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GENETIC DETERMINANTS OF VIRULENCE IN EMERGING VIRUSES OF NATURAL FELID POPULATIONS

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# GENETIC DETERMINANTS OF VIRULENCE IN EMERGING VIRUSES OF NATURAL FELID POPULATIONS

By

Meredith A. Brown

A DISSERTATION

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ABSTRACT

GENETIC DETERMINANTS OF VIRULENCE IN EMERGING VIRUSES OF NATURAL FELID POPULATIONS

By

Meredith A. Brown

Cats are a fascination. Whether our beloved pet cats providing companionship and play to our daily lives, or wild cheetahs questioning our choices as human impact continues to push wild species to extinction ...cats inspire us to advance the fields of wildlife conservation and veterinary health.

There is an additional developing rationale for studying disease ecology in the cat family. With the annotation of the complete cat genome, the field of comparative genomics has raised the prospects for developing the cat model as a comparative tool for human diseases. The members of the family *felidae* harbor many deadly infectious agents modeling human scourges including feline coronavirus, a relative to the human SARS virus; and feline leukemia virus and feline immunodeficiency virus, both retroviruses used as a model to study HIV AIDS and cancer in humans.

The diversity in habitat, behavior, and natural history of the 37 members of the cat family evolutionarily shape an array of host genetic determinant responses to these infectious diseases in terms of host-immune interactions in a natural setting. Here I use the tools of molecular genetics, virology, phylogenetics, and clinical pathology to better understand the mechanisms of pathogenesis in cat species infected with these deadly agents in nature. Through phylogenetic study, I describe the patterns of virulence in feline coronavirus infection (FCoV) in domestic cats, the emergence of feline leukemia virus (FeLV) in the previously naïve Florida panther population, and the first occurrence of feline immunodeficiency virus (FIV) in Asia in the wild Mongolian Pallas' cat population. My results unseat several paradigms relating to pathogenic mechanisms and highlight the opportunities afforded by studying natural cat models of deadly scourges using the tools of molecular genetics. The rigorous sampling strategy employed in these studies, including isolating FeLV and FIV virus from nondomestic cat populations, and isolating both virulent and avirulent strains of FCoV from cats, is an important lesson for studying viral dynamics of emerging diseases in a natural context.

## DEDICATION

I dedicate my thesis to
my husband, James, my sister, Allyson, my parents, Gail and Al
and my advisor, Dr. Stephen J. O'Brien.

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based on 1,698 bp of env sequences. B) Midpoint rooted maximum-likelihood phylogram based on 463 bp of 3′ long terminal repeat (LTR) sequences. Consensus FeLV-Pco sequences of clones generated from 5 env and 4 LTR panthers and reference domestic cat sequences are shown. The number of FeLV-Pco–cloned PCR products used in each consensus sequence is indicated in parentheses. The arrow indicates the monophyletic clade of all FeLV-Pco sequences. A similar topology, including the monophyletic clade, was obtained by using the different FeLV-Pco clone sequences rather than a consensus. The year of panther sampling is indicated as a suffix, e.g., Pco-1088-04 was sampled in 2004. Where maximum-likelihood tree was congruent with maximum parsimony tree, branch lengths are indicated below branches. Number of homoplasies is indicated after the branch length. Bootstrap values are shown (maximum parsimony/minimum evolution/maximum likelihood). The score (–ln likelihood) of the best maximum-likelihood tree was env 3615.01706, LTRs 1836.05922 (best tree found by maximum parsimony: env length = 221, consistency index [CI] = 0.941, retention index [RI] = 0.963; LTR length = 132, CI = 0.871, RI = 0.787).

## INTRODUCTION

Because of human pressures, it is estimated that sometime during the next century, the last wild free-ranging cats will disappear (Quamman 2003). Lions, leopards, pumas and their relatives will only exist behind chain-linked fences in zoos. Genetic attenuation of these increasingly docile captive animals will change the nature of these unique species forever and children will learn, at first in surprise and then in disbelief of the days when these creatures roamed free. However, a growing consciousness in fighting this trend of human-caused extinction has emerged and activists are engaging communities globally to promote tolerance of predators and find ways in which humans and predators can coexist. Nearly every member of the cat family *Felidae*, including 37 species, is considered endangered or threatened with extinction according to both CITES and IUCN. The loss of the vast land spaces necessary for wild cat species is thought of as the principal threat to their survival due to the continuous expansion of human development, marginalizing these species to smaller fragmented populations. Contact of predators with human settlements give rise to unacceptable threats to humans including livestock and human fatalities.

Besides acts of predations, the close contact between wildlife and human settlement also facilitate the cross-species spread of infectious agents among wildlife, domestic and human populations. The study of wildlife disease ecology has blossomed in the last thirty years, with agents such as the Ebola virus, *Borrelia burgdorferi* (agent of lymes disease), *Bartonella henselae* (agent of cat scratch fever), Hendra virus, West nile virus, Nipah virus, and SARS coronavirus

identified (Bender et al 2006). These pathogen discoveries, at the interface of wildlife ecology, veterinary and human medicine, stemmed mainly from outbreaks of new diseases affecting domesticated livestock and humans and later identified to have arisen from wildlife reservoirs (Heeney 2006). Besides the zoonotic potential, infectious agents can threaten the survival of endangered wildlife species as the free-ranging populations dwindle due to habitat loss and genetic depletion and exposure to new urban pathogens (Bradley et al 2007).

Pathogenesis from a virus in an individual and/or population is a complex event and is likely attributable to virus, host, and environmental factors. The recent advances in the study of genomics has given rise to specific abilities to now study these virus and host factors using molecular phylogenetic tools and begin to reveal the patterns of transmission and pathogenesis in naturally studied populations. In addition to the advances to conservation and veterinary population medicine afforded by such pursuits, these studies also allow a rare opportunity to study and document naturally occurring viral dynamics in a "natural laboratory" providing comparative genomics data in similar disease processes known to plague humans (O'Brien 2003). This thesis focuses on three molecular genetic studies of viral pathogens found in felids: feline coronavirus (FCoV), feline leukemia virus (FeLV), and feline immunodeficiency virus (FIV).

**FCoV:** Since the outbreak of SARS in 2003, the field of coronvirology, and specifically coronavirus genomics, has grown. GenBank now reports the full-length sequence of over twenty species of coronavirus, including two full-length sequence of FCoV (Dye and Siddell, 2005, 2007). Coronaviruses are a

large family of enveloped, single stranded, positive sense, non-segmented RNA viruses. Approximately 30 kilobases in length, this virus is the largest viral RNA genome known (Rottier 1995). The first 2/3 of the coronavirus genome encodes the replicase genes ORFs 1a and 1b. Proteolytic processing of these polyproteins, which are mediated by viral cysteine proteinases, produce non-structural proteins, some of which are responsible for replicating the viral genome and/or generating a nested set of subgenomic mRNAs to express all the other ORFs in the genome (Thiel et al., 2003; Ziebuhr, 2004). The ORFs for the structural proteins, spike (S), envelope (E), membrane (M) and nucleocapsid (N), are encoded in the remaining portion of the genome. Each coronavirus encodes different number of non-structural accessory proteins and the predicted sequences of these proteins do not share high level of homology (Rottier 1995). The SARS-CoV genome encodes the largest number of accessory proteins (eight) while humanHCoV-229E, pig transmissible gastroenteritis virus (TGEV), bird infectious bursitis virus (IBV), mouse hepatitis virus (MHV), and feline coronavirus (FCoV) encode two, two, four, two, and five, respectively. It is suspected that these non-structural accessory proteins (NSPs) are involved with virulence in SARS (Tan et al 2006; Akerstrom et al 2007).

Feline infectious peritonitis (FIP), first described in 1963 (Holzworth 1963), is an uncommon, fatal, and progressive viral disease of cats associated with FCoV. However, the pathogenesis of FIP is poorly understood and to date there is no diagnostic test, treatment, or effective vaccine available. FCoV infection is extremely common, with seroprevalence estimates from 40-100% of domestic

cats worldwide (Addie and Jarret 1992, Sparkes et al 1992, Addie 2000), the higher prevalence found typically in catteries and over-crowded shelter situations. Although most infections with FCoV are asymptomatic or limited to mild gastrointestinal disease (referred to as FECV biotype), a small percentage of FCoV infected cats develop the fatal multi-systemic, immune mediated disease of FIP (FIPV biotype).

Widely accepted, though never explicitly proven, is an *in vivo* mutation transition hypothesis (Poland et al 1996, Vennema et al 1998); also referred to as the 'internal mutation theory" (Dye and Siddell 2007) where *de novo* mutation in avirulent strains of FCoV give rise to virulent strains which are able to spread systemically and lead to FIP pathogenesis. Mutational transition in viral pathogenesis has been shown in HIV infection, where specific amino acid changes in the envelope gene determine which coreceptor (CCR5 or CXCR4) is used and hence virus success in cell entry (Hartley et al 2005). Similarly, key amino acid changes in the spike gene lead to virulence in transmissible gastroenteritis virus (TGEV) (Ballesteros et al 1997 Sanchez et al 1999) although the exact switch to pathogenesis in TGEV is still unresolved (Paul et al 1997; Saif 2006).

A dual circulating avirulent and virulent strains is an alternative hypothesis of viral pathogenesis. In this hypothesis, both benign and pathogenic strains of a virus circulate in a population, and those individuals exposed to the virulent strains, with the appropriate predisposition, develop disease sequelae. Dengue hemorrhagic fever is such an example, where it has been shown that four viral

strains circulate worldwide and individuals exposed for a second time to a the virus of a different strain, mount an inappropriate immune response and exhibit pathology consistent with immune-mediated vasculitis (Mongkolsapaya et al 2003). The zoonotic equine venezuelan encephalitis virus is another example where virulent and avirulent strains of the alphavirus have both been shown to circulate and ecological and epidemiological factors have been identified which contribute or constrain the frequency of disease sequela in equids and humans (Anishchenko et al 2006).

In chapter one, I test these two hypotheses: *in vivo* mutation hypothesis versus the dual circulating virulent and avirulent hypothesis, phylogenetic analysis of cloned viral sequences isolated from naturally occurring FIPV and FECV isolates was performed. Isolates were obtained from domestic cats sampled in Maryland catteries from 2004-2006 including virus sequence obtained from one cat (Fca-4590) seven months prior to and at the onset of FIP. Sequence from *Pol replicase*, *spike*, *membrane*, and non-structural proteins (NSP) 3a-c and 7b viral genes were obtained. Monophyletic groupings of the majority of FIPV and FECV biotypes were found in *membrane* and *NSP7b* in both nucleotide and amino acid phylogeny supporting a circulating avirulent and virulent strain hypothesis and failing to support the widely accepted *in vivo* mutation hypothesis (Poland et al 1996; Vennema et al 1998).

**FeLV:** First discovered in a cat colony in Scotland in 1964, the description of feline leukemia virus (FeLV) in nature provided the first evidence that a horizontally transmitted retrovirus could cause cancer (Hardy et al 1969, Jarrett

1970). Since then, FeLV has provided unique contributions to our understanding of the role of retroviruses in naturally occurring disease including early evidence that antiproliferative diseases appeared to be caused by retrovirus infection and the description of multiple oncogenes identified in naturally occurring cases of fibrosarcomas and lymphomas (Hardy et al 1973; Jarrett et al 1973). These important discoveries led to our current understanding of the retroviral etiology for AIDS (Mullins and Hoover 1990).

FeLV is a gammaretrovirus of the *retroviridae* family measuring 8.2 kilobase in full length mRNA (Mullins and Hoover 1990). The genomic structure contains the 5'- *gag- pol-env-3'* open reading frame genes flanked by two long terminal repeats (LTRs) found in all retroviruses. None of the accessory genes associated with the human and simian lentiviruses have been found to occur in FeLV (Hardy 1990).

FeLV occurs in both an exogenous and endogenous form in the domestic cat. Exogenous FeLVs are oncogenic retroviruses transmitted horizonatally while endogenous FeLV are transmitted vertically as part of the germline and are transmitted from parent to offspring as integral parts of chromosomes (Benveniste and Todaro 1975; Koshy et al 1980). Endogenous FeLVs are only found in wild cat species in the *Felis* family and are absent in species of other lineages within the Felidae. Thus, endogenous FeLVs are believed to have entered the germ line after the initial radiation of lineages in the cat family but before the radiation of the domestic cat lineage species (Reeves and O'Brien 1984; Roca et al 2004).

FeLV induces degenerative, proliferative, and malignant hematologic disorders in its natural host, the domestic cat (Mullins and Hoover 1990). Exogenous FeLV occurs in nature in four subgroups (A,B,C, and T) that are distinguished genetically by sequence differences in the *env* gene and functionally by requirements for cell entry (Overbaugh and Bangham 2001). FeLV-A is a weakly pathogenic highly transmissible subgroup that is thought to predominant in horizontal transmission in cat-to-cat spread in nature and associated with induction of thymic lymphomas of a T-cell origin (Phipps et al 2000a, Quackenbush et al 1990). FeLV-B, C, and T are associated with lymphomas (Donahue et al 1991), anemia (Neil et al 1991) and immunodeficiency disease (Overbaugh and Bangham 2001), respectively. FeLV B,C, and T are thought to arise from FeLV-A through recombination with the endogenous FeLV during viral replication in infected animals (Mullins and Hoover 1990).

A novel FeLV-A species, designated FeLV-945, has recently been identified at the predominant species in non-T-cell diseases in a temporal and geographic cohort of naturally infected cats isolated by veterinarian Dr. Murry Gardner in Pasedena, California from essentially one veterinary practice over a six year period (Chandhasin et al 2004). FeLV-945 has distinct sequence elements in both the LTR and envelope gene (Chanhasin et al 2005a,b). FeLV-945 LTR contains a unique sequence motif comprised of a single copy of a transcriptional enhancer followed downstream by a 21-bp sequence that is triplicated in tandem (Chandhasin et al 2004). The two junctions formed between

the three 21-bp sequences was shown to form a binding site for transcription factor c-Myb with the consequential recruitment of coactivator CREB-binding protein (Finstad et al 2004) in *in vitro* gene reporter assays. FeLV-945 envelope gene was shown to have a distinctive sequence. While clearly of exogenous origin and closely related to FeLV subgroup A, FeLV-945 envelope sequence was observed to have a different amino acid sequence from that of prototype FeLV subgroup A to a larger extent than any other known FeLV sequence. Through the engineering of recombinant virus chimeras and challenge *in vivo* studies, it has been shown that these changes in the envelope gene can alter the disease spectrum of FeLV (Chandhasin et al 2005a).

