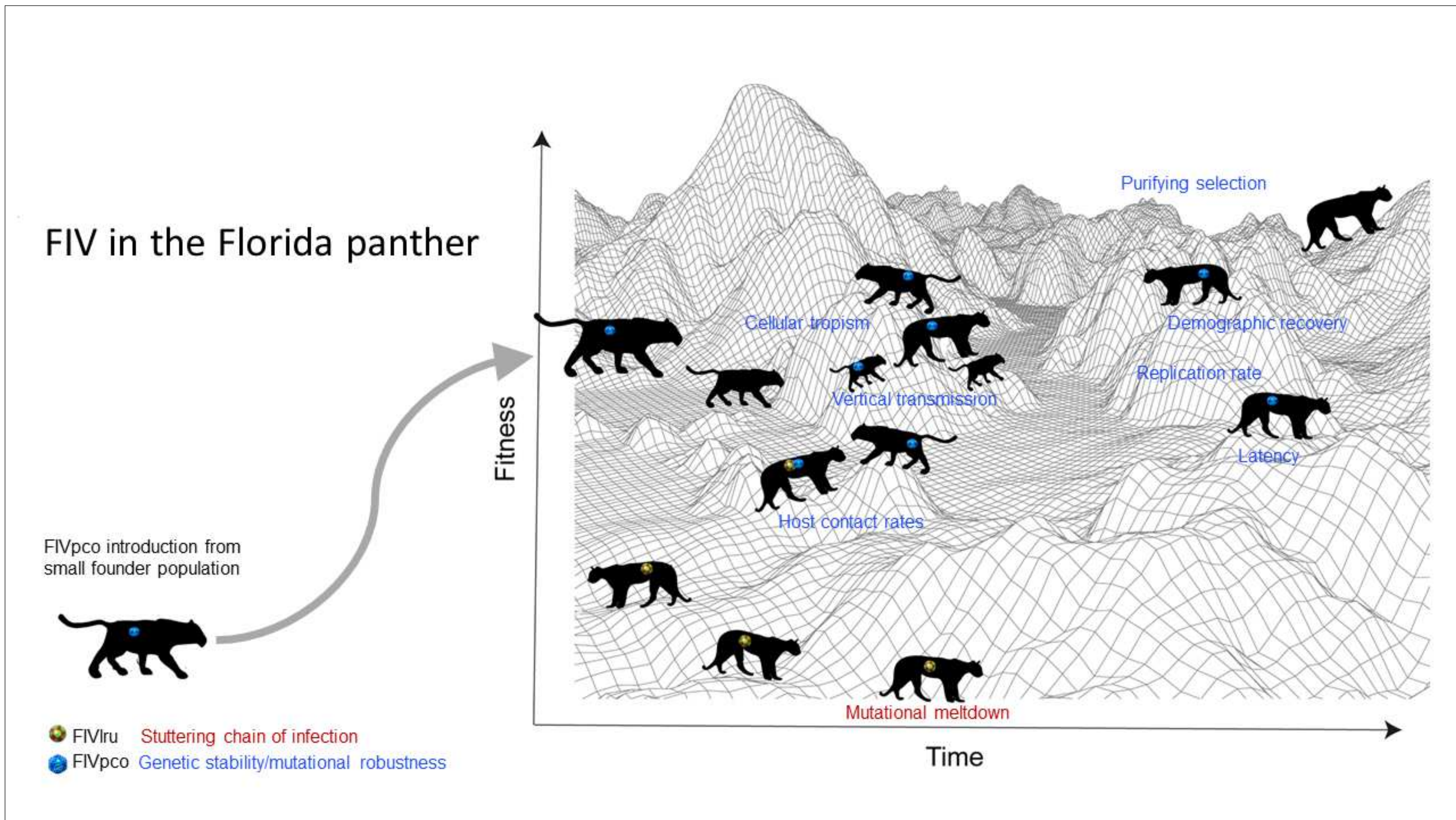


Figure 2.3. FIVpco replaced FIVlru as the dominant and highly 'fit' viral subtype in the Florida panther following cointroduction from Texas. Genetic stability and fitness of FIVpco have persisted through time, whereas poorly adapted subtype FIVlru was lost from the population. Ecological and molecular interactions determine the persistence and evolution of FIV in the panther.

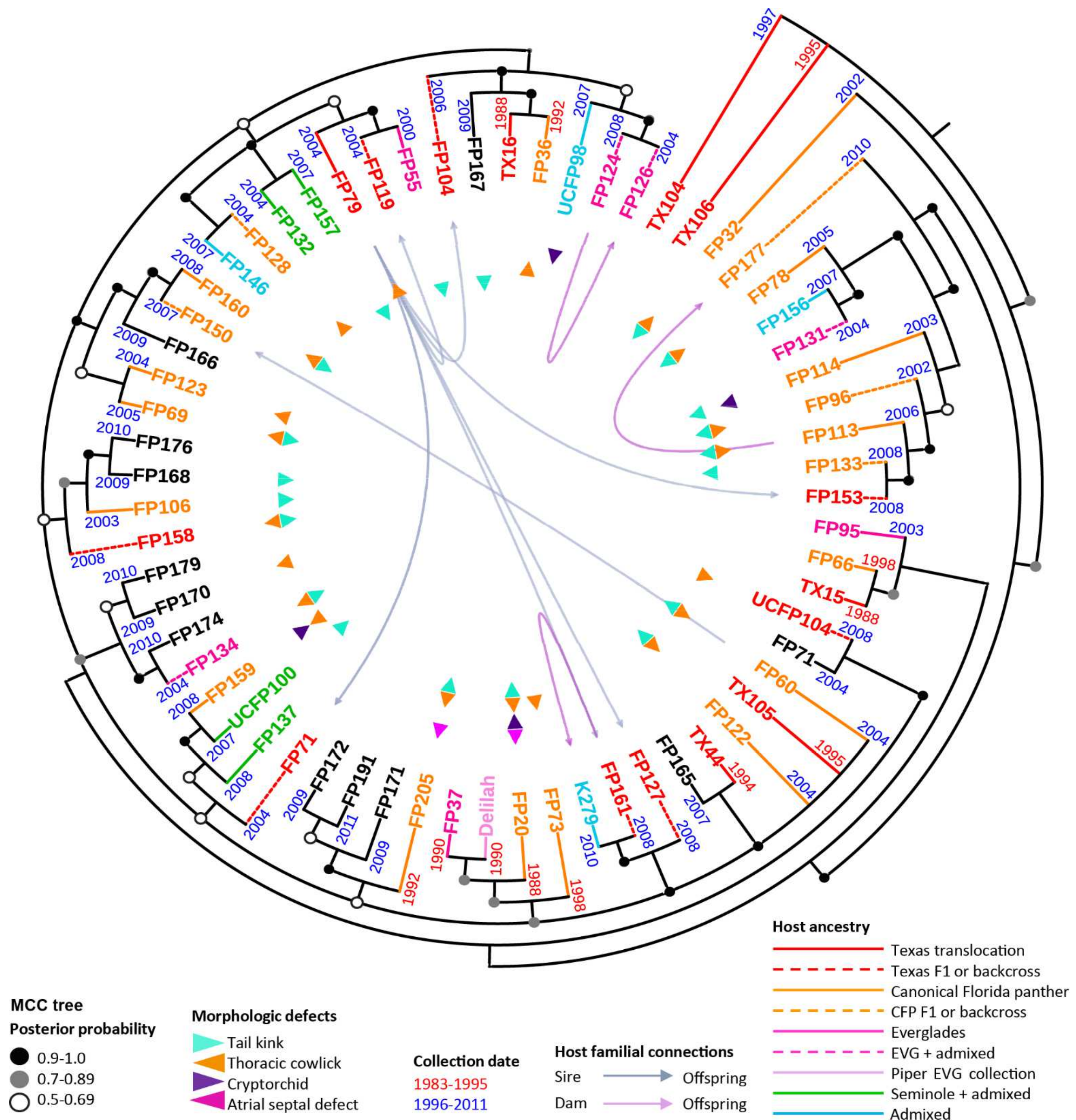


Figure 2.4: Two Texas FIVpco isolates are basal to all isolates recovered from Florida panthers, and all sampled isolates comprise a single FIVpco lineage with low genetic variation, independent of sample collection date. The maximum clade credibility tree was constructed from nearly whole genome sequences and converted to a cladogram for improved visualization due to short branch lengths. Host ancestry was assigned based on relationships reported by Johnson et al (169). FIVpco infections do not form separate clades based on host ancestry. Morphologic defects do not correlate with infection by a single viral clade. Two instances of presumed direct maternal transmission are identified, along with indirect familial transmissions, as based on sequences relatedness.

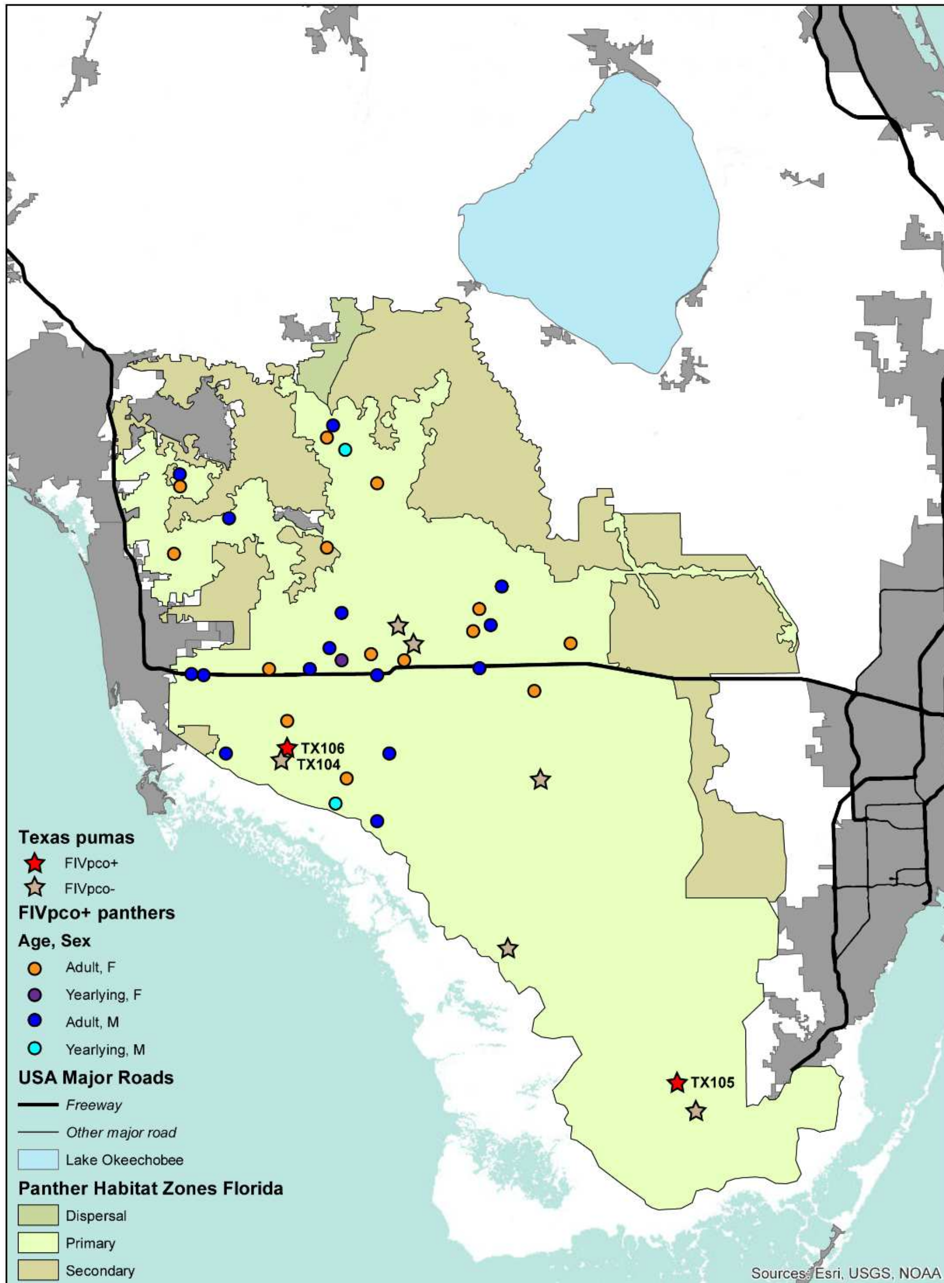


Figure 2.5: FIVpco is distributed across Florida panther habitat. Texas pumas 105 and 106 tested positive for FIVpco at the time of release in 1995, while puma 104 tested positive on recapture in 1997. Primary, secondary, and dispersal habitat zones were designated by Florida Fish and Wildlife Conservation Commission (2010). Grey shading represents urban corridors.

Figure 2.6: Florida panther FIVlru is easily distinguished from Texas derived FIVpco. Early FIV isolates (subtype FIVlru) recovered from Florida panthers are highlighted in red and distinguished by a 3 bp indel shown in the alignment at right. Contemporary FIVpco isolates from Florida panthers group with those from translocated Texas pumas and with two previously sequenced isolates originating from captive Texas pumas (highlighted in blue). A subset of the isolates from Figure 2.3 is included and highlighted in green. The maximum clade credibility tree is based on ~480 bp within the conserved region encoding the viral reverse transcriptase (RT-pol).

Table 2.1: Host demographic information for all sequenced isolates.

Lab ID	Field ID(s)	Collection Date	Ancestry	Sex	Age
X1409	PCO-424/FP48	2/18/2005	CFP	F	Adult
X1410	PCO-539/FP60	6/29/2004	CFP	M	Adult
X1411	PCO-901/FP69	1/3/2005	CFP	F	Adult
X1412	PCO-910/FP79	4/18/2004	HY	M	Adult
X1416	PCO-1000/FP104	3/10/2006	TX	F	Adult
X1419	PCO-1037/FP113	11/9/2006	CFP	M	Adult
X1424	PCO-1094FP127	2/5/2008	HY	F	Adult
X1425	PCO-977/FP128	2/18/2004	HY	M	
X1427	FP131	3/10/2004	EVG	M	Adult
X1428	PCO-1123/FP133	2/20/2008	HY	M	Adult
X1429	FP134/P1124	12/14/2004	EVG	M	Adult
X1432	FP137	12/12/2008	SEM	M	Adult
X1440	FP146	1/29/2007	HY	M	Adult
X1443	FP156	12/7/2007	HY	M	Adult
X1444	FP157	12/19/2007	SEM	M	Adult
X1445	FP159	1/29/2008	CFP	F	Adult
X1446	FP160	2/5/2008	CFP	M	Adult
X1447	FP165	11/24/2008	UNK	M	Yearling
X1448	FP166	1/30/2009	UNK	M	Adult
X1449	FP167	2/6/2009	UNK	F	Adult
X1450	FP168	2/9/2009	UNK	F	Adult
X1451	FP170	2/26/2009	UNK	F	Adult
X1452	FP172	11/19/2009	UNK	M	Adult
X1454	FP174	1/26/2010	UNK	M	Adult
X1455	FP176	2/8/2010	UNK	F	Adult
X1745	FP32/PCO-160	9/12/2002	CFP	F	Adult
X1749	PCO-733/TX106	2/16/1995	TX	F	Adult
X1765	PCO-732/TX104	1/23/1997	TX	M	Adult
X1826	PCO-71/FP20	8/24/1988	CFP	F	Adult
X1834	PCO-133/TX15	6/1/1988	TX	F	
X1841	PCO-174/FP36	1/8/1992	CFP	M	Adult
X1842	PCO-175/FP37	1/30/1990	EVG	F	Adult
X1845	PCO-178/Delilah	2/21/1990	EVG	F	Adult
X1855	PCO-190/FP205	10/11/1992	CFP	M	
X1877	PCO-534/TX44	3/8/1994	TX	F	Yearling
X1880	PCO-540/FP55	3/9/2000	EVG	F	Adult
X1892	PCO-739/TX105	6/1/1995	TX	F	Adult
X1895	PCO-902/FP66	7/16/1998	HY	F	Yearling
X1896	PCO-903/FP73	11/12/1998	HY	M	Adult
X1915	PCO-972/FP96	1/18/2002	CFP	F	
X1926	PCO-1002/FP106	2/20/2003	CFP	M	Adult
X1936	PCO-1033/FP119	11/17/2004	HY	F	Adult
X1937	PCO-1038/FP114	10/23/2003	CFP	F	Yearling
X1945	PCO-1079/FP150	2/8/2007	HY	M	Adult
X1948	PCO-1087/FP122	1/30/2004	CFP		
X1949	Pco-1088/FP123	2/2/2004	CFP	F	Adult
X1950	PCO-1091/FP124	2/27/2008	EVG	F	Adult
X1952	PCO-1093/FP126	5/28/2004	EVG	F	
X1953	PCO-1095/FP71	2/17/2004	TX	M	
X1955	PCO-1098/FP132	7/23/2004	SEM	F	Adult
X1957	PCO-1115/FP161	2/11/2008	HY	M	Kitten
X1966	PCO-1238/FP152	2/16/2007	HY	F	Adult
X1967	PCO-1239/FP153	3/3/2008	HY	M	Adult
X1980	PCO-1277/UCFP98	6/12/2007	HY	F	Adult

Table 2.2: All FIVpco isolates from Texas and Florida share high sequence homology across the genome, with pairwise identity averaging >99%. The number of isolates for each complete open reading frame is provided, with average pairwise identify for each gene (missing data excluded).

	gag	pol	vif	orfA	env
# isolates with no missing data	52	18	56	39	23
mean % pairwise identity	99.5	99.7	99.4	98.8	99.4

CHAPTER THREE

Differential degradation of puma APOBEC3 by geographically distinct variants of feline immunodeficiency virus

Introduction

Restriction factors are cellular proteins that inhibit viral infections and comprise an important arm of the innate immune system, serving as a first line of anti-viral defense. Nearly all phases of viral life cycles can be targeted by restriction factors, which are remarkably diverse both in structure and function. Genes encoding restriction factors tend to evolve under positive selection imposed by antagonistic interactions with the viruses they restrict (65). The evolutionary arms race is evidenced by gene duplications, losses, rearrangements, and length polymorphisms that have been characterized through comparative studies of restriction factors between species (16, 231, 232). In response to restriction by the innate immune system of the host, viruses have evolved auxiliary genes that effectively combat the restrictive activity of the host proteins in a gain of function fashion (64). The most thoroughly characterized restriction factors to date are those that antagonize HIV, a virus which has indeed evolved a number of accessory genes in response to intrinsic cellular restriction (40, 65).

The apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3; A3) family comprises cellular proteins found in clade-specific copy numbers and expressed constitutively in various cells and tissues of placental mammals. Best known for their robust capacity to restrict lentiviral infection, A3 proteins bind to single-stranded DNA or RNA and enzymatically edit nucleic acids of the target substrate via cytosine deamination. Target

substrates are constrained by the single-stranded specificity of A3 proteins, and antiretroviral activity is conferred by the ability of A3 to modify the template strand of cDNA during reverse transcription of the viral genome (233). When focused on specific genes and occurring under typical biological constraints, A3 enzymatic activity can have beneficial functions outside of viral restriction (234, 235). Off-target or unregulated A3 activity, however, can be genotoxic and oncogenic (236, 237), and therefore expression of A3 genes is tightly regulated under normal physiologic conditions (234, 238). Editing of viral genomes by A3 proteins is manifested as characteristic guanine-to-adenine (G-to-A) mutations in nascent proviral DNA, copied from cytosine-to-uracil modifications in the minus strand cDNA (86, 123, 239-241). In addition to the primary enzymatic editing function, other mechanisms of retroviral restriction have been described for A3 proteins, including impedance of reverse transcription and integration (242-246).