Between 2002 and 2005, Florida panthers (*Puma concolor coryi*) with ranges in or near the Okaloacoochee Slough State Forest experienced an outbreak of FeLV (Cunningham et al 2008). Following lymphadenopathy, anemia, septicemia, and/or weight loss, five panthers died. Panther genetic heritage (pure Florida panther versus Texas/Florida puma intercross) and FIV co-infection were not associated with disease outcome following FeLV infection. Genetic analysis of panther FeLV, designated FeLV-Pco, determined that the outbreak likely derived from a single cross-species transmission from a domestic cat. The FeLV-Pco virus was closely related to the domestic cat exogenous FeLV-A subgroup in lacking recombinant segments derived from endogenous FeLV. FeLV-Pco sequences were most similar to the well-characterized FeLV-945 strain, which is highly virulent and strongly pathogenic in domestic cats due to unique LTR and envelope sequences. These unique features may also

account for the severity of the viral outbreak following cross-species transmission to the Florida panther (Chapter two).

**FIV:** Feline immunodeficiency virus (FIV) is related to other lentiviruses known to infect primates (human and simian immunodeficiency virus), horse (equine infectious anemia), cattle (bovine immunodeficiency virus), and sheep and goats (caprine arthritis encephalitis virus). FIV was first discovered in 1987 in a cattery in California that had been experiencing morbidity and mortality of unknown origin (Pedersen et al 1987). Since this initial report, retrospective serosurvey of archived samples have revealed that the virus has been present in domestic cats since at least 1968 (Bendinelli et al 1995). FIV infection in domestic cats results in immune dysfunction, analogous to HIV infections in humans, characterized by early flu-like symptoms, chronic wasting disease, neurological disease, increased susceptibilities to opportunistic infections, and death (Bendinelli et al 1995,Willett et al 1997). A recent comprehensive serosurvey of serum and lymphocyte specimens found that 11 free-ranging species of cat are infected with FIV (Troyer et al 2005). The clinical effects of FIV in these wild cat species is less clear and the majority of FIV infection in wild felids appear clinically silent (VandeWoude and Apetrei 2006). However, recent studies of FIV infected pumas and lions have revealed CD4 depletion (Bull et al 2003; Roelke et al 2006), and documents clinical symptoms consistent with immunodeficiency (Roelke et al 2008 in preparation).

FIV is a lentivirus of the *retroviridae* family, distinctive from other *retroviridae* morphologically based on the presence of a cone-shaped core, or

nucleoid (Bendinelli et al 1995). The genome organization includes structural genes 5'- *gag- pol-env-3'* flanked by two long terminal repeats (LTRs) as well as a number of accessory genes occurring in various locations according to virus strain (Pecon-Slattery et al 2008, VanDeWoude and Apetrei 2006). Monophyly of FIV proviral sequence within various species of *Felidae* (Troyer et al 2005) suggests that FIV transfer between cat species is an infrequent event. FIV is endemic in the large African carnivores and most South American felids. The free-ranging Pallas' cat is the only known species from Asia that harbors a species-specific strain of FIV (FIV-oma) (Barr et al 1997; Troyer et al 2005). Phylogenetic analysis of proviral *RT-Pol* shown here from eight FIV-oma isolates suggests a recent emergence of the virus, possibly from the African cheetah or leopard, which harbor its closest known relatives, FIV-aju and FIV-ppa.

## CHAPTER ONE

## Investigating the viral genetic determinants of pathogenesis in feline infectious peritonitis: A study of free-ranging cat isolates

### Introduction

Feline infectious peritonitis (FIP) is an uncommon, fatal, progressive immune augmented disease of cats caused by infection with feline coronavirus (FCoV). Although FCoV is common in most domestic and feral cat and non-domestic cat populations world-wide (seroprevalence from 40-100%), less than 10% of FCoV seropositive cats will develop FIP (Addie 2000) (Kennedy et al 2002). FIP tends to occur most frequently in cats less than two years of age or, less commonly, in geriatric cats (Foley et al 1997a). The clinical manifestation of FCoV infection can present as one of two biotypes: (1) the pathogenic disease manifestation or FIP (FIPV) and (2) the more common, benign or mild enteric infection (FECV) (deGroot-Mijines et al 2005). Specific genetic determinants of these biotypes have yet to be discovered. There is no treatment, effective vaccine, nor effective diagnostic protocol that can discriminate the avirulent FECV from the pathogenic FIPV. Some cats infected with FCoV show no evidence of disease but are thought to be important carriers of virus, which can be pathogenic in other cats (Foley 1997b, Addie 2000).

FIP pathology is characterized typically by severe systemic inflammatory damage of serosal membranes and widespread pyogranulomatous lesions, occurring in lung, liver, lymph tissue, and brain (Weiss and Scott 1981).

Evidence suggests that the host immune system is crucial in the pathogenesis as profound T-cell depletion from the periphery and lymphatic tissues, changes in cytokine patterns are observed in end stage FIP (de Groot-Mijnes et al 2005; Kipar et al 2001; Kipar et al 2006) and the clinical finding of hypergammaglobunemia-associated FIP is indicative of virus-induced immune dysregulation (Hunziker et al 2003).

An outbreak of FIP occurred in 1982 in a population of cheetahs at an Oregon wildlife park and proved to be highly pathogenic, causing symptoms of fever, severe diarrhea, jaundice, and neurological spasms. The outbreak in cheetahs was far more virulent than expected, causing 90% morbidity and over 60% mortality (O'Brien et al 1985; Heeney et al 1990). Using archival samples saved from the 1982 outbreak, initial sequence phylogenetic analyses (Pearks Wilkerson 2004) showed the cheetah FIPV strains grouping with domestic cat FCoV and a close interspersed polyphyletic arrangement of the cheetah strains interspersed with domestic cat FCoV strains. Given this high genetic similarity between domestic cat FCoV and cheetah FIPV, and the fact that several lions (*Panthera leo*) at the wildlife park became infected with the virus simultaneously but did not succumb to FIP, the authors pointed to the paucity of genetic diversity, including monomorphism at the cheetah major histocompatibility complex (O'Brien and Yuhki 1999, O'Brien et al 1985) as a plausible determinant for the extremely high morbidity and mortality in cheetahs.

Comparative studies in other coronaviruses provide clues of molecular viral determinants of pathogenesis and plausible targets in the search for genetic

signatures correlating with FIPV pathogenesis. Murine hepatitis virus (MHV) causes hepatic and central nervous system diseases of varying severity depending on the strain and is therefore used as a model for hepatitis, viral encephalitis, and demyelination (Navas and Weiss 2003). Using a reverse genetics system whereby chimeric MHV viruses were engineered and virulence of specific genes was assayed, the nucleocapsid gene was implicated in hepatitis pathology (Navas-Martin et al 2007), the envelope gene in apoptosis (An et al 1999), and the spike gene in neurovirulence (Phillips et al 2002).

Determinants of FIPV pathogenesis have yet to be discovered. Phylogenetic study of the NSP 7b gene in a small group of cats exposed to FCoV, found relatedness a consequence of geographic locale, rather than clinical disease outcome (Vennema et al 1998). This, and comparative aforementioned study of *in vivo* mutational transition in the pig (Sanchez *et al.*, 1999) led to an *in vivo* mutation hypothesis (Vennema et al 1998; Poland et al 1996), also called the "internal mutation hypothesis" (Dye and Siddell 2007) whereby *de novo* virus mutation occurs *in vivo* giving rise to virulence. Together with *in vitro* studies previously describing the FIPV biotypes affinity for macrophages (Stoddard and Scott 1989) in contrast to FECV biotypes, the hypothesis was extended to propose that the enteric coronavirus (FECV) undergoes a mutational shift in the gastrointestinal system, thus allowing infection of macrophages and systemic dissemination and fatal disease manifestation. Subsequently, in the last decade, elegant reverse-genetics systematic studies have engineered chimeric viruses based on this paridigm

(Haijema et al 2003), in the hopes of revealing the key transitional viral determinants of pathogenesis in FIP. As of yet, these pursuits have been unsuccessful. Further, it has been shown that circulating virus has been found systemically in cats clinically displaying the FECV biotype (Can-Sahna et al 2007) and recent comparative genomic analysis of structural and non-structural viral genes isolated from the intestinal tract and the liver of an infected cats was essentially identical (Dye and Siddell 2007).

This study aims to phylogenetically test the *in vivo* mutation versus the circulating virulent/avirulent hypotheses in the pathogenicity of FIP in the cat. I develop a study of naturally occurring FECV and FIPV using molecular genetic tools by collecting samples from field cases of FECV and FIPV. Additionally, I survey the viral genetic diversity and dynamics and determine genetic signatures associated with pathogenesis in FIP. Phylogenetic analysis of viral gene sequences would be paraphyletic for FIPV and FECV biotypes if the *in vivo* mutation hypothesis is supported, and monophyletic if the circulating virulent/avirulent hypothesis is supported (Figure 1).

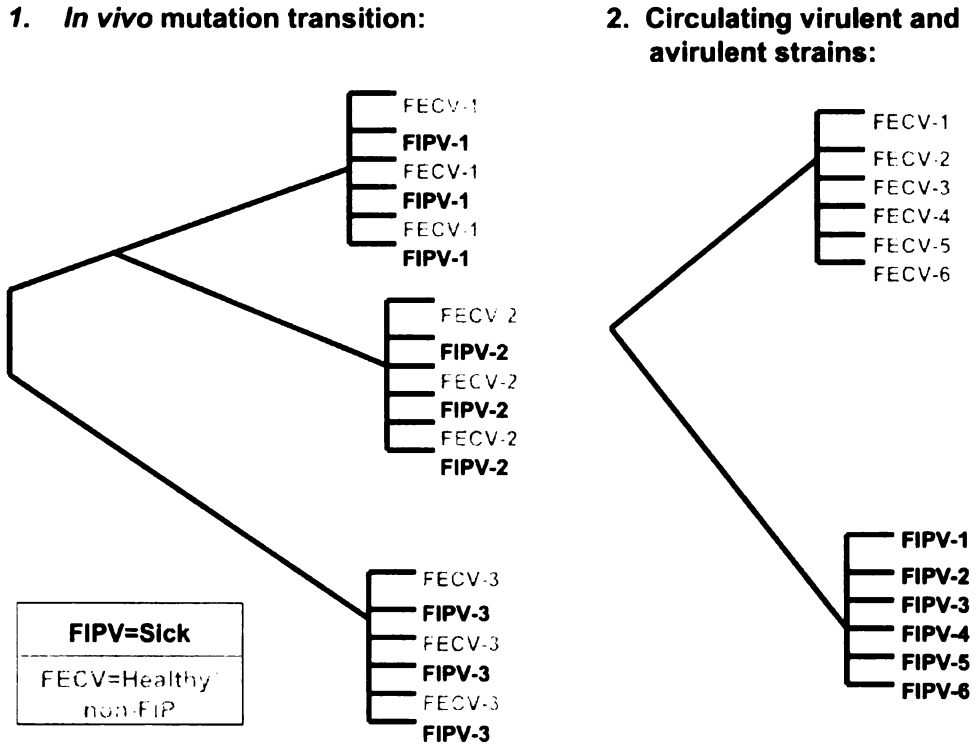



**Figure 1:** Theoretical phylogenetic relationships in the *in vivo* mutation hypothesis versus the dual circulating virulent/avirulent hypothesis. Number represents individual cat with either FIPV (sick) or FECV (healthy/non-FIP) biotype. Evidence in this paper supports the circulating dual virulent and avirulent strains.

## Materials and Methods

**Sampling**: A total of 56 live, euthanized, or recently deceased domestic cats were examined and sampled through Maryland veterinary hospitals from 2004-2006 (Table 1). Physical examination of the live cats including body condition score, auscultation of heart and lungs, palpation of abdomen, and evaluation of external eyes, ears, nose, and mouth including mucous membrane quality was performed. Ocular and nasal discharge was a common clinical clinical finding

and considered normal. The only abnormal findings (AB) consisted of heart murmurs (Fca-4596 and Fca-4609) noted in 2004. Blood (3-6 mls) was collected by local veterinarians via venipuncture from manually restrained or anesthetized domestic cats. Feces was obtained from the rectum via cotton swab and frozen in 0.5 cc PBS (approx 10% fecal suspension). Cats from the Weller farm were microchipped by attending veterinarian for identification for repeat fecal sampling of individual cats.

For the euthanized and recently deceased cats, gross necropsy examination and sample collection was performed at times ranging from a few minutes to two hours after death. Fluid accumulation was noted present (effusive) or absent in either abdominal or thoracic cavity. Samples from liver, spleen, mesenteric lymph node, kidney, jejunum, and colon were taken, fixed in 10% buffered formalin, and routinely embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin (HE). Tissues were also flash frozen in liquid nitrogen (-220°C) for RNA extraction and stored at either -220°C or -70°C (Table 1).

**Table 1: Clinical, demographic, and FCoV viral RT-PCR success data from 56 domestic cats sampled in Maryland from 2004-2006**

FIPV cases are shaded in grey in the cat ID column.

αFCAC=Frederick County Animal Shelter; NM=New Market Animal Shelter

^M=male; F=female; *Age in years unless otherwise stated; mo.=months

Grey shading in the columns listed 2004, 2005, and 2006 indicate sampling event. Within these shaded blocks, RT-PCR and cloned sequencing success, producing atleast one sequence, is noted for 5 genes in this study: membrane (M), NSP 7b (7), RT-Pol (P), Spike (S), and NSP3a-c (3)

Status in "07: E=euthanized; H=healthy; D=dead

#FCoV=feline coronavirus; IHC=immunohistochemistry; histo=histopathology; neg=negative; pos=positive. SI=small intestine

Cats that died during the study period and were excluded from an FIPV diagnosis are shaded in the pathology column. Dz=disease; FECV=feline enteric coronavirus biotype; WNL=within normal limits.