All lentiviruses, with the exception of equine infectious anemia virus (EIAV), have evolutionarily adapted the expression of an accessory gene known as viral infectivity factor (*vif*), which functions to prevent incorporation of A3 proteins into progeny virions during encapsidation (247). Pressures imposed by Vif have driven evolution and expansion of the mammalian A3 repertoire in species-specific patterns (16), while concurrent adaptations of *vif* have emerged in response (124, 182, 248). As such, A3-Vif interactions exemplify the molecular arms race that drives genetic variation through coevolution of pathogens alongside the intrinsic immune system of the host (231).

A3-Vif interactions play a role in lentiviral host specificity

Lentiviruses are typified by species-specific patterns of infection and restriction by A3 proteins is known to comprise an important barrier to cross-species transmission (63). The

adaptative potential of lentiviral Vif is thus a decisive determinant in the outcome of lentiviral transmission to a nontarget host (249). The critical role of Vif adaptation to a nontarget host is best exemplified by spillover of simian immunodeficiency virus (SIV) from chimpanzees to humans, where interactions between Vif and A3 haplotypes conceded productive infection, marking the inception of the HIV-1 pandemic (97, 250). Numerous exceptions to the species-specific norms of A3-Vif interactions have been documented, and restriction activity of A3 proteins against nontarget lentiviruses, especially those of closely related hosts, is not fully understood (249). Several studies have documented restriction of HIV-1 by feline A3s (106, 249, 251, 252), while activity against feline A3 has been reported for SIV Vif derived from macaques (SIVmac) (253).

Within the Felidae family, FIV spillover infections from bobcats (*Lynx rufus*) to pumas (*Puma concolor*) are relatively common, indicating a relaxation of A3-imposed host barriers between closely related feline species (114). Further, several studies have shown that domestic cat FIV Vif is successful in opposing A3 activity of most species of larger cats, demonstrating a generalist adaptation of domestic cat FIV (FIVfca) (125). In contrast, domestic cat infection with FIV of pumas (FIVpco) is abortive and displays signatures of A3 restriction including characteristic G-to-A lethal hypermutation (137). Adaptations of feline lentiviral Vif to A3 repertoires of target and nontarget hosts therefore provide a unique opportunity to investigate the evolutionary pathways governing species-specific tropisms of lentiviral infections (254). Importantly, feline A3-Vif interactions represent a naturally-occurring, biologically relevant system for the study of complex interactions that are most commonly investigated in artificial *in vitro* systems that often fail to reproduce all biological aspects required to deliver meaningful results.

Retroviral infections have shaped mammalian A3 loci

Pressures imposed by viruses and retroelements have shaped the genomic structure of the mammalian A3 locus in a species-specific manner (16, 255). A3 gene duplications, losses, and polymorphisms vary across mammalian clades, with evolutionary convergence on the function of viral restriction (16). All proteins of the A3 family are characterized by distinct zinc-binding domains and can be classified accordingly based on catalytic motif (A3Z1, A3Z2, or A3Z3) (256, 257). The genome of the Felidae family is characterized by 3 copies of A3Z2 (A3Z2a, A3Z2b and A3Z3c), a single copy of A3Z3, and a notable absence of the A3Z1 gene observed in the canine counterpart of the order Carnivora (Figure 3.1A) (16, 106). An additional transcript containing a 'linker region' is produced via read-through transcription and alternate splicing, resulting in the double domain protein A3Z2-Z3 (Figure 3.1A). Variants A3Z2b-Z3 and A3Z2c-Z3 have been identified (106). A3Z3 is the feline ortholog to human A3H, for which crystal structure has been solved (Figure 3.1B) (258). The A3Z3 locus has been extensively characterized in the domestic cat (*Felis catus*) (106, 259), and partially characterized in the African lion (*Panthera leo bleyenberghi*), two tigers subspecies (*Panthera tigris sumatrae* and *Panthera tigris corbetti*), leopard (*Panthera pardus japonensis*), Eurasian lynx (*Lynx lynx*), and puma (*Puma concolor*) (106, 260). Extensive haplotype surveys, however, have not been performed for most species of the Felidae family.

Pumas are host to multiple retroviral infections

Pumas are natural hosts of several retroviral infections, including feline immunodeficiency virus (FIV) of the Lentivirus genus, feline leukemia virus (FeLV) of the Gammaretrovirus genus, and feline foamy virus (FFV) of the Spumavirus genus. As apex predators that occasionally prey on smaller felids such as bobcats (*Lynx rufus*) and domestic cats

(*Felis catus*), pumas have been shown to acquire viral infections of closely related mesocarnivores with relative frequency. For example, transmission of the bobcat subtype of FIV (FIVlru) to pumas has been documented with relative frequency in California and Florida (56, 114), while spillover of FeLV from domestic cats has resulted in epidemics among Florida panthers (*Puma concolor coryi*), an endangered subspecies of puma (187). Further, molecular analyses have recently revealed that FFV is readily transmitted from domestic cats to pumas with relative frequency (Kraberger unpublished data). Feline retroviruses therefore represent a naturally-occurring system for the study of Vif-A3 interactions as determinants of retroviral susceptibility within and between closely related host species.

A3 restriction of infection is virus specific

In the domestic cat, adaptations to evade A3 activity have been elucidated for FIV and FFV. Accessory proteins Vif and Bet oppose A3 restriction to permit FIV and FFV infection, respectively (106, 125, 261-263). Similar to HIV Vif, FIV Vif targets A3 for poly-ubiquitination and degradation through recruitment to an E3 ubiquitin ligase complex comprised of Cullin 5 (CUL5) and Elongin B & C (ELOB and ELOC) (Figure 3.2A) (264). By virtue of the ubiquitin/proteasome-dependent pathway, degradation abrogates the packaging of A3 into nascent viral particles (Figure 3.2B) (233). In contrast, FFV Bet evades A3 restriction through a degradation-independent mechanism involving putative formation of insoluble Bet-A3 complexes to circumvent virion encapsidation of A3 (261-263). While anti-FIV activity is conferred by A3Z3 and A3Z2-Z3 (67, 106, 125, 253), anti-FFV activity is primarily attributed to A2Z3(a-c) (261, 262). A3Z3 and A3Z2-Z3 have a lesser impact on the infectivity of Bet-deficient FFV (261, 262). Interestingly, a counter mechanism directed against A3 activity has not been identified for FeLV despite the finding that A3Z2-Z3 significantly reduces FeLV infectivity

in vitro (106). A mild inhibitory effect on FeLV infectivity has been demonstrated for A3Z3, while A2Z3(a-c) does not alter infectivity (106). It has been hypothesized that FeLV may evade A3 activity via a tropism for cells with low A3Z2-A3 activity, as has been proposed for Equine Infectious Anemia Virus (EIAV), the only lentivirus lacking the Vif protein (125). An alternate hypothesis proposes FeLV evasion of A3 activity via expression of a glycosylated form of Gag (Glyco-Gag), as has been demonstrated for murine leukemia viruses (MuLVs) (265).

A3 polymorphisms correlate with susceptibility to retroviral infections

In primates and other mammals, polymorphisms can impact the anti-viral properties of A3 proteins by altering stability and localization (68, 266). Human A3 haplotypes and allele frequencies vary across individuals of European, Asian, and African descent, and polymorphisms that alter A3 activity and/or expression have been identified in association with altered risk and progression of HIV infection (97, 267, 268). Similarly, A3 polymorphisms in the domestic cat have been identified in correlation with susceptibility to FIV and FeLV (269, 270). Specifically, seven haplotypes of A3Z3 have been identified in the domestic cat, and resistance to Vif-mediated proteasomal degradation has been documented for haplotype V (270). Haplotype V is effectively packaged into nascent virions during replication yet evades proteasomal degradation in the presence of Vif. This resistant haplotype is differentiated from other domestic cat A3Z3s by residue I65, where the functional difference is related to the size of the surface-exposed side chain rather than hydrophobicity (270). A polymorphism encoding either an arginine or lysine at position 65 was also identified in the Indochina subspecies of tiger (106). Amino acid position 65 is a positively selected site in feline A3Z3, and reflects a putative gain of function in response to pressures imposed by FIV (270).

Diverse FIVpco genotypes circulate in pumas

Geographically distinct genotypes of FIVpco circulate in puma populations across North America (120, 142). Susceptibility of puma populations to each genotype is dependent in part on the competence of FIVpco Vif to evade A3 anti-viral activity (137). However, the A3 locus has not been extensively characterized in pumas, and the relationship between Vif genotypes and A3 interactions has not been thoroughly investigated. In this work, we aimed to characterize the diversity of puma A3Z3 and FIVpco Vif across North America and investigate relationships between FIVpco genotypes and puma A3 haplotypes as determinants of infection. Specifically, we aimed to: (1) thoroughly characterize the A3 locus in pumas; (2) demonstrate the capacity for FIVpco Vif to bind A3 and promote proteasomal degradation; (3) identify possible associations between A3 variation and susceptibility to FIVpco infection; and (4) explore the mechanistic interactions between geographically distinct FIVpco genotypes and puma A3 proteins as related to outcome of lentiviral infection. Somewhat surprisingly, we found that A3Z3 is conserved in pumas, while FIVpco is characterized by diverse *vif* genotypes with variable efficacy against A3-mediated restriction. Importantly, we confirmed a naturally occurring, geographically distinct FIVpco genotype (PLV-1695) circulating in Western Canada that is defective in prompting A3Z3 proteasomal degradation (Figure 3.3A). The survival of this genotype and the absence of detectable signatures of A3-induced hypermutation suggest a Vif-independent mechanism of A3 evasion. We further identified 10 sites in FIVpco *vif* evolving under episodic diversifying (positive) selection (Figure 3.3B and 3.4A) and hypothesize that one or more of these sites may determine Vif counteractivity to A3-imposed restriction, setting the stage for further mechanistic investigation into the unique relationships of FIVpco Vif and puma A3.

Methods

Sample collection and nuclei acid extraction

Blood and tissue samples were collected from pumas across North America from 1988 to 2017. Capture of free-ranging animals involved the use of baited cage traps or trained tracking hounds, as previously described (146). Capture and handling protocols followed approved Institutional Animal Care and Use Committee guidelines, as well as applicable local government regulations. Additional samples were opportunistically collected during routine postmortem examinations by local government agencies. Aliquots of blood and tissue samples were sent to Colorado State University for analyses as described below. Multiple tissue types were analyzed as available with priority assigned to lymphoid organs (i.e. spleen, followed by lymph node). DNA was extracted from tissue, whole blood, or peripheral blood mononuclear cells (PBMCs) using an adapted version of the DNeasy Blood and Tissue protocol (Qiagen Inc., Valencia, CA.) Tissues were homogenized using the benchtop FastPrep-24 cell and tissue homogenizer (MP Biomedicals, LLC., Santa Ana, CA.). Blood samples were incubated in lysis buffer at 56℃ overnight. Table 3.1 provides the collection animal ID and location for each sample selected for A3Z3 sequencing.

Amplification and sequencing of A3Z3

Coding regions of feline A3Z3 were amplified from puma blood and tissue DNA using primers described in Table 3.2. Three separate reactions were performed for each sample (exon 2, exons 3 and 4, and exon 5). Reactions of 25 µL were prepared using KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA) and run on a C1000 touch thermocycler (Bio-Rad, Hercules, CA) using the following conditions: initial denature 95℃ for 3 min, 40 cycles of 98℃ for 20 s, 65℃ for 15 s, and 72℃ for 15 s, followed by a final extension at 72℃ for 1 min.

DNA sequencing was performed by Macrogen (Rockville, MD) and sequences and chromatograms were analyzed in Geneious (155).

Amplification and sequencing of FIVpco vif

FIVpco *vif* sequences were recovered from Florida panthers as described for whole genome sequencing here in Chapter Two. Sequencing of FIVpco whole genomes from other geographic regions was performed previously (120) and Vif coding regions were obtained from GenBank. Newly sequenced, additional *vif* isolates were amplified using primers 1695_19L (ATTGGGGAGAAGGATCAGTGTTAATT) and 1695_21R (TTTTGGGTTCCTTGTGTCGCAA) at 10 µM concentration (Table 3.2). Reactions of 25 µL were prepared using Q5 High-Fidelity DNA Polymerase Enzyme (New England Biolabs Inc., Ipswith, MA) and touch-down cycling conditions. Specifically, thermocycling was initiated at 68□ and decreased by 0.5□ for 6 cycles, followed by 34 additional cycles at 65□ for a total of 40 cycles. Negative samples and no template reactions were included and were consistently negative.

We predicted that the observed divergence of genotype PLV-1695 compared to other FIVpco isolates could be principally attributed to *in vitro* mutagenesis in cell culture. We therefore amplified and sequenced *vif* of genotype PLV-1695 from primary cells and supernatant collected at various stages of *in vitro* infections. Specifically, we sequenced *vif* from puma PBMC cultures at days 10, 16, 20, and 27 post-infection and from supernatant collected from domestic cat PBMC culture at day 10. We then compared the Vif amino acid sequences to the prototypical PLV-1695 in a multiple sequence alignment, seeking evidence for adaptive evolution in cell culture systems.

Because PLV-1695 originated from Western Canada (specifically Vancouver Island) we sought to further investigate genotypes of FIVpco circulating in Western Canada. We therefore screened 19 spleen and 26 blood samples recently collected from pumas in this region using primers and cycling conditions described above. FIVpco was detected in 3/26 blood samples based on products of the expected size (~1000 bp) visualized using gel electrophoresis. Of these, Sanger sequencing yielded full *vif* sequence for one sample (x2838), partial *vif* sequence for another, and no high-quality sequence data for the third.

FIVpco vif phylogenetic analysis

Nucleotide sequences of FIVpco *vif* were codon aligned using the Clustal W algorithm and adjusted manually in MEGA6 (148). The multiple sequence alignment was then screened for recombination using GARD through the Datamonkey interface of the HyPhy package (225). No evidence of recombination was detected. Codon aligned nucleotide sequences were then translated to amino acid sequences and model selection was performed using jModelTest (226). The multiple sequence alignment was subjected to Bayesian analysis using the MrBayes 3.2.6 Geneious plugin (227) with gamma-distributed rate variation under the JTT substitution model. Four heated chains of 1,100,000 iterations were run with a subsampling frequency of 200 and the initial 10% discarded as burn-in. A domestic cat FIV isolate obtained from GenBank was used an outgroup (accession number AF474246).

FIVpco whole genome hypermutation analysis

A multiple sequence alignment of all available FIVpco whole genome sequences was generated using MEGA6 (148). Nucleotide sequences were codon aligned using the Clustal W algorithm and manually adjusted. Ancestral sequences of FIVpco whole genomes were computationally extrapolated independently using MEGA6 (148) and FastML (271). Integrating

both indels and characters, the most likely state at each node was extrapolated for a maximum likelihood phylogenetic tree, and the sequence from the deepest internal node (N1) was inferred as the single shared common ancestor of all isolates. Mutational biases were then investigated using Hypermut 2.0 (272) with the ancestral sequence as a reference and enforcing dinucleotide preference of A3 proteins (Table 3.2). Fisher's exact test was employed in Hypermut to determine statistical significance. Mutations details are provided in Table 3.3.

FIVpco Vif selection analysis

The previously described codon aligned FIVpco *vif* multiple sequence alignment was analyzed using a mixed effects model of evolution (MEME) (273) via the Datamonkey 2.0 interface (274) for the HyPhy package (275). The domestic cat FIV outgroup included in the maximum clade credibility tree described above was removed from the multiple sequence alignment. Sites identified as evolving under positive selection were those with a nonsynonymous (dN) to synonymous (dS) substitution ratio >1 and a p value >0.1.

Vif amino acid signatures patterns

To further investigate residues in FIVpco Vif potentially associated with attenuated A3Z3 degradation, we compared the amino acid signature pattern in Vif from a query set of sequences (those from Western Canada) to a background/control set of sequences (those from all other regions) using Viral Epidemiology Signature Pattern Analysis (VESPA), available at HIV Los Alamos database (http://www.hiv.lanl.gov./content/sequence/VESPA/vespa.html). Due to low numbers of sequences from Western Canada and the requirement for relatively equal numbers of query and background sequences necessary to reach statistical significance, we did not perform statistical testing, but rather interpreted the pattern in the context of previous studies along with findings of the selection analysis described above in order to propose sites for future assessment.

A3 degradation analyses

In vitro A3 degradation analyses were performed at Heinrich Heine University Düsseldorf and the related data shown here were provided courtesy of Carsten Münk and Zeli Zhang. Briefly, co-transfections of puma-derived A3s and FIVpco Vif expression plasmids were performed in 293T cells, as described in (253). Constructs expressed A3 as a C-terminal HA-tag and Vif as a C-terminal V5-tag fusion protein. Immunoblots of lysates from cells expressing both A3 and Vif were used to assess degradation of corresponding A3 proteins, as detailed in (253). Tubulin control proteins were additionally detected via the above method.

Results

The puma A3Z3 locus is conserved across North America

Recent reports of multiple A3Z3 haplotypes in domestic cats that correlate with susceptibility to infections provide evidence for shaping of immune genes by molecular interactions with ancestral retroviruses (269, 270). Pumas are known to harbor not only species-specific retroviral infections, but also those native to other feline species (56, 114). To investigate the influence of positive selection imposed by retroviral infections on the innate immune system of the puma, we sequenced the coding regions of A3Z3 for 51 pumas and identified a single synonymous mutation (c381t) in exon 3. Two SNPs were additionally identified within introns. We found no evidence of the c392t polymorphism reported by Konno et al. 2018.

Vif competency for A3 degradation varies across genotypes

The capacity for Vif to oppose A3 activity is a primary determinant of lentiviral replication potential (66). However, partial resistance to Vif counteractivity has been reported in

primates (276-278) and more recently in the domestic cat (270). To investigate the capacity for diverse FIVpco Vif genotypes to promote proteasomal degradation of A3Z3, we performed immunoblot assays using two reporter viruses: PLV-1695 and a consensus Vif derived from available sequences (Figures 3.2A and 3.2B). We found that puma A3Z3 is not degraded in the presence of genotype PLV-1695 Vif (Figure 3.2A) as previously reported (279). In contrast, we found that puma A3Z3 is efficiently degraded in the presence of FIVpco consensus Vif, as illustrated in Figure 3.2A. We hypothesized that this difference could be attributed to *in vitro* mutagenesis of genotype PLV-1695 and sought to document adaptation of this isolate to cell culture systems. Interestingly, we found that *in vitro* changes in the nucleotide sequences of PLV-1695 *vif* are minimal, with 100% conservation of amino acid homology across all sequenced isolates.