**PE=physical examination. WNL=within normal limits; AB=abnormal.

βFeline coronavirus antibody titre; pos=positive; neg=negative

μ FIPV=pathogenic feline infectious peritonitis virus biotype, FECV=benign feline enteric coronavirus biotype. See appendix Table 7 for additional clinical biochemistry and serology results.

| cat ID | Farmα | Sex^ | Age* | 2004 | 2005 | 2006 | Status'07 | IHC/Histo# | Pathology | PE** | FCoV ab β | FIPV/FECVμ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **4549** | Weller | M | 1.5 | M7PS3 | | | D | pos | effusive | | pos 1:400 | FIPV |
| **4561** | FCAC | F | 3 | MP3 | | | E | neg(pos SI) | FECV | WNL | neg 1:400 | FECV |
| **4562** | Palmer | M | 1 | | | | E | neg | early viral dz | WNL | neg 1:400 | FECV |
| **4563** | Palmer | M | 1 | | | | E | neg | early viral dz | WNL | pos 1:400 | FECV |
| **4564** | Palmer | M | 1 | | | | E | neg | early viral dz | WNL | neg 1:400 | FECV |
| **4566** | Weller | M | 1.5 | M7PS3 | | | D | pos | effusive | AB | pos 1:25 | FIPV |
| **4580** | Weller | F | 1 | | | | H | | | WNL | pos 1:25 | FECV |
| **4581** | Weller | F | 1 | 7 | | | H | | | WNL | pos 1:25 | FECV |
| **4582** | Weller | F | 1 | M | M | | H | | | WNL | pos 1:25 | FECV |
| **4583** | Weller | M | 1 | | M | 7 | H | | | WNL | | FECV |
| **4584** | Weller | F | 2 | 7 | | 3 | H | | | WNL | pos 1:25 | FECV |
| **4585** | Weller | M | 1 | M | | M | H | | | WNL | pos 1:25 | FECV |
| **4586** | Weller | F | 1 | M7S3 | | | H | | | WNL | pos 1:25 | FECV |
| **4587** | Weller | M | 1 | | | | H | | | WNL | pos 1:25 | FECV |
| **4588** | Weller | M | 1 | M | | | H | | | WNL | pos 1:25 | FECV |
| **4589** | Weller | M | 1 | M7 | | | H | | | WNL | pos 1:25 | FECV |
| **4590** | Weller | M | 2 | M7 | M73 | | D | pos | effusive | AB | pos 1:1600 | FIPV |
| **4591** | Weller | F | 1 | M7SP | M | | H | | | WNL | pos 1:25 | FECV |
| **4592** | Weller | F | 1 | | M | | H | | | WNL | pos 1:25 | FECV |
| **4593** | Weller | M | 1 | 7P | M | M | H | | | WNL | pos 1:25 | FECV |
| **4594** | Weller | F | 1 | M7P | | M7 | H | | | WNL | pos 1:25 | FECV |
| **4595** | Weller | F | 1 | M7P3 | | | H | | | WNL | pos 1:25 | FECV |
| **4596** | Weller | F | 1 | | | | H | | | AB | pos 1:25 | FECV |
| **4597** | Weller | F | 1 | P | | M73 | H | | | WNL | pos 1:25 | FECV |
| **4606** | Weller | F | 4 | MS | | M7 | H | | | WNL | pos 1:25 | FECV |
| **4607** | Weller | M | 3 | | | | H | | | WNL | pos 1:25 | FECV |
| **4608** | Weller | F | 3 | | | | H | | | WNL | neg 1:25 | FECV |
| **4609** | Weller | F | 1 | 7 | | | H | | | AB | pos 1:25 | FECV |
| **4611** | Weller | M | 7 | | | | H | | | WNL | pos 1:25 | FECV |
| **4612** | Weller | F | 1 | 73 | | | H | | | WNL | pos 1:25 | FECV |
| **4613** | Weller | F | 4 | | | | H | | | WNL | pos 1:25 | FECV |
| **4614** | Weller | M | 5 | | | | H | | | WNL | pos 1:25 | FECV |
| **4615** | Weller | F | 7 | | | | H | | | WNL | pos 1:25 | FECV |
| **4616** | Weller | F | 6 | | | | H | | | WNL | pos 1:25 | FECV |
| **4618** | Weller | M | 1 | M7 | | | D | pos | effusive | AB | pos 1:1600 | FIPV |
| **4620** | Weller | M | 6 | | | | D | neg | pancreatitis | AB | neg 1:400 | FECV |
| **4623** | Weller | UK | 0 | | | | D | neg | perinatalWNL | AB | neg 1:25 | FECV |
| **4624** | Seymour | F | 2 | | MP37 | | E | neg | lymphoma | AB | | FECV |
| **4625** | Weller | F | 1 | PS | | | D | lymphoma | lymphoma | AB | | FECV |
| **4626** | Ambrose | F | 11 | | | | H | | | WNL | | FECV |
| **4627** | Ambrose | M | 11 | | | | H | | | WNL | pos 1:25 | FECV |
| **4628** | Ambrose | F | 7 | | | | H | | | WNL | neg 1:400 | FECV |
| **4629** | Ambrose | F | 7 | | | | H | | | WNL | pos 1:400 | FECV |
| **4630** | Ambrose | M | 4 | | | | H | | | WNL | pos 1:400 | FECV |
| **4631** | Ambrose | M | 2 | | | | H | | | WNL | pos 1:25 | FECV |
| **4653** | Ambrose | F | 4 | | | M7PS3 | D | pos | effusive | AB | pos 1:1600 | FIPV |
| **4654** | NM | M | 2 mo. | | | | D | neg | SI enteritis | AB | pos 1:25 | FECV |
| **4655** | FCAC | F | 2 | | | | E | neg | early viral dz | AB | pos 1:25 | FECV |
| **4656** | FCAC | M | 6 we. | | 7M3 | | E | neg | early viral dz | WNL | | FECV |
| **4657** | FCAC | M | 6 we. | | M7 | | E | neg | early viral dz | WNL | pos 1:25 | FECV |
| **4658** | FCAC | M | 3 we. | | | | E | neg | early viral dz | WNL | pos 1:25 | FECV |
| **4659** | FCAC | M | 6 we. | | M73 | | E | neg | early viral dz | WNL | pos 1:25 | FECV |
| **4660** | FCAC | M | 8 we. | | | | E | neg | early viral dz | WNL | pos 1:25 | FECV |
| **4662** | Weller | M | 4 mo | | | M7P3 | E | pos | effusive | | | FIPV |
| **4663** | Weller | F | 4 mo. | | | M7P3 | E | pos | effusive | | | FIPV |
| **4664** | NM | M | 6 mo. | | | M73 | E | pos | effusive | | | FIPV |

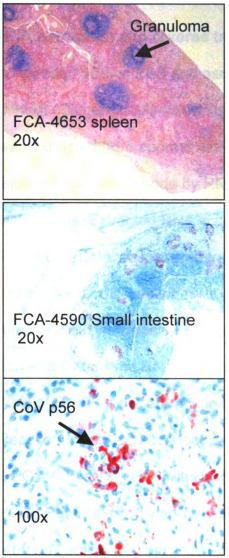
**Table 1: Clinical, demographic, and FCoV viral RT-PCR success data from 56 domestic cats sampled in Maryland from 2004-2006**

**Clinical hematology and biochemistry:** For complete blood counts, fresh (< 12 hr) whole-blood samples were assessed by Antech veterinary diagnostic laboratory using an automated cell counter (Avid Cell-Dyn 3500, Abbott Laboratories, Abbott Park, Illinois, USA). Biochemistry analysis (Hitachi 717 Clinical Chemistry Analyzer, Roche Diagnostics, Indianapolis, Indiana, USA) and enzyme-linked immunoassays (ELISA) for feline immunodeficiency virus (FIV; Petchek FIV ELISA, Idexx Laboratories, Westbrook, Maine, USA), and coronavirus (Virachek CV, Synbiotics Corp., San Diego, California, USA) antibodies (Table 1) were also performed.

**Pathology:** HE slides of spleen, liver, lymph node, intestine, and kidney sections were evaluated by Dahlem Smith, a board-certified pathologist (National Cancer Institute Laboratory Animal Sciences Program Frederick, Maryland, USA) for evidence of granulomatous and pyogranulomatous lesions. Alternative diagnosis, based on histopathology changes were noted in non-FIP cases, consistent with early viral disease, pancreatitis, or lymphoma (Table 1).

**Immunohistochemistry:** Formalin-fixed sections (3 μm thick) were cut from paraffin blocks and placed on glass slides for immunohistochemistry (IHC). Sections were deparaffinized in a clearant and placed in absolute alcohol, and then brought to water through graded alcohols. Indirect peroxidase, peroxidase-antiperoxidase, and avidin-biotin-peroxidase complex methods were performed as previously described (Kipar et al 1998a; Kipar et al 1998b). CoV p56, a cross-reacting antibodies for the demonstration of feline coronavirus (both FECV and FIPV biotypes) was applied. Following rinse, sections were counterstained 4

minutes with Mayer hemetoxylin and mounted. Known positive and negative control tissues were used (Washington Animal Disease Diagnostic Laboratory Washington State, USA) (Figure 2).




| Cat ID # | Histopath | IHC |
|---|---|---|
| Fca-4549 | Pos | Liver, Colon, Jejunum |
| Fca-4554 | Neg | Neg |
| Fca-4561 | Neg | Jejunum |
| Fca-4562 | Neg | Neg |
| Fca-4563 | Neg | Neg |
| Fca-4564 | Neg | Neg |
| Fca-4566 | Pos | Liver, Spleen, Lymph node |
| Fca-4590 | Pos | Lung, Jejunum, Lymph node |
| Fca-4618 | Pos | Liver, Lymph node |
| Fca-4620 | Neg | Neg |
| Fca-4623 | Neg | Neg |
| Fca-4624 | Neg | Neg |
| Fca-4625 | Neg | Neg |
| Fca-4653 | Pos | Liver,Spleen, Colon |
| Fca-4655 | Neg | Neg |
| Fca-4656 | Neg | Neg |
| Fca-4657 | Neg | Neg |
| Fca-4658 | Neg | Neg |
| Fca-4659 | Neg | Neg |
| Fca-4660 | Neg | Neg |
| Fca-4662 | Pos | ND |
| Fca-4663 | Pos | ND |
| Fca-4664 | Pos | ND |

**Figure 2:** Histopathology and Immunohistochemistry (IHC) results of 23 necropsied cats (Table 1). Liver, lung, spleen, colon, jejunum, stomach, heart, kidney, lymph node were evaluated by IHC. Cases highlighted in grey are designated FIPV in this study. Representative cases from Fca-4653 spleen (histopathology) and Fca-4590 (Immunohistochemistry) are shown at magnification shown. Red dye indicates binding of coronavirus antibody (CoV p56). Pos=positive; Neg=negative; ND=not done.

**RNA extraction and reverse transcription**: RNA from 160 ul frozen feces suspended 10% in PBS and ascites fluid was extracted by QIAamp virus RNA mini kit (Qiagen, USA) following the manufacturers instructions. RNA from tissue was extracted from approximately 60 mg of frozen liver, lung, spleen, colon, jejunum, and lymph node by RNAeasy (Qiagen, USA) following manufacturer's instructions. Extracted RNA was eluted in 35 uL of RNase-free water and stored at -70°C. cDNA was reverse transcribed using 9 uL of eluted RNA (10 pg-5 ug) in an initial 12 uL reaction mixture containing 50 ng of random hexamers and 0.5 mmol of deoxynucleoside triphosphate per liter. After incubation at 65°C for 5 min to denature the RNA, 10 mmol of dithiothreitol per liter, 5x Synthesis Buffer, 40 U of RNaseOUT, and 15 units of Thermoscript RT were added on ice. Reaction mixtures were incubated in thermocycler at 25°C for 10 min, followed by 50°C for 30 min. cDNA was stored at -20°C.

**PCR**: Primers amplifying 7b (786 bp), Membrane protein (655 bp), Polymerase (400 bp), spike (230 bp), and spike NSP3abc (1018 bp) (Table 1 Figure 3A) were designed based on available feline coronavirus sequence (Poland et al 1996; Vennema et al 1998; Addie et al 2000). 2 μL of cDNA was amplified in a 50 μL reaction mixture containing PCR was performed using approximately 50 ng of genomic DNA in a 50 μL reaction containing 50 mM KCl, 10 mM Tris-Hcl (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM concentrations of dATP, dCTP, dGTP, and dTTP, 2 mM concentrations of each primer, and 2.5 units of Platinum Taq DNA polymerase (Invitrogen, USA). PCR was run on a geneAmp PCR system 9700

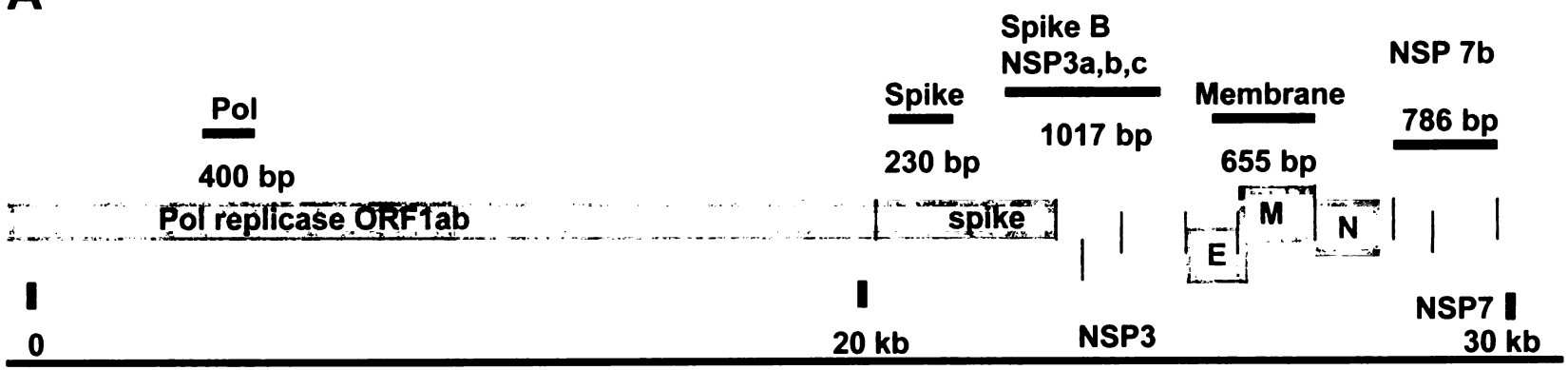
thermocycler (Applied Biostystems, USA) with the following touchdown conditions: 2 min at 94°C then a touch down, always starting with 20 sec at 94°C, then 30 sec of 60°C(3 cycles), 58°C (5 cycles), 56°C (5 cycles), 54°C (5 cycles), 52°C (22 cycles), and then 1 min at 72°C for extension, and with a final extension at 72°C for 7 min and hold at 4°C. PCR products were visualized on a 1% agarose gel and primers and unincorporated dNTPs were removed by Microcon YM (Millipore Billerica, MA).

**Cloning and sequencing:** Representative PCR products were cloned and sequenced (Figure 3B). Cloning was performed with a TOPO-TA cloning kit (Invitrogen, USA) according to the manufacturer's instructions. Plasmid DNA was isolated from 1-47 clones from each reaction product (Agencourt CosMCPrep, Agencourt Bioscience Corporation, USA). The presence of the correct sized insert was verified via restriction digest (Eco R1) and sequences were obtained from clones with the correct insert using standard ABI BigDye terminator reactions (Applied Biosystems, USA).