FIVpco Vif genotypes form strongly supported clades corresponding to geographic region, with occasional putative recombination events and/or complex genetic relationships that cannot be fully resolved (Figure 3.4A). In addition to the whole genome sequences of FIVpco previously published and obtained from GenBank, we additionally sequenced *vif* from 56 Florida panther samples, 2 samples from Western Canada, and 14 additional samples from Colorado. Phylogenetic relationships are depicted in Figure 3.4. The 2 newly sequenced isolates from Western Canada include one sample from Vancouver Island collected in 1995 (‘Clem’) and one sample collected in Alberta in 2018 (‘X2838’). PLV-1695 also originated from a Vancouver Island sample collected in 1995. These sequences, along with cell culture isolates derived from PLV-1695 *in vitro* infections, form a monophyletic clade with high support (Figure 3.4). One previously sequenced isolate from British Columbia (accession number EF455615) is paraphyletic to the Western Canada clade and is most genetically similar to isolates from

Montana. Interestingly, isolate X2838 is most divergent due to a 34bp insertion in the C-terminus.

Within geographic regions, amino acid homology of Vif can be high, as demonstrated in Florida where 53/56 Vif sequences share 100% pairwise identity (Figure 3.4). Across broad geographic space, however, sequence divergence ranges from ~75-90% in most cases, with increased genetic distance of X2838 due to the previously mentioned insertion. The percent pairwise identity between the FIVfca Vif used as an outgroup and FIVpco Vif is notably low (~35%), highlighting the remarkable divergence between species-specific subtypes of FIV.

Escape from A3 activity is independent of Vif

Based on reports that *vif*-deficient FIV is unable to replicate both *in vitro* and *in vivo*, in cell culture systems (280) and experimental domestic cat models (281), respectively, antagonism of A3 editing activity by FIV Vif is a putative prerequisite for production viral infection. We therefore predicted that the genome of PLV-1695 would show evidence of hypermutation relative to Vif-competent FIVpco genotypes. We thus quantified the number of G-to-A substitutions across the PLV-1695 genome using a computationally extrapolated ancestral FIVpco sequence as a reference. We found no evidence of hypermutation across the genome of PLV-1695 relative to the ancestral reference sequence (Table 3.3). In fact, A-to-G substitutions were more common than G-to-A substitutions. This lack of evidence for hypermutation suggests that PLV-1695 has escaped A3Z3 editing activity via a mechanism that is independent of Vif.

FIVpco vif is subject to episodic diversifying selection

To investigate the evolutionary pressures imposed by the host immune system on FIVpco Vif, we performed a gene-wide selection analysis to define the ratio of nonsynonymous (dN) to synonymous (dS) mutations (273). While most codons in FIVpco Vif evolve under purifying

selection, we found evidence for episodic diversifying selection at 10 sites (p value <0.1). These sites clustered within the N terminus (N=3) or the C terminus (N=7) of the protein, within regions previously identified as important sites for direct interaction with A3 (282) or the CUL5-ELOB-ELOC (283, 284), respectively (Figures 3.2C and 3.3B). Of particular interest is the finding that the 3 sites under positive selection in the N terminus differ between PLV-1695 and the consensus sequence used as a reporter virus in the A3 degradation assays (Figure 3.3B). Additionally, 5 of 7 sites under positive selection differ between the two reporter viruses, and two of the sites are located within the KCCC motif identified as the putative sites for CUL5 binding (Figures 3.2A, 3.2C, and 3.3B) (284). Collectively, these findings suggest that one or more of these sites may determine the capacity for Vif to route packaged A3Z3 proteins to the proteasome for degradation during viral replication.

PLV-1695 genotype has a unique amino acid signature

To further investigate the residues that differ between PLV-1695 Vif and other FIVpco genotypes in the context of Vif-mediated degradation of A3Z3, we searched for signature amino acids in the PLV-1695 (Western Canada) clade that differed from isolates from all other regions. Using the Western Canada group as a query and all other isolates shown in Figure 3.4 as background controls, we used VESPA (http://www.hiv.lanl.gov./content/sequence/VESPA/vespa.html) to identify 39 sites at which the amino acid signature of the Western Canada clade differs from all other background sequences (Figure 3.5). Many of these sites had relatively low % conservation across the entire sequence alignment; however, some sites were relatively conserved across most geographic regions, differing only in the Western Canada clade. Four of the sites (146, 152 192 and 227) identified as

important signature sites of the Western Canada genotype were also identified as evolving under episodic diversifying selection (Figure 3.5B).

Discussion

In primates, a loss of A3 function in response to lentivirus-imposed selective pressure has been implicated in the emergence of HIV-1 following spillover of SIVcpz to humans (96, 285). In contrast to the loss function in primates, recent reports provide evidence for feline A3 gain of function as a putative response to ancestral lentivirus infections in the domestic cat (270). While domestic cat A3-Vif interactions have been thoroughly investigated (106, 253, 282, 284), little is known about coevolution of A3 and FIV in most feline species. Given that pumas are host to a species-specific lentivirus (FIVpco) and additionally acquire lentiviral infections from sympatric feline species with relative frequency (56, 114), we aimed to fully characterize the puma A3Z3 locus through an extensive haplotype survey. We further aimed to characterize the diversity of FIVpco *vif* and investigate the functional relationship between puma A3Z3 haplotypes and FIVpco *vif* genotypes.

We sequenced the A3Z3 locus from 51 pumas spanning their geographic range in North America (Table 3.1). We found one silent mutation in exon 3 and two intronic SNPs but did not find evidence for multiple A3Z3 haplotypes. We found that residue 65, a polymorphic site in tigers (106) and domestic cats (106, 270), is conserved in the puma.

Based on observations that *vif*-deficient FIV is unable to replicate in cell culture (280) and in experimentally infected domestic cats (281) in the presence of A3Z3, Vif-mediated degradation of A3Z3 is a putative prerequisite for productive viral infection. We found that one genotype of FIVpco *vif* (PLV-1695) is unable to degrade puma A3Z3 *in vitro* (Figure 3.3A). We

predicted that the observed resistance to degradation was due to *in vitro* mutagenesis of PLV-1695 in cell culture, and we therefore amplified and sequenced *vif* of genotype PLV-1695 from primary cells and supernatant collected at various stages of *in vitro* infections. We then compared the Vif amino acid sequences to the prototypical PLV-1695 in a multiple sequence alignment, seeking evidence for adaptive evolution in cell culture systems. Surprisingly, we found that Vif amino acid sequences were 100% conserved between *in vitro* isolates and the prototypical PLV-1695. This led us to surmise that PLV-1695 is a geographically unique FIVpco variant circulating in Western Canada, and we sought to sequence additional isolates from this region.

We recovered FIVpco *vif* sequences from 2 additional pumas in Western Canada, referred to as 'Clem' and 'X2838', and obtained one additional sequence from GenBank (accession number EF455615). Pairwise identity was high between PLV-1695 and Clem (98% at the amino acid level) (Figure 3.4), which is not surprising given that both isolates were recovered from pumas inhabiting Vancouver Island in 1995. The genetic distance between X2838 and other Western Canada isolates was relatively large due to a 34 bp insertion in the C-terminus of X2838. The British Columbia isolate from GenBank (EF455615) shares highest sequence homology with the Montana subgroup (~97-98% amino acid pairwise identity) and is more divergent from the Vancouver Island isolates (~84% amino acid pairwise identity) (Figure 3.4).

Based on collective knowledge of A3Z3-Vif interactions, the inability of PLV-1695 Vif to degrade A3Z3 should theoretically render the virus vulnerable to lethal hypermutation. We therefore looked for evidence of A3Z3 editing activity across the whole genome sequence of PLV-1695 by quantifying G-to-A mutations relative to control (background) mutations in the context of the dinucleotide preferences described for human (Table 3.3). Importantly, we found

no evidence of G-to-A hypermutation in the PLV-1695 genome or any other isolate of FIVpco (Table 3.3).

In contrast to FIV in the domestic cat and some lentiviruses of primates, FIVpco has not been shown to produce symptomatic disease in the puma (reviewed in (134)). The resistance of pumas to clinical disease associated with lentiviral infection could underlie the absence of apparent A3Z3 evolution in pumas. In fact, it has been reported that Vif-A3 interactions play a key role in controlling replication and pathogenicity of FIV in native host species (66). One study reported a relative replication advantage of a high virulence subtype of FIVfca conferred by the enhanced anti-A3 activity of highly efficient Vif, concluding that Vif can mediate FIV replication potential and associated virulence in the domestic cat (66). Another study reported attenuated anti-A3 activity in low virulence FIVfca subtype B (286) . Indeed, subtype specific differences in domestic cat FIV virulence have been characterized (287, 288) and may depend on transcription and stability of Vif in addition to A3 counteractivity.