**Figure 3: A:** Feline coronavirus genome indicating PCR products obtained. Structural proteins are shaded in grey; non-structural proteins are shaded in light grey. **B**: Forward and reverse primers used to amplify virus segments listed in 5' to 3' orientation. The number of source cats and cloned sequences generated (**#** of unique clones in parenthesis) from FIPV and FECV biotypes.

Figure 3

A




B

| Gene | Forward Primer sequence | Reverse Primer sequence | #cats FECV | #cats FIPV | FECV | FIPV |
|---|---|---|---|---|---|---|
| Pol replicase | CTCACAAGTTATACTCAAGGGC | TCAGTGGTTTACCAGCTTGCA | 9 | 5 | 50(14) | 68(28) |
| Spike A | GCAAGTTACGAGCACAATCAT | AGTTGTTGGTGGTTATTACCCTACA | 46 | 9 | 48(42) | 18(14) |
| Spike B NSP 3a-c | AATGGCCTTGGTATGTGTGG | CCTGAGAAAAGGCTGCATTGT | 6 | 6 | 43(20) | 27(24) |
| Membrane | TCTTGCTAACTGGAACTTCAGCTGG | TGACGCGTTGTCCCTGTGTG | 27 | 8 | 154(96) | 100(67) |
| NSP 7b | GGGTTTTCCTGCTATACATTG | CACTAGATCCAGACGTTAGCTC | 20 | 8 | 140(95) | 157(96) |

Anticontamination measures were taken at all steps of RT-PCR amplification and post-PCR processing. Pre-PCR setup was performed in a laminar flow hood, RNA or cDNA was added in a free-standing containment hood in a separate room, and all post-PCR manipulations were performed under a fume hood in a third room. All surfaces were washed with a 10% bleach solution, and each hood was exposed to UV light for 30 min before and after use. PCR tubes with individual lids, rather than 96-well plates, were used and kept closed except when reagents and RNA or cDNA were being added or aliquots were extracted for use. Tubes were only opened in their designated hoods, and, to avoid cross-contamination, RNA and cDNA tubes were never open simultaneously. Water was run with every reaction as the negative control.

**Phylogenetic analysis:** Sequences from *Pol replicase, spikeA, spikeBNSP3a-c, membrane, and NSP7b* were analyzed separately. Nucleotide sequences were compiled and aligned for subsequent phylogenetic analysis by ClustalX (Thompson et al 1997) and verified visually (Maddison and Maddison 1995) by the following methods: minimum evolution, maximum parsimony, and maximum likelihood in PAUP (Swofford 2002). Modeltest (Posada and Crandall 1998) was used to estimate the optimal model of sequence evolution, and these settings were incorporated into subsequent analyses. Minimum evolution trees were constructed from models of substitution specified by Modeltest, with starting trees

obtained by neighbor joining followed by application of a tree-bisection-reconnection (TBR) branch-swapping algorithm during a heuristic search for the optimal tree. Maximum parsimony analysis employed a heuristic search of starting trees obtained by stepwise addition followed by TBR. Maximum likelihood parameters specified by Modeltest selected the general time-reversible model of substitution included empirical base frequencies and estimated rate matrix and corrected for among-site rate variation (gamma distribution). A bootstrap analysis using 1, 000 iterations was performed for maximum parsimony and minimum evolution and 100 iterations using the NNI branch-swapping algorithm for maximum likelihood. Amino acid residue alignments were generated using MacClade 3.05 (Maddison and Maddison 1995) and ClustalX. Variable sites and parsimoniously informative sites were computed in Mega 3.0 (Kumar et al 2004). Pairwise comparisons of genetic distances were performed in PAUP and the mean and range of genetic distances were calculated in Excel (Microsoft, USA).

## Results

A total of 56 domestic cats from Maryland were sampled during 2004-2006 from farms and veterinary hospitals with suspected FIP or exposure to infected FIP cats (Table 1). Cats were found to be healthy or recently deceased or euthanized. All cats tested were FCoV antibody positive on screening (>1:12) except Fca-4620, Fca-4562, and Fca-4608 (Table 1). The majority of cats (34) sampled were on the Weller farm where many individuals were sampled once per year for the 2-3 year study period. Necropsies were performed on nine cats

from the Weller farm having died or were euthanized due to severe illness over the study period. Both healthy and recently deceased cats were included from the Ambrose farm (7), one shelter at the Palmer veterinary hospital (3), Frederick County Animal Shelter (7), and independent cats from the New Market animal hospital (3). Fca-4590 from the Weller farm is an important case because samples were obtained on 5/20/04 when the cat was clinically healthy (pre-disease) and then again on 12/22/04 when the cat died of FIP (post-disease).

Thirteen of the twenty necropsied cats grossly exhibited abdominal and/or thoracic effusion (Table 1; Figure 2). Eight cats were classified as FIPV biotypes based on these results (Fca-4549, Fca-4566, Fca-4590, Fca-4618, Fca-4653, Fca-4662, Fca-4663, Fca-4664). Liver was the only tissue that was consistently positive via IHC. One case (Fca-4561) was IHC positive only in the jejunum and negative by histopathology on all tissues, therefore it was classified as an FECV biotype. Two of the necropsied cases (Fca-4624, Fca-4625), were FCoV antibody positive, IHC negative and diagnosed lymphoma based on histopathology, and classified as FECV biotype with respect to coronavirus infection. Similarly, the FCoV antibody positive necropsied cases with absence of characteristic FIPV histopathology and IHC lesions were classified as FECV biotype (Table 1; Figure 2). Healthy cats were classified as FECV biotypes based on normal physical examinations, FCoV antibody positive (>1:25), but not lymphopenic (<1.5 cells/uL), and/or were monitored until 2007 and known to be free of FIP disease (Table 1).

RT-PCR was attempted with 5 primer pairs (Figure 3B). Of the 79 samplings of the 51 FCoV antibody positive cats, 23 samples amplified virus with atleast one primer pair yielding a 29% rate of recovery of viral sequence from FCoV antibody positive cats (Table 1). All eight FIPV biotype cats amplified virus while many of the FECV biotype cats from the Weller Farm and Frederick County Animal Shelter also tended to amplify, in contrast to the Ambrose and Palmer populations. Once amplified, the viral gene amplicons were cloned and sequenced yielding from 1-47 cloned sequences each (Figure 3B).

Phylogenetic analysis of the cloned virus sequences from the Weller farm sampled from 2004-2006 revealed specific patterns of viral dynamics. First, healthy cats infected with coronavirus displayed a different genotype array than cats diagnosed with FIP in both the *membrane* and *NSP 7b* genes (Figure 4 A-D pink versus green). Second, virus variation from different tissues within each cat diagnosed with FIP is minimal, as evidenced by the phylogenetic grouping of cloned sequences from individual cats, regardless of tissue source ((Fca-4549, 4653, and 4663) (Figure 4) in both the membrane and NSP 7b genes. This suggests that cats infected with FIPV shed the FIPV strain in their feces. However, in two cases (Fca-4662 and Fca-4664; Figure 4A), cloned viral sequence from differing tissue compartments yielded distinct phylogenetic lineage, although both of a FIPV nature, suggesting that these two cats were super-infected with two strains of circulating FIPV. There were no FECV isolates which were super-infected by more than one virus strain (Figure 4). Third, cloned virus sequences of the *membrane protein* gene from six FECV cats

sampled in 2004 and then again in either 2005 or 2006 (Figure 4A-B, serial samples ⊙, ●) reveals changes in the predominant virus infecting individual cats over time consistent with a natural occurrence of persistence (Fca-4585) or a clearing of virus from the gastrointestinal tract, and re-infecting with new strains endemic to geographic location (Fca-4582,4591,4593, and 4606) (Foley et al 1997b). Lastly, phylogenetic analysis and genotype designation of Fca-4590, in which cloned virus sequence was obtained when the cat was clinically healthy and then a different genotype was present seven months later when the cat died of FIP, supports the circulating virulent/avirulent hypothesis, rather than the *in vivo* mutation hypothesis (boxed sequence 4A-D). The virus sequence pre-disease and post-disease occupy clearly distinct phylogenetic clades with strong bootstrap support.

When additional cases of FIPV and FECV from other farms in Maryland as well as an archived case of FIPV in a cheetah (Pearks-Wilkerson et al 2004), phylogenetic analysis of the cloned virus sequences still shows monophyletic groupings correlative to disease biotype (FIPV versus FECV) in membrane protein and NSP7b (Figure 4A and C), however there is also additional structure possible relating to year or sampling and/or geographic location.

**Figure 4:** Mid-point rooted maximum likelihood phylogenetic tree of unique *membrane* and *NSP 7b* FCoV gene sequences showing monophyly correlating to disease status. Cloned sequences from FIPV biotypes are shown in pink; FECV biotypes in green. **(A) *membrane*** 655 bp sequences (ML -ln L=3086.20787 best tree found by MP: length =493, CI=0.551724, RI= 0.0926505) **(B) *membrane* Weller Farm only** (ML -ln L=2646.84352 best tree found by MP: length =270, CI=0.789, RI= 0.971), **(C) *NSP 7b*** 736 bp sequences ML -ln L=4556.60497 best tree found by MP: length =452, CI=0.608, RI= 0.942;) **(D) NSP *7b* Weller farm only** ML -ln L=3997.98885 best tree found by MP: length =411, CI=0.791, RI= 0.981;), FCoV sequence from cats only from the Weller farm are shown in Figure B and D. The number of cats followed by the number of clones is indicated in parenthesis in the key. Each sequence is labeled as follows: four-digit cat identification number, tissue source (fe=feces, af=ascites fluid, co=colon, li=liver, sp=spleen, in=intestine, je=jejunum, ln=lymph node), and 2 digit year (eg. 04=2004). The number of clones for each sequence is indicated after the sequence label in parenthesis in 4A and 4C. Where maximum likelihood tree was congruent with maximum parsimony tree, branch lengths are indicated below branches; the number of homoplasies is in parenthesis after the branch length in Figure 4A and 4C. Bootstrap values are shown (maximum parsimony/minimum evolution/maximum likelihood) above branches. Virus sequence obtained from cat 4590 in May 2004 and at the time of death due to FIP in December 2004 is indicated by box. The two distinct virus genotypes isolated from this case pre and post disease in both the ***membrane*** and ***NSP 7b*** genes are consistent with the dual circulating virulent and avirulent strains in FCoV pathogenesis.

# Figure 4A: Membrane 655 bp ML

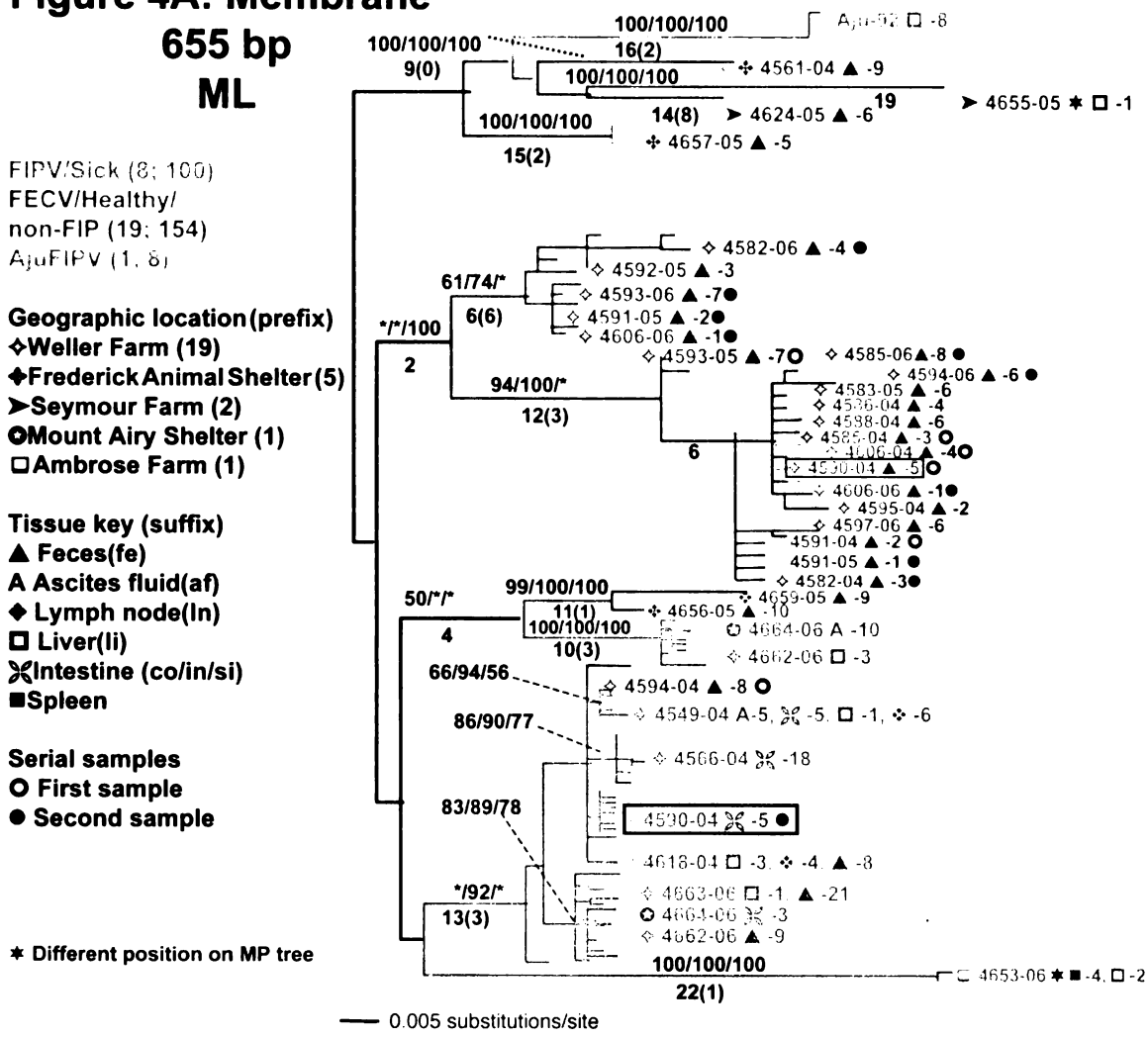
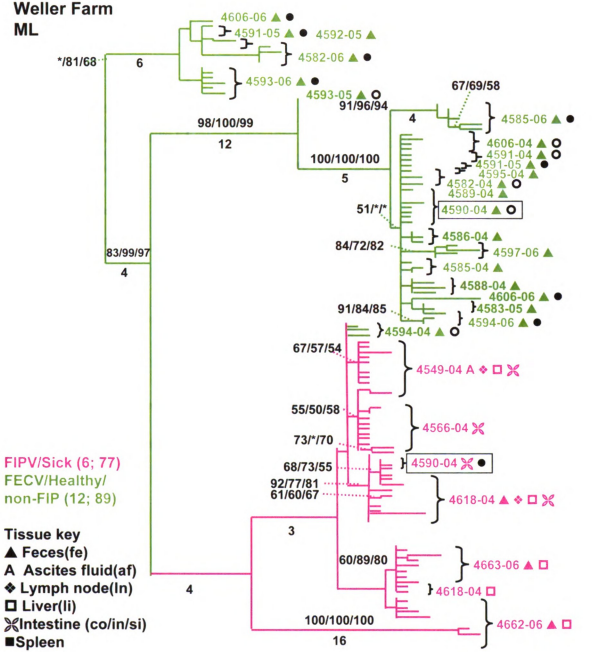
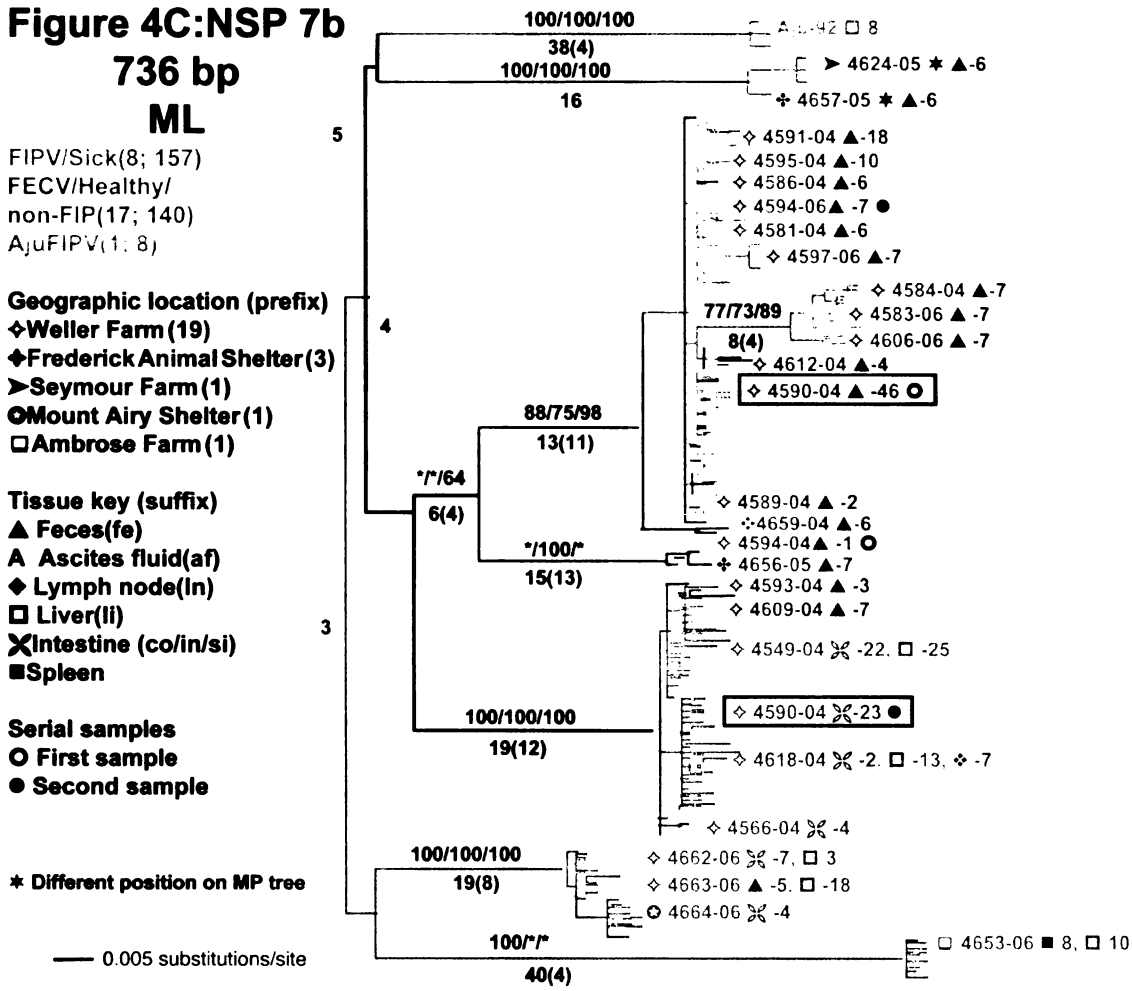


**Figure 4B: Membrane**
**655 bp**
**Weller Farm**
**ML**



# Figure 4C:NSP 7b 736 bp ML



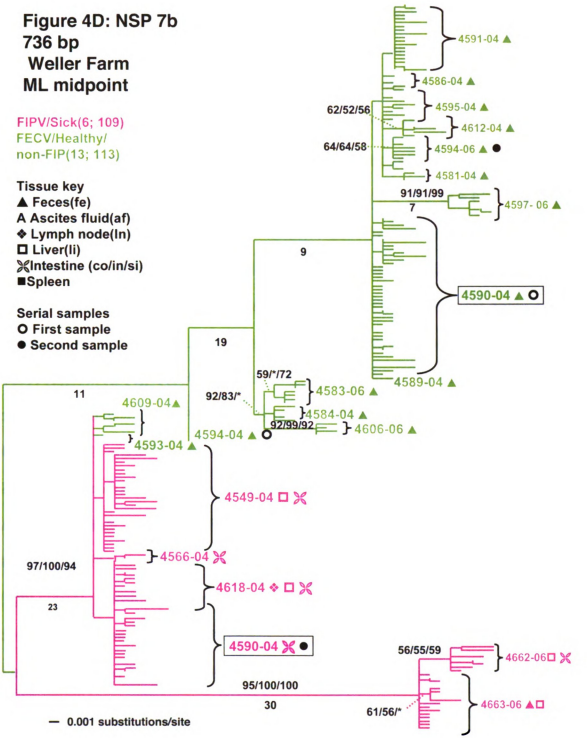


Figure 4D: NSP 7b
736 bp
Weller Farm
ML midpoint
FIPV/Sick(6; 109)
FECV/Healthy/
non-FIP(13; 113)
Tissue key
▲ Feces(fe)
A Ascites fluid(af)
❖ Lymph node(ln)
□ Liver(li)
Intestine (co/in/si)
■Spleen
Serial samples
O First sample
● Second sample
4591-04 ▲
4586-04 ▲
4595-04 ▲
4612-04 ▲
4594-06 ▲ ●
4581-04 ▲
4597- 06 ▲
4590-04 ▲ O
4589-04 ▲
4583-06 ▲
4584-04 ▲
4606-06 ▲
4609-04▲
4593-04 ▲
4594-04 ▲
4549-04 □
4566-04
4618-04 ❖ □
4590-04 ●
4662-06□
4663-06 ▲□
62/52/56
64/64/58
91/91/99
7
9
19
59/*/72
92/83/*
92/99/92
11
97/100/94
23
95/100/100
30
56/55/59
61/56/*
— 0.001 substitutions/site

Nucleotide sequences of the membrane protein and NSP 7b generated in this study were translated to amino acid sequences (Figure 13). Five informative amino acid sites were determined in the membrane protein at positions 108, 120, 138, 163, and 199 (based on reference sequence for TGEV GenBank NP058427) giving rise to six genotype arrays diagnostic of FIP versus non-FIP in cats infected with coronavirus infection (Table 2). For the total of 8 FIPV cases, 19 FECV cases sampled 25 times, and1 FIPV case from cheetah there were six genotype arrays based on these 5 diagnostic sites. All cats diagnosed with FIP by pathology and/or immunohistochemistry displayed the amino acid genotype of either "YIVAL" (1) or "YIIAL"(2) while cats infected cats without clinical FIP had "HIIVI"(3), "HIIVL"(4), "HVIAL"(5), "YVVAL(6)", or "YIVAL"(1). Three cats without clinical FIP but with the FIP-specific genotype "YIVAL" (Fca-4594, 4624, and 4657; Table 2) are the exception: Cat 4594 was sampled twice (2004-2006), the 2004 signature YIVAL-FIPV and in 2006 HIIVI-FECV, suggesting that this cat was able to clear the virulent FIPV virulent strain and become re-infected with an avirulent strain. Cat 4624 and 4657 were euthanized at the time of sampling (light green), therefore it is unknown if, given more time, FIP may have developed in these two cats. No informative sites, in terms of FIP diagnostics, were found in the *NSP 7b* nucleotide or amino acid alignments.

**Table 2**:Genotype array of 8 FIPV and19 FECV Maryland domestic cats sampled a total of 25 times at five variable amino acids in the FCoV *membrane* protein.

Genotype array of 8 FIPV (pink), 19 FECV (green) Maryland domestic cats sampled a total of 25 times at five variable amino acids (genbank reference sequence number P04135) in the *membrane* protein. FCoV infected healthy cats (dark green) and cats excluded of FIP diagnosis at euthanasia (light green) (table 1) have a different genotype array than cats diagnosed with FIP.
Column one: original number of nucleotide sequenced clones, column two: number of corresponding unique amino acid sequences.
*Cat identification number (see Table 1)
**Source of cloned virus (af=ascites fluid, fe=feces, li=liver, ln=lymph node, co=colon) (as in Figure 4), year of sampling, and farm where sampled is listed.
^Distinct genotype composite array correlative with FIPV/FECV designation. Viral genotype was consistent in individual cats from various tissue compartments except in cat 4662. Viral genotype from healthy cats sampled at more than one time point is shown (4606,4591,4582,4593, 4585, and 4594) and 4590, which was sampled in May 2004 when healthy and again in December of 2004 at death from FIP.
Genotype array of FCoV-Aju (orange), Cell line (Pedersen et al 1984) and reference sequences for SARS-CoV, MHV-1, IBV-Beu, BVC-K, HcoV-229E, TGEV-Purdue, and FCoV 79-1146 (GenBank accession numbers P59596, AB587268, P69602, BAF75636, P15422, PO4135, and P25878, respectively) are also shown.

**Table 2**:Genotype array of 8 FIPV and19 FECV Maryland domestic cats sampled a total of 25 times at five variable amino acids in the FCoV *membrane* protein.

| # cl. | # aa | FCA ID* | source** | 108 | 120 | 138 | 163 | 199 | Year | Farm | Gen^ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | 10 | 4549 | af,li,ln,co | Y | I | V | A | L | 2004 | Weller | 1 |
| 18 | 7 | 4566 | je | . | . | . | . | . | 2004 | Weller | 1 |
| 17 | 12 | 4618 | li,ln,fe | . | . | . | . | . | 2004 | Weller | 1 |
| 3 | 3 | 4662 | fe | . | . | . | . | . | 2006 | Weller | 1 |
| 13 | 5 | 4663 | fe,li | . | . | . | . | . | 2006 | Weller | 1 |
| 3 | 2 | 4664 | je | . | . | . | . | . | 2006 | Mt Airy | 1 |
| 12 | 1 | 4590 | si | . | . | . | . | . | Dec-04 | Weller | 1 |
| 6 | 3 | 4653 | sp,li | . | . | I | . | . | 2004 | Ambrose | 2 |
| 3 | 3 | 4662 | li | . | . | I | . | . | 2006 | Weller | 2 |
| 11 | 7 | 4664 | af,li | . | . | I | . | . | 2006 | Mt Airy | 2 |
| 5 | 2 | 4590 | fe | H | . | I | V | I | May-04 | Weller | 3 |
| 13 | 2 | 4582 | fe | H | . | I | V | I | 2004 | Weller | 3 |
| 6 | 2 | 4583 | fe | H | . | I | V | I | 2005 | Weller | 3 |
| 3 | 6 | 4585 | fe | H | . | I | V | I | 2004 | Weller | 3 |
| 9 | 3 | 4585-06 | fe | H | . | I | V | I | 2006 | Weller | 3 |
| 2 | 3 | 4586 | fe | H | . | I | V | I | 2004 | Weller | 3 |
| 5 | 3 | 4588 | fe | H | . | I | V | I | 2004 | Weller | 3 |
| 15 | 1 | 4591 | fe | H | . | I | V | I | 2004 | Weller | 3 |
| 6 | 2 | 4594-06 | fe | H | . | I | V | I | 2006 | Weller | 3 |
| 2 | 1 | 4595 | fe | H | . | I | V | I | 2004 | Weller | 3 |
| 6 | 4 | 4597 | fe | H | . | I | V | I | 2006 | Weller | 3 |
| 3 | 2 | 4606 | fe | H | . | I | V | I | 2004 | Weller | 3 |
| 1 | 1 | 4593 | fe | H | . | I | V | . | 2004 | Weller | 4 |
| 2 | 2 | 4606-06 | fe | . | V | I | . | . | 2006 | Weller | 5 |
| 9 | 4 | 4561 | fe | . | V | I | . | . | 2004 | FCAC | 5 |
| 6 | 2 | 4582-06 | fe | . | V | I | . | . | 2006 | Weller | 5 |
| 3 | 1 | 4591-05 | fe | . | V | I | . | . | 2005 | Weller | 5 |
| 3 | 2 | 4592 | fe | . | V | I | . | . | 2005 | Weller | 5 |
| 7 | 6 | 4593-06 | fe | . | V | I | . | . | 2006 | Weller | 5 |
| 10 | 3 | 4656 | fe | . | V | I | . | . | 2005 | FCAC | 5 |
| 9 | 9 | 4659 | fe | . | V | I | . | . | 2005 | FCAC | 5 |
| 1 | 1 | 4655 | fe | . | V | . | . | . | 2005 | FCAC | 6 |
| 6 | 2 | 4594 | fe | . | . | . | . | . | 2004 | Weller | 6 |
| 6 | 4 | 4624 | fe | . | . | . | . | . | 2005 | Seymour | 6 |
| 6 | 5 | 4657 | fe | . | . | . | . | . | 2005 | FCAC | 6 |
| 8 | 8 | Aju-92 | li | . | V | . | . | . | 1982 | WSP | 6 |
| | | FIPV79-1146 | genbank | . | V | . | . | . | 1991 | Wash | 6 |
| | | FIPV79-1683 | genbank | . | V | I | . | . | 1991 | Wash | 5 |
| | | SARS | genbank | . | . | F | L | C | 2003 | | |
| | | MHV-1 | genbank | . | . | I | M | F | 2007 | | |
| | | IBV-Beu | genbank | P | . | I | T | C | 2001 | | |
| | | BCV-K | genbank | . | . | I | M | Y | 2007 | | |
| | | HCV-229E | genbank | W | F | F | V | V | 1990 | | |
| | | TGEV | genbank | . | . | I | . | M | 1986 | | |

**Table 3:** A summary of feline coronavirus genes and their phylogenetic characterstics in this study

| | pol | spike | NSP3a-c | Mem | NSP 7b |
|---|---|---|---|---|---|
| gene length | 11897 | 4358 | 1167 | 788 | 620 |
| PCR length | 386 | 234 | 883 | 575 | 736 |
| Variable sites | 98 | 60 | 206 | 238 | 402 |
| Pars. inform sites | 49 | 42 | 145 | 134 | 240 |
| Mean gen. dist. | 2.4 | 7.8 | 4.9 | 4.7 | 4.9 |
| Gen. dist. range | 0.26-8.0 | 0.44-12.5 | 0.11-10.6 | 0.17-9.0 | 0.27-11.0 |
| no. cats | 14 | 20 | 12 | 13 | 11 |
| no. seq | 118 | 33 | 70 | 254 | 297 |
| no. unique seq | 42 | 17 | 44 | 163 | 191 |

pol=RT-polymerase, NSP=non structural protein, mem=mebrane. Gene length, PCR product length obtained, variable sites, parsimoniously informative sites, mean genetic distances and range, and number of cats, sequences and unique sequences used in analyses are shown.