The survival of a Vif-deficient FIVpco genotype in Western Canada without evidence for hypermutation suggests escape of A3 editing via a Vif-independent mechanism. Other studies have documented incomplete A3 counteractivity by Vif (123, 278, 289-291), and *in vitro* studies have documented the emergence of A3-resistant HIV-1 variants in cell culture when T cells expressing A3 are infected with Vif-deficient HIV (292-294). Evidence for a novel A3 tolerance mechanism in which resistant HIV-1 variants packaged less A3 and accumulated fewer G-to-A mutations has been reported (292, 293). Another study found that evasion of lethal hypermutation in Vif-deficient HIV-1 mutant viruses is conferred by several point mutations in Env that attenuate fusogenicity, which in turn promotes increased Gag-Pol packaging and faster viral replication (294). The increased rate of viral replication ultimately minimizes the time

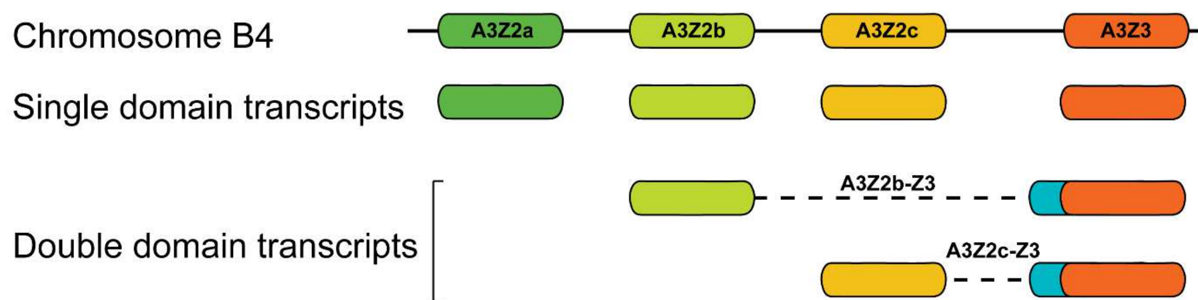
during which viral genomes exist in the single-stranded state targeted for deamination by A3 proteins (294). Additionally, adaptations in FIVfca Pol have recently been elucidated that allow for cleavage of A3 by the viral protease in released virions in absence of competent FIVfca Vif (286). Collectively, these findings suggest that although Vif is considered critical to productive lentiviral infection in the presence of restrictive A3, adaptations in alternate genes can compensate for deficient Vif in the molecular interplay between virus and cellular proteins.

Our findings suggest a naturally-occurring emergence of Vif-defective, A3-resistant FIVpco variants which evade lethal hypermutation by an unknown mechanism. Interestingly, the coevolutionary history of many felid species and their respective lentiviruses is evidenced by multiple A3Z3 haplotypes with differential resistance to Vif-mediated degradation. In the puma, however, A3Z3 is relatively divergent from other felids, and conserved under putative purifying selection, while FIVpco Vif has diversified across geographic regions and co-occurring FIVpco subtypes. In contrast to lentiviruses of domestic cats and primates, evidence suggests that some genotypes of FIVpco have adapted novel mechanisms for evading lethal hypermutation that have not measurably altered the A3Z3 locus. It is also possible that A3Z3 editing activity is unusually low in FIVpco infections, and restriction is principally achieved through other cellular proteins, negating the requirement for Vif-mediated degradation of A3Z3 and thereby explaining the absence of measurable anti-A3Z3 activity.

Elucidating mechanisms of host-pathogen coevolution remains a critical undertaking in the study of infectious disease emergence, cross-species transmission, and adaptation. In this work, we have documented that the puma A3Z3 locus is conserved despite frequent infections with FIVpco and other feline lentiviruses. We show that four residues within Vif evolving under episodic diversifying selection comprise part of a signature pattern of the PLV-1695 genotypes

circulating in Western Canada (Figure 3.5B). These four residues may be of particular interest to future functional studies investigating the relationship between A3Z3 and FIVpco Vif. We also recovered a contemporary FIVpco vif sequence with a 34 bp insertion in the C-terminus. Given that the C-terminus comprises known functional sites for interacting with the E3 ubiquitin ligase complex that mediates A3 degradation in the proteasome (Figures 3.2A and 3.2C), the insertion is an interesting finding that should be analyzed for its impact on anti-A3 activity. Additional genotypes, such as the highly homologous Florida FIVpco subgroup, should also be analyzed in functional assays to further elucidate relationships between puma A3Z3 and FIVpco Vifs. In conclusion, our findings set the stage for further probing of the A3-Vif system in the naturally occurring puma model, providing valuable insights into the coevolution of lentiviruses and host restriction factors.

A.



B.

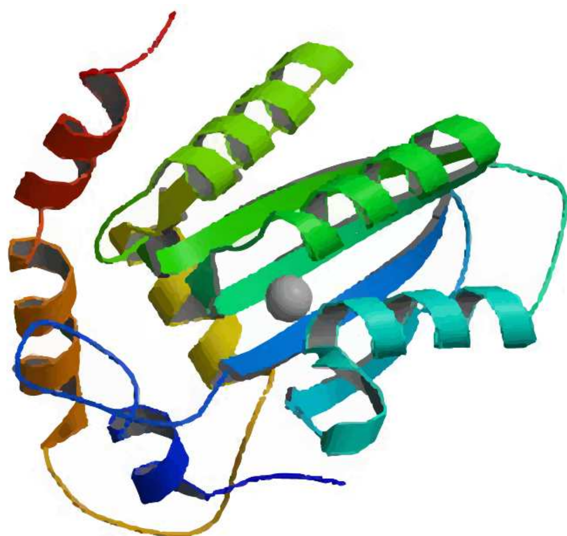


Figure 3.1A: The feline A3 locus is characterized by three copies of A3Z2 and one copy of A3Z3. Alternate splicing produces two 'double domain' variants that include a linker region, shown in blue. 3.1B: The A3Z3 protein is the feline ortholog of human A3H, crystal structure image derived from Ito et al. 2018 (258).

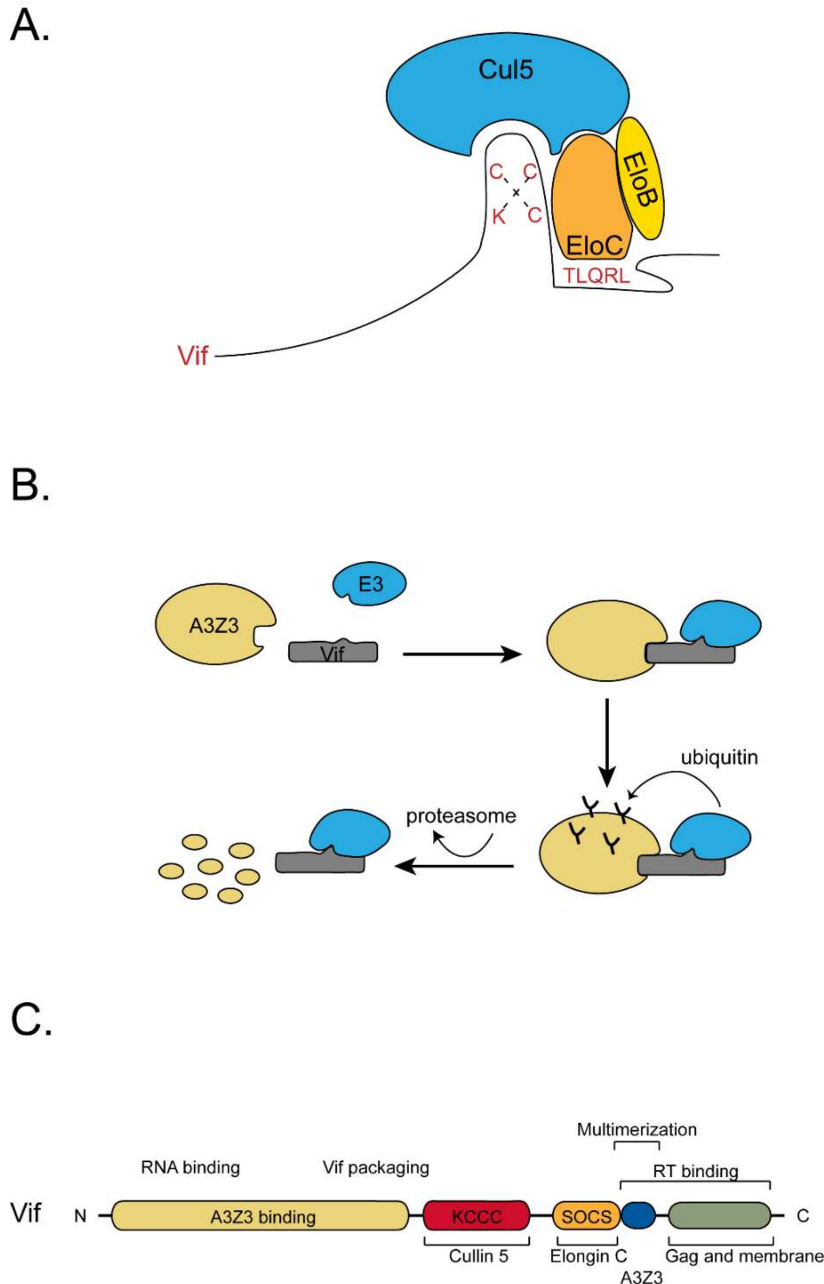


Figure 3.2: Vif recruits A3Z3 to the E3 ubiquitin ligase complex for proteasomal degradation. A. E3 ubiquitin ligase complex. Cul5 binds to FIV Vif through the conserved KCCC motif. ElocC binds through the SOCS (BC box) to the conserved TLQ motif of FIVpco Vif. B. A3Z3 proteasomal degradation occurs through Vif-mediated recruitment to the E3 complex. C. A3Z3 binding to Vif occurs through the N-terminus, while sites important for E3 recruitment are distributed in the C-terminus. Adapted from and Goila-Gaur, Ritu, and Klaus Strebel. "HIV-1 Vif, APOBEC, and intrinsic immunity." *Retrovirology* 5.1 (2008): 51 (2.3A and 2.3B) and Batisse, Julien, et al. "APOBEC3G impairs the multimerization of the HIV-1 Vif protein in living cells." *Journal of virology* (2013): JVI-03494 (2.3C).

A.