In contrast to the monophyletic findings in the *membrane* and *NSP 7b* genes, cloned viral sequences of *pol replicase, spike,* and *NSP3a-c* were paraphyletic in terms of disease phenotype (Figure 12). The lack of phylogenetic signal in these genes may be partially explained by the relatively low number of variable sites, parsimoniously informative sites, and the relatively low mean genetic distance in *pol replicase*, the short PCR product obtained in the *spike* gene (Table 3). However, it is not clear why there is a lack of phylogenetic signal in the NSP 3a-c genes. *pol replicase, spike*, and *NSP3a-c* are therefore not indicated as informative gene regions correlating with FIP pathogenesis, although additional sequencing of these genes may provide further insight.

## Discussion

Infection with FCoV is common in cats throughout the world, although in most cases the virus causes no clinical signs or only mild self-limiting gastrointestinal disorders. However, in some cases, infection is associated with the development of the progressive and fatal disease manifestation of FIP. FIP is arguably the most serious viral infection in cats not only because of its fatal nature, but also because of the difficulties in diagnosing FIP antemortem and controlling the spread of FCoV. Here we have presented a molecular virology study of naturally occurring feline coronavirus infection and phylogenetic analysis of the cloned virus sequences obtained from the *membrane, NSP 7b, spike, pol replicase, and NSP3a-c* genes isolated from domestic cats located in Maryland catteries infected with FCoV from 2004-2006. We have shown monophyletic clustering of strains correlating with disease phenotype in both NSP 7b and *membrane* genes indicative of a circulating virulent/avirulent strain hypothesis necessary for FIP pathogenesis; rather than the previously postulated and widely accepted *in vivo* mutation theory.

Since phylogenetic study of the sequences evaluated *in pol replicase, spike, and NSP3a-c* were paraphyletic, it is not clear if these genes are correlative uniquely to FIP pathogenesis. *Pol replicase* is the largest gene of 20 kb occupying the 5' two thirds of the coronavirus genome encoding a large protein complex for independent viral replication. *Pol replicase* has been excluded as a factor in viral pathogenesis in murine hepatitis virus (Navas-Martin 2007) and the mean genetic distance calculated for *Pol replicase* is the lowest of

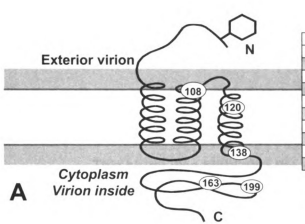
all genes in this study (Table 3). The spike gene (4358 bp) encodes a large glycoprotein which forms spikes on virion surfaces, binds to specific cellular receptors, induces neutralizing antibody, and elicits cell-mediated immunity (Rottier 1995). Spike has been implicated in as a determinant of virulence in the TGEV (Ballesteros et al 1997 Sanchez et al 1999), and neurological MHV (Phillips et al 2002), but not in FCoV, SARS, or IBV (Tan et al 2006). The fact that both FCoV serotype I and II have the ability to cause FIP yet serotype II is a recombinant virus, encoding the spike genome of canine coronavirus, suggest a more complex pathogenesis. From this study, however, only 230 base pairs of *spike* genome were studied because of technical difficulties in designing RT-PCR primers to amplify this variable region from field samples. With the additional sequence data from FCoV serotype I isolate now available (Dye and Siddell 2007), it will be possible to design more specific primers in the spike region. FCoV contain 5 non- structural proteins (*NSP*) in 2 distinct gene clusters: *3a-c* and *7a-b*. The function of these proteins is largely unknown. SARS has the largest number of NSP and it has been suggested that some of all of these eight SARS NSP play critical roles in SARS pathogenesis (Tan et al 2006). The indels in NSP 7b and 3c, previously implicated as determinants of virulence in FCoV (Vennema et al 1998), were not confirmed in our study at the same gene region. As has been suggested, these indels are likely artifacts of cell-culture adaptation (de Groot-Mijnes et al 2005).

We have shown that cats become re-infected with new strains of FCoV from external sources, rather than *in vivo* mutations. We have shown that cats

are generally infected with a predominant virus strain and not superinfected with multiple strains of both FECV and FIPV. The exception to this finding in our study were two cases of FIPV (Fca-4662 and 4664) whereby the virus sequence isolated from the gastrointestinal tract (feces or intestine) differs from the systemic virus (liver and/or ascites fluid) indicating that *in vivo* super-infection does occur sometimes, but that the super-infected virus tends to be of the same viral genotype and segregate in the gastrointestinal compartment versus the systemic circulation (figure 4A, Table 3).

The demonstration of six naturally occurring composite genotypes based on five variable sites in the membrane protein amino acid alignment highly correlative with disease phenotype (Table 3) offer specific opportunities for the management of this disease. If confirmed by extending this study to additional cat populations, the development of an antemortem screening tool for the discrimination of virulent versus avirulent strains of FCoV will be possible. Further, a role of the membrane protein in FIP pathogenesis seems likely given the known functions in other coronaviruses. The membrane protein is the most abundant structural protein with important functions in virus budding (Rottier 1995). The membrane protein also interacts with cell-mediated host immunity (Rottier 1995), is known to both induce alpha interferon (Laude et al 1992) and induce apoptosis (Chan et al 2007, Zhao et al 2006). The specific functions of the membrane protein amino acid sequences have been determined in SARS-CoV (He et al 2005). Aligning the sequences from this study with the annotated SARS-CoV, the first diagnostic amino acid site (108) aligns to a site just

upstream from the second transmembrane helice. Tyrosine at position 108, which is found in all FIPV biotypes and shared among SARS-CoV, MHV-1, TGEV, and BCV-K (bovine coronavirus) has a neutral polarity (in contrast to a histidine there, found in the majority of FECV biotypes, which has a positive polarity) may be of significance to the stability of the virus within the membrane. Site 120 aligns within the third transmembrane helice, site 138 aligns just downstream to the transmembrane helice, site 163 aligns within the C-terminus which projects inside the virus particle, and finally site 199, also within the C-terminus domain, aligns within a defined SARS-immunodominant epitope (He et al 2005) (Figure 5).




B

| residue | symbol | amino acid | polarity | hydro |
|---|---|---|---|---|
| 108 | H | histidine | positive | philic |
| 108 | Y | tyrosine | neutral | philic |
| 120 | V | valine | non-polar | phobic |
| 120 | I | isoleucine | non-polar | phobic |
| 138 | I | isoleucine | non-polar | phobic |
| 138 | V | valine | non-polar | phobic |
| 163 | V | valine | non-polar | phobic |
| 163 | A | alanine | non-polar | phobic |
| 199 | I | isoleucine | non-polar | phobic |
| 199 | L | leucine | non-polar | phobic |

**Figure 5:** A: Diagram of membrane protein containing three transmembrane helices, an external N terminus and an internal carboxy-terminus. Approximate position of five variable diagnostic amino acid sites (Table 3) as determined by sequence comparison to SARS-CoV (He et al 2005). B: Amino acid residue, polarity, and hydrophobicity or hydropholicity is stated.

Fca-4594, which was infected with the disease-associated genotype without succumbing to FIP, suggests alternate requirements for viral pathogenesis. As has been suggested in the outbreak of FIP in a colony of captive cheetahs (Heeney et al 1990), host immune genetics may contribute to FIPV pathogenesis. Candidate genes such as IL-12 (Kipar et al 2006), IL-10 (Kipar et al 2006,Ward et al 2005), IL-6 (Kipar et al 2006), MCP-1/CCL-2 (De Albuquerque et al 2006), IFN-gamma (De Albuquerque et al 2006, Ward et al 2005), TNF-alpha (De Albuquerque et al 2006), CXCL10 (Ward et al 2005), RANTES (Ward et al 2005), IL-8 (Ward et al 2005), L-SIGN (Jeffers et al 2004), DC-SIGN (Yang et al 2004), Ace2 (Li et al 2003), and fAPN (Tresnan et al 1996) have been identified as associated with coronavirus-disease findings in cat, mouse, and human cases. Further study of these genes in parallel with the viral genotypes described here will likely advance our understanding of the complex disease pathogenesis of FIP, including both viral and host factors, and aid in the diagnosis, treatment, and prevention of this fatal disease.

This study also highlights the importance of a rigorous sampling strategy when investigating viral outbreaks in natural populations. By collecting biological samples from the healthy cats infected with coronavirus, we were able compare and contrast viral genes and discern the viral dynamics relating to pathogenesis. By carefully archiving these specimens with the appropriate clinical information, we've also created a study cohort which will be available for hypothesis driven study of viral and host genetic factors relating to coronavirus pathogenesis and susceptibility in the future. In order to apply a similar

hypothesis-led research effort to viral outbreaks in the future, it is imperative to obtain samples from individuals, both symptomatic and asymptomatic, exposed to the disease-causing agent and document in detail the clinical course of the individuals. For example, less than 10% of those infected with SARS-CoV developed SARS (Chinese SMEC 2004, Drosten et al 2003). However, although there are over 200 full-length SARS-CoV sequences available on GenBank, there are no sequences from avirulent cases. Some researchers have been able to obtain virus sequence from fecal samples from SARS patients exhibiting only mild symptoms (Zhao 2007). Although it is particularly difficult to obtain viral sequence from asymptomatic infected individuals, advancements in concentrating RNA viruses by centrifugation (Christopherson et al 1998; Anton et al 2003) employed in HIV research for quantitative viral measures, should provide useful in such pursuits.

CHAPTER TWO

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## CHAPTER TWO

## Genetic characterization of feline leukemia virus from Florida panthers

### Introduction

The Florida panther (*Puma concolor coryi*) is the only remaining puma (also called cougar or mountain lion) population east of the Mississippi River in North America. This population, which is confined to a small portion of southern Florida, was originally described as 1 of 30 subspecies of puma (Culver et al, 2000). By the 1970s, Florida panther numbers diminished to »30 because of hunting and habitat destruction. Since the early 1980s, the population has been studied extensively by monitoring a large proportion of adults by radio telemetry (Roelke et al 1993a,b, Maehr 2002, Cunningham et al 2008). In the early 1990s, concern over the fate of the population increased as signs of inbreeding and loss of genetic diversity were reported. These observations included low levels of genetic variation, high levels of sperm abnormalities, and increased incidence of heart defects relative to other puma populations and felids in general (Roelke et al 1993a,b). In 1995, faced with the compounding effects of reduced genetic variation, probable depression of numbers from inbreeding, and evidence of compromised health, wildlife managers released 8 female Texas pumas into southern Florida to increase genetic variation and ameliorate the physiologic effects of inbreeding. Subsequently, increases were noted in the population of individuals of mixed genetic heritage, genetic variation, and population size; a decrease was noted in incidence of deleterious physiologic traits in crosses between the pure Florida panthers and the Texas females (Maehr 2002).

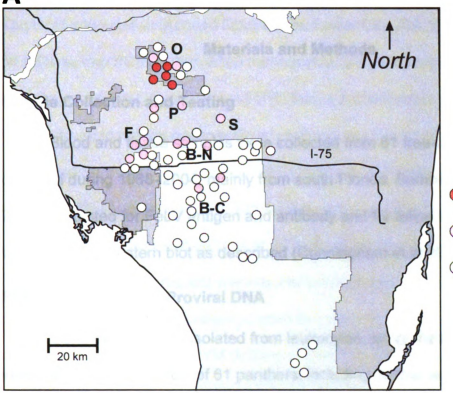
The Florida panther population, as well as other North and South American puma populations, has historically tested negative for exposure to or infection by feline leukemia viruses (FeLVs). A serosurvey of 38 free-ranging Florida panthers sampled during 1978–1991 reported complete absence of FeLV antigen (Roelke 1993b). However, since early 2001, 23 panthers (>33% of the population) were found to be positive for FeLV antibodies, and at least 5 adult panthers were positive for FeLV antigen and subsequently died. In the 3 panthers available for necropsy, evidence was found of diseases compatible with FeLV infection (Cunningham et al 2008). We describe the molecular genetic characterization of circulating FeLV strains isolated from the 2001–2005 outbreak and compare them with FeLV strains isolated from domestic cats.

FeLV is transmitted horizontally among domestic cats through body secretions (Hardy et al 1973) and was the first retrovirus shown to cause both neoplastic and degenerative disorders (Jarrett et al 1964; Mullins and Hoover 1990). Like other retroviruses, FeLV induces immunosuppression in its host. Although the mechanism of immunopathogenesis is unclear, viral envelope proteins may be involved (Denner 2000). FeLV envelope (*env*) and the long terminal repeat (LTR) sequences have been suggested as being involved in determination of disease sequelae, virus transactivation, and virus replication (Abujamra et al 2003; Chandhasin et al 2005a; Chandhasin et al 2005b; Finstad et al 2004). There are 4 naturally occurring viral subgroups of exogenous FeLV (A, B, C, and T) that are distinguished genetically by sequence differences in the *env* gene and functionally by receptor interactions required for cell entry

(Overbaugh and Bangham 2001). FeLV-A is the predominant subgroup circulating in feral cats and is often only weakly pathogenic (Phipps et al 2000b). FeLV-B, -C, and -T subgroups arise *in vivo* through recombination between exogenous FeLV strains and domestic cat endogenous FeLVs (Mullins and Hoover 1990, Stewart et al 1986). The endogenous feline leukemia provirus sequences are present in the genome of the domestic cat and are transmitted vertically as integral components of the germline (Okabe et al 1976). Endogenous feline leukemia virus sequences by themselves do not produce infectious virus. However, the pathogenic subgroups, FeLV-B, -C, and -T are generated by recombination in the *env* region between exogenous subgroup A virus and endogenous proviral sequences (Mullins and Hoover 1990). FeLV-A, -B, -C, and -T are often associated with, respectively: thymic lymphoma of T-cell origin (Neil et al 1991), tumor formation (Donahue et al 1991), aplastic anemia and bone marrow dysfunction (Neil et al 1991), and lymphoid depletion and immunodeficiency disease (Overbaugh and Bangham 2001). We used viral genome sequence and phylogenetic analyses to identify and characterize the virulent and pathogenic FeLV in Florida panthers and compare it with FeLV strains in the domestic cat.

**Figure 6**. A) Prevalence and distribution of 19 Florida panthers, sampled 1999–2005, showing evidence of feline leukemia virus (FeLV) exposure. All antigen-positive panthers (red) are clustered in the Okaloacoochee Slough State Forest (O). PCR-positive and/or antibody-positive (pink) pumas were found there also, as well as in the surrounding areas including Florida Panther National Wildlife Refuge (F), private lands (P), Big Cypress Seminole Indian Reservation (S), and Big Cypress North and South (BC-N, BC-S respectively). All but 2 infected panthers were found north of Interstate 75. B) Information on affected panthers. Gray shading indicates timeline for monitoring of individual panthers until death. Symbols within gray boxes indicate presence (+), absence (–), or no data (*) for FeLV antigen in serum, FeLV sequence recovered by PCR, or presence of antibodies against FeLV in serum, respectively. FP-122 was antigen negative when tested 1 month previously (§). LGD ID, Laboratory of Genomic Diversity identification number; FP ID, Florida panther identification number; GH, genetic heritage; FIV, feline immunodeficiency virus; GEO, geographic locale; C, canonical (pure) Florida panther; H, Texas hybrid.

**A**




**B**

| LDG ID | FP ID | Sex | 1999 | 2000 | 2001 Jan – Jun | 2001 Jun – Dec | 2002 Jan – Jun | 2002 Jun – Dec | 2003 | 2004 Jan – Jun | 2004 Jun – Dec | 2005 | GH | FIV | GEO |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pco-1058 | FP-115 | F | | | | | | +,+,+ | +,+,* | | | | C | + | O |
| Pco-1022 | FP-109 | M | | | | +,+,+ | –,*,– | | +,+,+ | | | | C | – | O |
| Pco-1087 | FP-122 | F | | | | | | | | +[§],+,– | | | C | – | O |
| Pco-1088 | FP-123 | M | | | | | | | | +,+,– | +,+,* | | H | + | O |
| Pco-1098 | FP-132 | M | | | | | | | | –,*,– | +,+,* | | H | – | O |
| Pco-722 | FP-167 | F | *,*,– | | | | –,–,+ | | | | | | C | – | P |
| Pco-908 | FP-78 | F | *,–,– | | | –,–,+ | | | | | | | C | + | F |
| Pco-962 | FP-82 | F | | *,*,– | | | | –,–,+ | | | | | C | – | O |
| Pco-972 | FP-96 | M | | | –,+,– | –,+,+ | *,+,* | | | | | | C | – | O |
| Pco-990 | FP-99 | F | | | *,–,+ | *,*,+ | | | | | | | C | + | F |
| Pco-1016 | UCFP43 | M | | | | –,–,+ | | | | | | | H | + | P |
| Pco-994 | FP-108 | M | | | | –,–,+ | | | | | | | H | + | B-C |
| Pco-1060 | FP-118 | F | | | | | | | –,+,– | | | | H | – | S |
| Pco-1000 | FP-104 | M | | | –,–,+ | | | *,*,– | | | | *,*,– | H | + | B-N |
| Pco-991 | FP-100 | M | | | –,+,+ | | | | | –,+,* | | | C | – | B-N |
| Pco-971 | FP-107 | F | | | | –,–,+ | | | | | *,*,– | | C | + | F |
| Pco-984 | FP-110 | F | | | | | *,–,+ | –,+,– | | | | | C | – | O |
| Pco-1064 | FP-119 | M | | | | | | | –,+,– | | –,–,– | | C | + | B-C |
| Pco-901 | FP-69 | F | | | | | | | | | | –,–,+ | C | + | B-N |

## Materials and Methods

### Sample Collection and Testing

Blood and tissue samples were collected from 61 free-ranging pumas captured during 1988–2004, mainly from south Florida. Samples were stored at –70°C and tested for FeLV antigen and antibody and for feline immunodeficiency virus (FIV) by Western blot as described (Cunningham et al 2008) (Figure 6).

### PCR Amplification of Proviral DNA

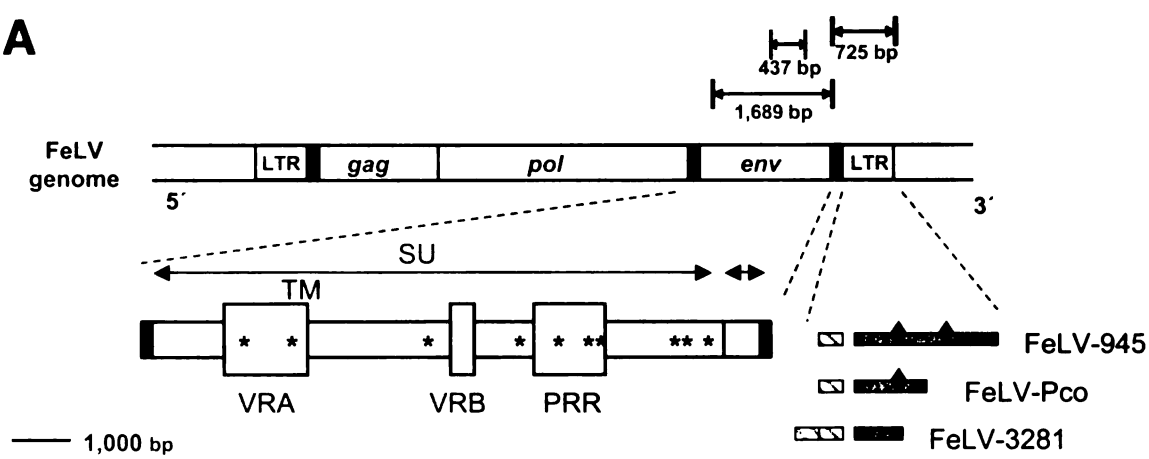
Genomic DNA was isolated from leukocytes, lymph nodes, spleen, intestines, or bone marrow of 61 panthers, including all that were positive for FeLV antigen and antibody. Proteinase K digestion was followed by standard extraction using the QIAGEN DNeasy tissue DNA extraction kit (#69504; QIAGEN, Valencia, CA, USA). Isolated DNA was visualized by electrophoresis on a 1% agarose gel and quantified by using a UV spectrophotometer (Bio-Rad, Hercules, CA, USA). PCR primers were designed from the conserved regions of *env* and LTR sequences of domestic cat FeLV (GenBank accession nos. M18247, M18248, M12500, AY374189, X00188, M14331, M23025, AY364318). PCR primers amplifying *env* (437 bp and 1,700 bp) and *env*/LTR (725 bp) are listed in Figure 7. The forward *env*/LTR primer (LTR4) was designed by using panther FeLV (FeLV-Pco) envelope sequence additionally.

PCR was performed by using □50 ng of genomic DNA in a 50-μL reaction with 50 mmol/L KCl; 10 mmol/L Tris-HCl (pH 8.3); 1.mmol/L $MgCl_2$; 0.25 mmol/L

each of dATP, dCTP, dGTP, and dTTP; 2 mmol/L of each primer; and 2.5 U of Taq Gold polymerase (Applied Biosystems, Foster City, CA, USA). PCR was run on a GeneAmp PCR system 9700 thermocycler (Applied Biosytems) under the following conditions: 9 min 45 s at 95°C; then a touchdown of annealing temperatures to reduce nonspecific amplication, always starting with 20 s at 94°C; then 30 s at 60°C (3 cycles), 58°C (5 cycles), 56°C (5 cycles), 54°C (5 cycles), 52°C (5 cycles), or 50°C (22 cycles), and then 30 s (437-bp *env)*, 1 min (LTR) or 2 min 20 s (1,698-bp *env*) at 72°C for extension; and a final extension at 72°C for 7 min. PCR products were examined after electrophoresis on a 1% agarose gel. Primers and unincorporated deoxynucleotide triphosphates were removed by using Microcon YM (Millipore, Billerica, MA, USA) technology or exonuclease I and shrimp alkaline phosphatase (Amersham, Piscataway, NJ, USA) (Figure 2). Representative PCR products from independent amplifications were cloned and sequenced. For the *env* and LTR sequences, products were cloned from 4 PCR products each (Figure 7). Cloning was performed with a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was isolated from 6 to 16 clones from each reaction product by using a QIAGEN Miniprep Kit. Sequences were obtained from clones by using internal primers in standard ABI BigDye terminator (Applied Biosystems) reactions. Anticontamination measures were taken at all steps of PCR amplification and after PCR processing. Pre-PCR setup was performed in a laminar flow hood, DNA was added in a free-standing containment hood in a separate room, and all post-PCR manipulations were performed under a fume

hood in a third room. All surfaces were washed with a 10% bleach solution, and each hood was exposed to UV light for 30 min before and after use. PCR tubes with individual lids, rather than 96-well plates, were used and kept closed except when reagents and DNA were being added or aliquots were extracted for use. DNA tubes were opened only under their designated hoods; to avoid cross-contamination, tubes were never open simultaneously. Water and a sample from an FeLV-negative puma were run with every reaction as negative controls. Positive controls of known sequence were also run for each reaction: 1 from a domestic cat, 1 from a known seropositive panther (FP-115 or FP-122), or both.

**A**




**B**

| Forward Primer (3´→5´) | Reverse Primer (3´→5´) | gene | size | Name |
|---|---|---|---|---|
| AACARAAGTAAAGACTGTTGG | GCYTGGTGGGTCTTAGGAA | *env* | 437 bp | PfeF6/PfeR6 |
| TCTATGTTAGGAACCTTAACCGATG | TTAAGGCTTGTACCACAGATATTCTG | *env* | 1689 bp | PEF2/R14 |
| AAGTCCCCCTGGCTTACAAC | GGAGACCTAGTTCAGGGGTCTT | *env*/LTR | 725 bp | LTR4F/2R |

**Figure 7 (A)** Diagram of the FeLV genome showing the PCR products obtained from FeLV-Pco *env* and LTR genes. Envelope gene surface (SU) and transmembrane (TM) subunits, variable regions A and B (VRA and VRB) and the proline-rich region (PRR), 3' LTR enhancer element(s) (hatched rectangle), signature 21 bp repeat(s) (grey shade) and putative c-Myb binding sites (black triangles) (Chandhasin et al 2004) are depicted for FeLV-945, FeLV-Pco, and FeLV-3281A . Unique signature amino acid residues found only in FeLV-945 and FeLV-Pco are marked by asterisks (see Figure 5). **(B)** Primers used for PCR amplifications are reported in 5' to 3' orientation.

**Phylogenetic Analysis**

Sequences from *env* and LTR were analyzed separately. For analysis relative to known domestic cat FeLV sequences, we included FeLV-945, FeLVA-3281, FeLVA-61E, FeLVA-Glasgow-1, FeLVC-Sarma, FeLVB-Rickard, SM-FeSV, enFeLV-AGTT (accession nos. AY662447, M18248, M18247, M12500, M14331, X00188, M23025, AY364318 respectively) (*env*) and FCA-945, FCA-934, FeLVA-3281, and FeLVA-Glasgow-1 (accession nos. AY374189, AY374184, M18248, and M12500, respectively) (LTR). Nucleotide sequences were compiled and aligned for subsequent phylogenetic analysis by ClustalX (Thompson et al 1997) and verified visually (Maddison and Maddison*1995*). MODELTEST (Posada, and Crandall 1998) was used for *env* and LTR analysis to estimate the optimal model of sequence evolution; these settings were incorporated into subsequent analyses. Minimum-evolution trees were constructed from models of substitution specified by MODELTEST; starting trees were obtained by the neighbor-joining method, followed by application of a tree-bisection-reconnection branch-swapping algorithm during a heuristic search for the optimal tree. Maximum-parsimony analysis used a heuristic search of starting trees obtained by stepwise addition and followed by tree-bisection-reconnection. Maximum likelihood parameters specified by MODELTEST selected the general time-reversible model of substitution; they included empirical base frequencies and estimated rate matrix and corrected for among-site rate variation (□ distribution). A bootstrap analysis that used 1,000 iterations was performed with each method. Amino acid residue alignments were generated by using MacClade

3.05 (Maddison and Maddison1995) and ClustalX. Sequences were inspected for homoplasies. Nucleotide sequences were translated to protein, and genetic distances were calculated in MEGA 3.0 (Kumar et al 2004) by using the Tajima-Nei (nucleotide) and Dayhoff (amino acid) algorithms. The sequences of FeLV-Pco *env* and LTR were deposited in GenBank under accession nos. EU189489–EU189498.

## Results

### FeLV Serosurvey and PCR Amplification

The first sign of an emerging outbreak of FeLV in the free-ranging Florida panther population was the 2001 detection of FeLV antibodies, FeLV proviral PCR, or both, in 8 pumas from the Florida Panther National Wildlife Refuge, private lands, or the northern range of Big Cypress Swamp (Figure 6). Antigen-positive results and documented death compatible with FeLV infection first occurred in FP-115 in 2002 near the Okaloacoochee Slough State Forest (Cunningham et al 2008). With the exception of FP-108 and FP-119, found in the central region of Big Cypress National Park, all 19 other FeLV-exposed panthers were found north of Interstate 75 (Figure 6) (Cunningham et al 2008). During the next 2 years, 4 additional antigen-positive panthers died; FeLV-related disease was suspected for 2 (FP-123 and FP-132) and confirmed for 2 (FP-109 and FP-122). Additionally, 8 panthers (FP-67, FP-78, FP-82, FP-96, FP-99, UCFP43, FP-108, FP-118) that were antigen-negative but seropositive or PCR positive for

FeLV died during the outbreak, but their deaths were not attributed to FeLV (Cunningham et al 2008).