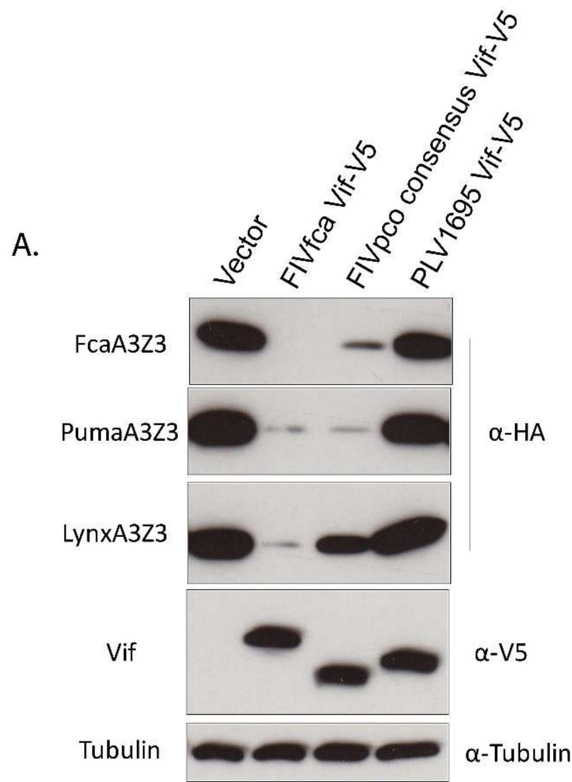
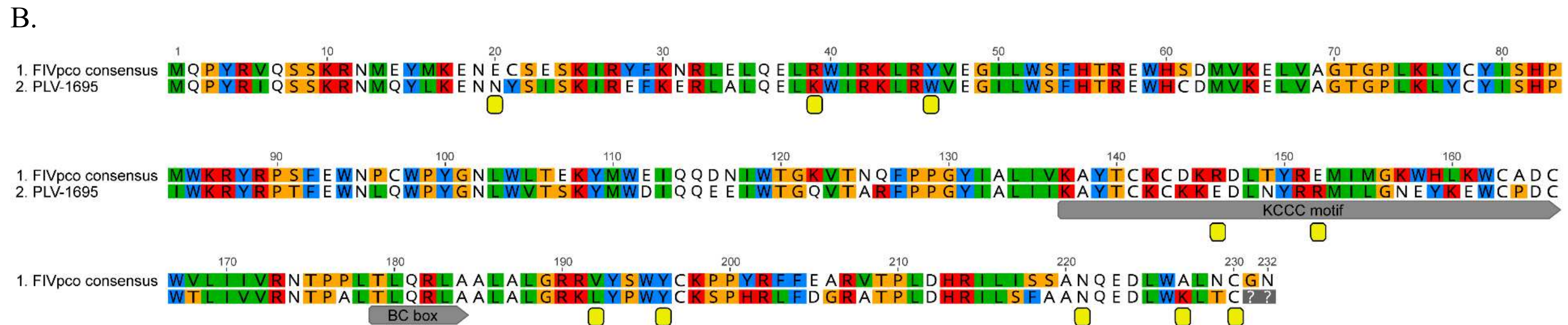


Figure 3.3: Interactions with A3Z3 differ between FIVpco genotypes. A. Immunoblot provided by Zeli Zhang and Carsten Münk. 293T cells were transfected with expression plasmids for domestic cat (Fca) A3Z3, puma A3Z3, and lynx A3Z3, together with FIVfca Vif, FIVpco consensus Vif or PLV1695 Vif. A3Z3s, Vifs and tubulin were visualized by immunoblot using anti-HA, anti-V5 and anti-tubulin antibodies. FIVfca Vif is active against A3Z3 of all three species. FIVpco consensus Vif is active against Puma A3Z3 and domestic cat A3Z3, but shows minimal activity against Lynx A3Z3. In contrast, PLV1695 Vif shows no anti-A3Z3 activity. B. FIVpco consensus Vif and PLV1695 Vif are compared in a pairwise amino acid alignment. Sites evolving under episodic diversifying selection as determined by the mixed effects model of evolution (MEME) are annotated with yellow squares. Functional sites for interactions with the E3 ubiquitin ligase complex are annotated in grey.

B.



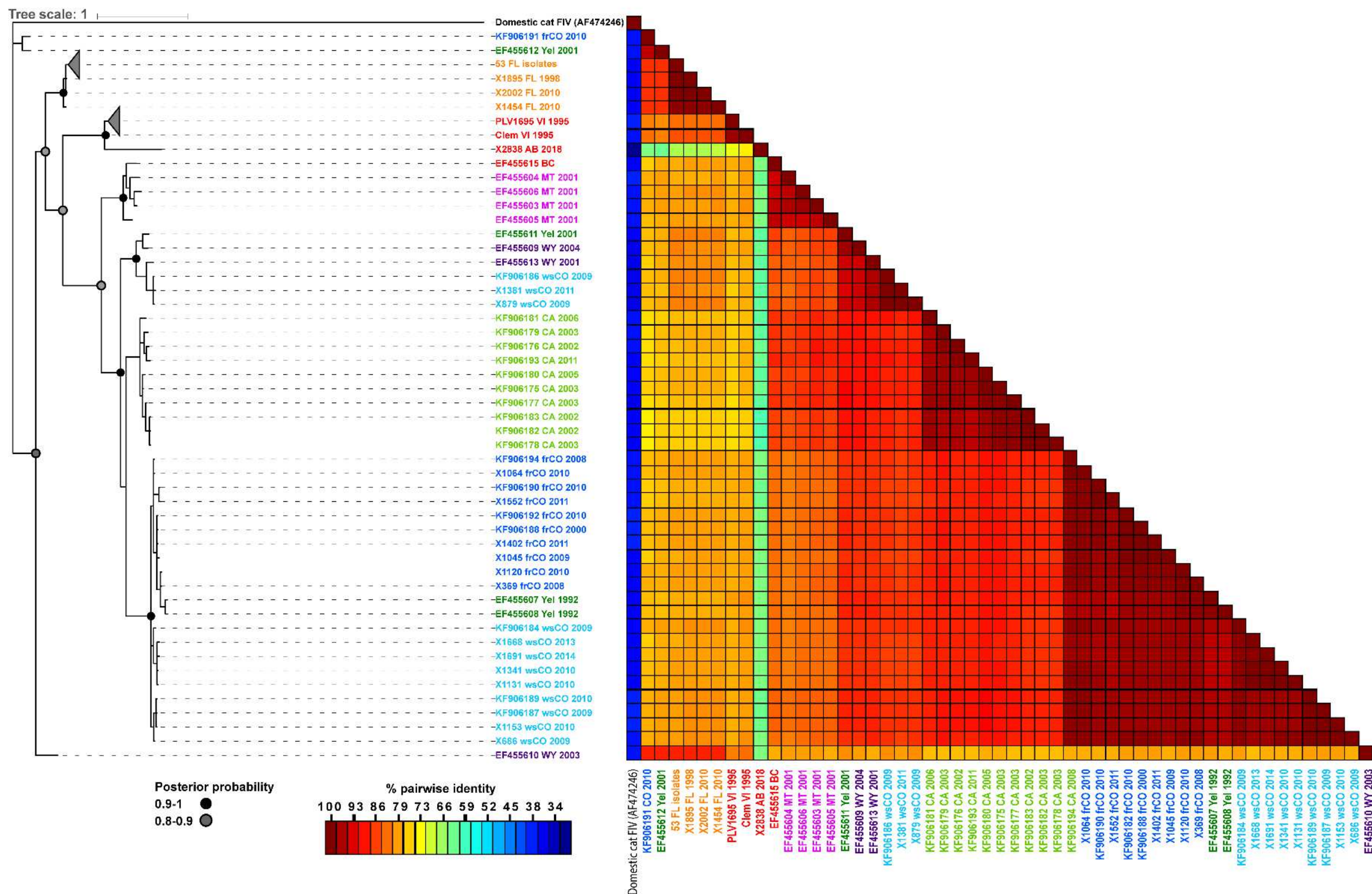
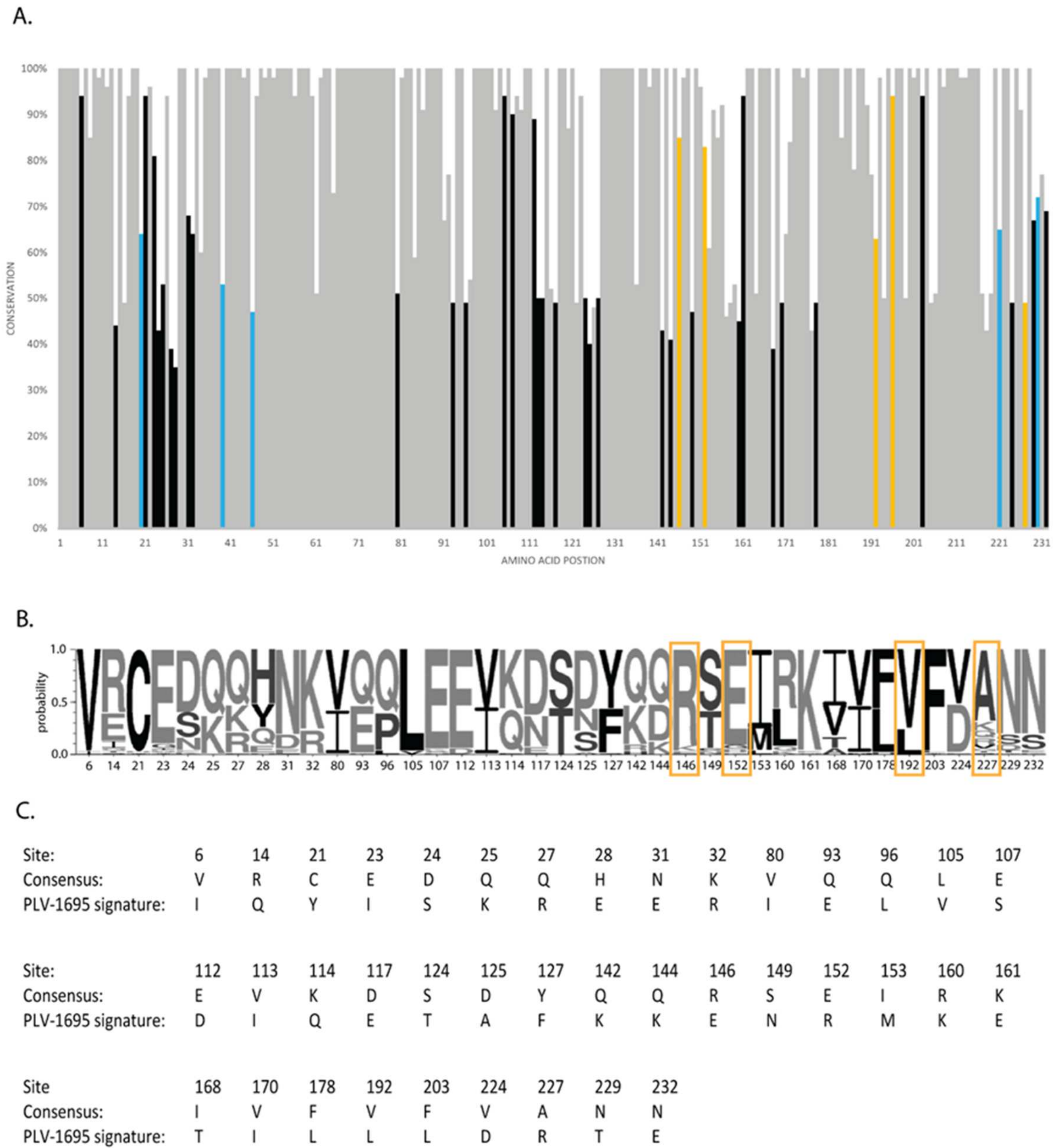


Figure 3.4: FIVpco Vif isolates comprise geographically associated subgroups. A Bayesian phylogenetic tree was constructed from Vif amino acid sequences recovered from 105 pumas. 53 highly homologous (>99% pairwise identity) sequences from Florida were collapsed into a single group. In vitro isolates from cultured PBMCs infected with PLV1695 were also collapsed. Pairwise identity is highest within geographic regions and ranges from ~65-100% across all groups. The large genetic distance between X2838 and other isolates is due to a 34 bp insertion in the C-terminus.



Site:	6	14	21	23	24	25	27	28	31	32	80	93	96	105	107
Consensus:	V	R	C	E	D	Q	Q	H	N	K	V	Q	Q	L	E
PLV-1695 signature:	I	Q	Y	I	S	K	R	E	E	R	I	E	L	V	S

Site:	112	113	114	117	124	125	127	142	144	146	149	152	153	160	161
Consensus:	E	V	K	D	S	D	Y	Q	Q	R	S	E	I	R	K
PLV-1695 signature:	D	I	Q	E	T	A	F	K	K	E	N	R	M	K	E

Site	168	170	178	192	203	224	227	229	232
Consensus:	I	V	F	V	F	V	A	N	N
PLV-1695 signature:	T	I	L	L	L	D	R	T	E

Figure 3.5: A signature pattern of genotype PLV1695 is detectable across the amino acid alignment of FIVpco Vif. A. Conservation of amino acids across the multiple sequence alignment is plotted for each site. Sequences from Western Canada were queried against all other 'background' sequences in the alignment. Sites at which the most common character in the query set differs from that in the background set are shown in black. Sites evolving under episodic diversifying selection based on MEME analysis are shown in blue. Sites under diversifying selection also identified as signature pattern sites by VESPA are highlighted in orange. B. Graphical representation of amino acid diversity across the multiple sequence alignment for signature sites detected by VESPA. Orange boxes highlight the four sites identified both by VESPA (signature pattern) and MEME (positive selection). C. Comparison of consensus versus PLV1695 residues at signature pattern sites.

Table 3.1 Animal ID and location for A3Z3 sequences.

Animal ID	Location	Animal ID	Location
FL_zoo	FL	x1587	CA
x2783	TX	x1589	CA
x2787	TX	x1643	CO
x1131	CO	x1679	CO
x114	CA	x1756	NM
x1182	CA	x1757	NM
x128	CO	x1815	FL
x132	CO	x1818	FL
x1349	NV	x1827	FL
x1350	NV	x1833	FL
X1351	NV	x1834	FL
x1386	NV	x2015	FL
x140	CO	x2018	FL
x1402	CO	x2045	FL
x1408	CO	x2048	FL
x1433	FL	x218	CA
x1448	FL	x2809	AB
x1554	CO	x2815	AB
x1565	NV	x2816	AB
x1580	CA	x2817	AB
x1582	CA	x2819	AB
x1585	CA	x2820	AB
x2841	AB	x2825	AB
x686	CO	x2834	AB
x879	CO	x2838	AB
x2840	AB		

Table 3.2: Primers used for sequencing of A3Z3 exons

A3Z3 coding region	Primer Name	Primer Sequence
Exon 2	Z3ex2-F1	5'-AGGAGTTGGGTTCAGGAGGT-3'
	Z3ex2-R1	5'-GCATTCTGCTGGAGGGAAC-3'
Exons 3&4	Z3ex34-F2	5'-CAGCAGGGACAGCTTCTCA-3'
	Z3ex34-R2	5'-CTGCCTTGTAACCAAAAATTC-3'
Exon 5	Z3ex5-F5	5'-TCTCATCAGCTTTCCGTTTCC-3'
	Z3ex5-R5	5'-CGACGAAATGATCCAGCTATTCT-3'

Table 3.3: Isolate PLV-1695 does not show evidence of A3Z3-mediated hypermutation despite the inability of Vif to degrade A3Z3. A. A3Z3-induced mutations are described as sites where G→A is preceded by any upstream nucleotides and followed by downstream RD (‘R’ = G or A; ‘D’ does not = C). Control mutations are defined by G→A preceded by any upstream nucleotides and followed by downstream YN|RC (‘Y’ = C or T; ‘N’ = any of AGCT). Rate ratio ((muts/potential muts)/(controls/potential controls)) <1.0 reveals that mutation patterns consistent with A3Z3 editing activity are less frequent than control mutations across the PLV-1695 genome. B. G→A mutations are not disproportionately high in PLV-1695 relative to the average number occurring in all FIVpco whole genomes analyzed.

A.

Pattern	**Upstream**	**From →**	**Downstream**
‘Mut'	…	G → A	RD…
‘Control	…	G → A	YN|RC…

	Muts:	**Out of Potential Mut**	**Controls:**	**Out of Potential Controls**	**Rate Ratio**	**Fisher Exact p-**
PLV-1695:	46	1082	44	769	0.74	0.9398

B.

Mutation	PLV-1695 count	Mean
GG->AG	11	28
GA->AA	43	65
GC->AC	18	23
GT->AT	20	21
G->A	93	139
G->C	13	18
G->T	15	21
A->G	214	255
A->C	57	88
A->T	104	168
C->A	35	58
C->T	71	80
C->G	12	16
T->A	69	144
T->C	138	147
T->G	25	29
GAPS	209	190

CONCLUSIONS

Persistent nonpathogenic infections such as FIV in pumas provide a unique window into the adaptive relationship between a pathogen and its host. While viruses exist as measurably evolving populations, there is great variation even within viral families among evolutionary rates, determined by endless variables interacting at minute to vastly broad scales. Ancient viral infections have the potential to elucidate the life history of mammalian hosts, serving as a useful tool in the study of broadly ranging, solitary carnivores such as pumas of North America. However, viral phylogenetics and other molecular analyses cannot be decoupled from the ecological covariates of host-pathogen dynamics, and such studies are best approached through an interdisciplinary framework that unifies the diverse perspectives of ecologists, evolutionary biologists, wildlife managers, and microbiologists.

The collective results from this work provide evidence for ancient host-pathogen coevolution of the puma and FIVpco. In contrast, FIVlru is poorly adapted to the puma and transmitted primarily through spillover from bobcats. We conclude that FIV genetic diversity varies across North American puma populations, and likely correlates with migrations and dispersals, which facilitate movement of FIV genotypes and permit recombination in coinfected individuals. We provide evidence that FIV dynamics in the Florida panther have been altered by active management of this endangered subspecies and show that the effective number of FIVpco infections historically increased in correlation with population expansion of the host. Lastly, we provide evidence for a novel yet unidentified means of evasion of intrinsic viral restriction in at least one genotype of FIVpco. Future aims of this work will expand analyses to incorporate additional populations, such as modern Texas pumas, and more thoroughly investigate genotype variation in Vif-A3Z3 interactions. We will additionally explore intrahost diversity of FIV in

pumas by adapting the techniques described here to viral quasispecies and single nucleotide variant analyses.

Although our questions were specific to pumas, the approaches described here can be applied to other systems and models that aim to link macro- and mircoevolutionary processes at the interface of pathogen and host. Methodologies developed and adapted for this work can be applied to other field studies, especially those rooted in opportunistic collection of both modern and historic biological data. The approaches described here can help address limitations such as DNA degradation and sequencing of low copy number templates from archival samples. We hope that this work will inform additional studies that seek to elucidate determinants of host-pathogen interactions in naturally-occurring systems across diverse ecosystems and broad spatiotemporal scales.

Each of these chapters began with a question and ended with a somewhat surprising answer that generated deeper questions. I have learned that unexpected results are drivers of intelligent thought and scientific design born out of much collaborative effort. In the face of constant change, it seems the only certainty is to contemplate uncertainty. Darwin's tangled bank lives on. "There is grandeur in this view of life", after all.

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