Retrospective screening of 6 panthers (FP-67, FP-78, FP-82, FP-109, FP-122, and FP-132) for antibody or antigen or by PCR demonstrated that they had not had FeLV infection before this outbreak. FP-96 in the Florida Panther National Wildlife Refuge was one of the first to have documented FeLV exposure; this panther displayed a latent infection, being PCR positive in 2001 and in 2002. Three panthers (FP-104, FP-107, FP-119) likely cleared the virus; after initial positive test results, they were seronegative on follow-up testing. Positive FIV antibody results by Western blot were found for 11 of the 19 FeLV-exposed and 2 of the 5 clinically affected panthers (Figure 6). An analysis of 21 microsatellites (short tandem repeats) showed that 6 of the 19 FeLV-exposed and 2 of the 5 antigen-positive panthers were crosses with some Texas heritage and that the rest were pure Florida panthers (W.E. Johnson et al., unpub. data).

**Phylogenetic Analysis**

An alignment of FeLV-Pco, FeLV-A, FeLV-B, and endogenous *env* nucleotide sequence (not shown) established the concordance of FeLV-Pco with subgroup A and found a lack of recombination of FeLV-Pco with endogenous FeLV-Pco sequence. The absence of endogenous sequences was not unexpected because pumas and other cats outside of the genus *Felis* do not carry endogenous FeLV sequences (Benveniste and Todaro 1975; Reeves and O'Brien 1984). The FeLV-Pco was classified as subgroup A on the basis of this lack of evidence for recombination with endogenous FeLV across 1,794 bp of

FeLV-Pco *env* sequence and on the basis of in vitro receptor utilization studies (Cunningham et al 2008). The aligned sequences of the LTRs and the *env* region were analyzed as separate datasets. For both datasets, phylogenetic analyses identified the FeLV-Pco sequences as monophyletic (Figure 8). Each had strong bootstrap support for a clade containing all FeLV-Pco but none of the previously sequenced domestic cat FeLVs (Figure 8). This pattern was consistent with a recent and focal introduction of the virus. Furthermore, the 376-bp nucleotide *env* sequence obtained from the earliest cases of FeLV exposure (Pco-972 and Pco-991, found respectively in the Florida Panther National Wildlife Reserve and northern Big Cypress National Preserve) were identical in sequence to the later FeLV cases found in the Okaloacoochee Slough State Forest (Appendix Figure 14). On the basis of >50 cloned envelope sequences (Figure 8; Table 4), the FeLV-Pco viruses associated with this outbreak were highly conserved. The mean percentage nucleotide and amino acid sequence differences of the FeLV *env* gene among FeLV-Pco sequences were 0.4% (nucleotide) and 0.1% (amino acid). Of published FeLV sequences available in GenBank, the closest strain was the domestic cat virus FeLV-945, according to LTR and *env* sequence comparisons (Figure 8); calculated differences were only 1.5% (nucleotide) and 3.5% (amino acid) between FeLV-Pco and FeLV-945 *env* sequences (Table 4).

**Figure 8**. Phylogenetic trees of panther feline leukemia virus (FeLV-Pco) and domestic cat FeLV nucleotide sequences. A) Midpoint rooted maximum-likelihood phylogram based on 1,698 bp of env sequences. B) Midpoint rooted maximum-likelihood phylogram based on 463 bp of 3′ long terminal repeat (LTR) sequences. Consensus FeLV-Pco sequences of clones generated from 5 env and 4 LTR panthers and reference domestic cat sequences are shown. The number of FeLV-Pco–cloned PCR products used in each consensus sequence is indicated in parentheses. The arrow indicates the monophyletic clade of all FeLV-Pco sequences. A similar topology, including the monophyletic clade, was obtained by using the different FeLV-Pco clone sequences rather than a consensus. The year of panther sampling is indicated as a suffix, e.g., Pco-1088-04 was sampled in 2004. Where maximum-likelihood tree was congruent with maximum parsimony tree, branch lengths are indicated below branches. Number of homoplasies is indicated after the branch length. Bootstrap values are shown (maximum parsimony/minimum evolution/maximum likelihood). The score (–ln likelihood) of the best maximum-likelihood tree was env 3615.01706, LTRs 1836.05922 (best tree found by maximum parsimony: env length = 221, consistency index [CI] = 0.941, retention index [RI] = 0.963; LTR length = 132, CI = 0.871, RI = 0.787).

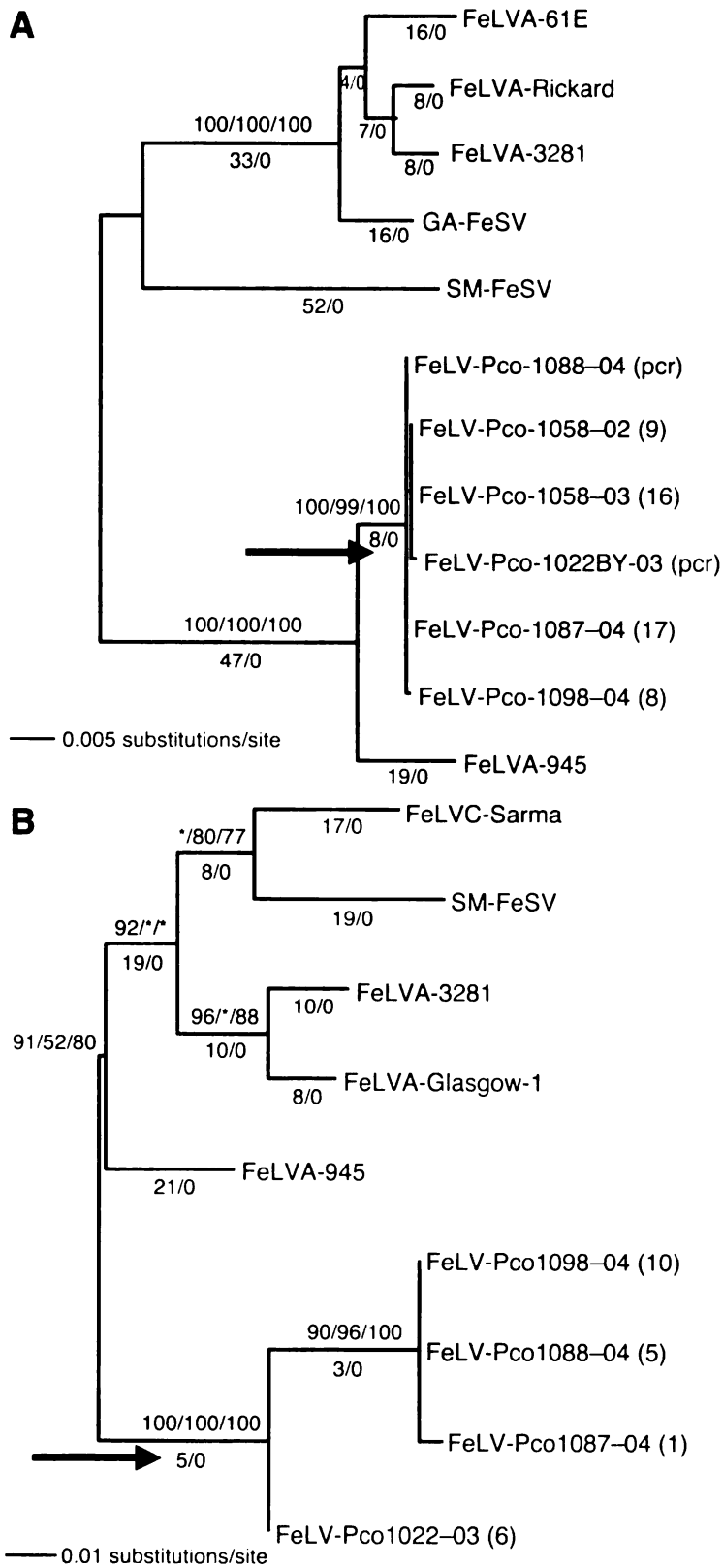

A
FeLVA-61E
16/0
4/0
FeLVA-Rickard
8/0
100/100/100
7/0
33/0
FeLVA-3281
8/0
GA-FeSV
16/0
SM-FeSV
52/0
FeLV-Pco-1088–04 (pcr)
FeLV-Pco-1058–02 (9)
100/99/100
FeLV-Pco-1058–03 (16)
8/0
FeLV-Pco-1022BY-03 (pcr)
100/100/100
FeLV-Pco-1087–04 (17)
47/0
FeLV-Pco-1098–04 (8)
0.005 substitutions/site
FeLVA-945
19/0
B
FeLVC-Sarma
*/80/77
17/0
8/0
SM-FeSV
19/0
92/*/*
19/0
FeLVA-3281
10/0
96/*/88
91/52/80
10/0
FeLVA-Glasgow-1
8/0
FeLVA-945
21/0
FeLV-Pco1098–04 (10)
90/96/100
FeLV-Pco1088–04 (5)
3/0
100/100/100
FeLV-Pco1087–04 (1)
5/0
FeLV-Pco1022–03 (6)
0.01 substitutions/site

**Figure 8**

**Table 4**. Mean percent amino acid and nucleotide *env* sequence differences of feline leukemia virus subgroups, FeLV-945, and FeLV-Pco strains*

| | FeLV A† | FeLV B‡ | FeLV C§ | FeLV-945¶ | FeLV Pco# |
|---|---|---|---|---|---|
| FeLV A | **1.8**, 3.8 | 10.3 | 6.6 | 6.4 | 6.1 |
| FeLV B | **19.1** | NA | 13.2 | 14 | 13.3 |
| FeLV C | **16.3** | **28.7** | **14.2**, 6.2 | 7.3 | 7.4 |
| FeLV 945 | **15.2** | **30.1** | **16.4** | NA | 1.5 |
| FeLV Pco | **14.3** | **28.2** | **16.7** | **3.5** | **0.4**, 0.1 |

*FeLV, feline leukemia virus. Shaded gray boxes contain mean percent sequence differences within strain. **Boldface** indicates mean percent amino acid *env* sequence differences, 1,689 bp (see Figure 2). Nucleotide differences are above diagonal.
†M18247, M18248, M12500, AF052723.
‡X00188.
§M14331, M23025
¶AY374189.
#Pco-1058-02 (9 cloned sequences), Pco-1058-03 (16 cloned sequences), Pco-1087-04 (17 cloned sequences), Pco-1098-04 (8 cloned sequences).

Because FeLV-945 is well characterized and highly virulent in the domestic cat (Chandhasin et al 2005a; Chandhasin et al 2005b), sequence elements associated with disease determination (*env*) and transcription enhancement (LTR) in FeLV-945 were examined in FeLV-Pco. In the envelope protein, 10 signature amino acid residues (found within the surface glycoprotein) that were shared between FeLV-Pco and FeLV-945 were distinctive from other strains of FeLV (Figure 9). Of these synapomorphic sites, 2 were in variable region A, which in FeLV-945 defines the specificity required for viral binding to receptors (Chandhasin et al 2005b). Three of the sites were within the proline-rich region, which in FeLV-945 encodes for conformational changes required for FeLV cell entry (Chandhasin et al 2005b). The FeLV-Pco LTR sequences had 1 copy of a 40-bp enhancer element that has been characterized in FeLV-945 (Appendix Figure 15; Finstad et al 2004). Finally, the exogenous domestic cat

FeLV-945 isolate, which FeLV-Pco strains resemble displays unusual repeat junctions where the transcription factor c-Myb is known to bind in FeLV-945, possibly accelerating the rate of transcription of the virus (Figure 7; Appendix Figure 15) (Finstad et al 2004). FeLV-Pco also contains 1 copy of this repeat junction, which supports the conclusion that FeLV-Pco is derived from a strain closely related to and perhaps from the pathogenic FeLV-945 domestic cat strain. FeLV-945 is unusual in that its severe pathogenicity does not involve recombination with endogenous FeLV in domestic cats. That FeLV-Pco pathogenesis in pumas is due to a virus similar to FeLV-945 that was not derived from endogenous recombination is consistent with the complete lack of endogenous FeLV sequences in the puma genome.

**Figure 9.** Variable sites in the amino acid alignment of panther feline leukemia virus (FeLV-Pco) and domestic cat FeLV env sequences (1,689 bp). Surface glycoprotein (SU), transmembrane (TM), variable region A and B (VRA and VRB), and proline-rich region (PRR) locations are indicated. Horizontal line separates sequences of puma (above) and domestic cat (below). The 10 amino acid residues in this region unique to FeLV-945 and FeLV-Pco sequences are shaded in gray. Matches to the reference sequence are indicated by dots; gaps are indicted by dashes.

<----SU----
----VRA----
---VRB---
---PRR--
A H I A D I V L D N K H G S K Y V G M H Q E N T S N S E G K V R G M T V T S T T D E A R P
Puma
FeLV-Pco-1022-03
FeLV-Pco-1058-02
FeLV-Pco-1058-03
FeLV-Pco-1087-04
FeLV-Pco-1088-04
FeLV-Pco-1098-04
Domestic cat
FeLVA-945
FeLVA-61E
FeLVA-3281
FeLVA-Glasgow-1
FeLVB-Rickard
SM-FeSV
FeLVC-Sarma
----PRR----
----TM----->
T N T S T P G P A M S G I I L T L K N K Q T A A T V D T L T M V E E R K K L F

Figure 9

## Discussion

We genetically characterized the FeLV that emerged in the previously naive free-ranging Florida panther population. According to the retrospective longitudinal antibody and antigen results and the virus' geographic distribution, the virus was likely introduced into the Florida panther population in 2001 (Figure 6) (Roelke et al 1993b). From the earliest detected panthers with FeLV (2001) to the most recent (2005), the FeLV-Pco *env* sequences were nearly identical, which indicates that the source of infection was likely a single domestic cat. FP-96, resident in the Florida Panther National Wildlife Reserve area in January of 2001, was the first panther with exposure detectable by PCR. The virus then spread north and east through the population, affecting individual panthers in Big Cypress (FP-100, FP-119), Seminole Indian Reservation (FP-118), and Okaloacoochee Slough (FP-109, FP-108, FP-115, FP-122, FP-123, FP-132) (Figure 6). Texas genetic heritage did not protect infected pumas from developing disease associated with FeLV; pure Florida panthers and pumas died after having symptoms compatible with FeLV (Figure 6).

Among characterized strains of FeLV, domestic cat FeLV-945 was closest in sequence to FeLV-Pco in the panthers. FeLV-945 in domestic cats was originally isolated as the predominant FeLV species from a geographic cohort of 21 infected domestic cats and is known to cause non–T-cell diseases characterized by degenerative and proliferative changes of myeloid and erythroid

origin (Chandhasin et al 2004). Although FeLV-945 is included among FeLV subgroup A isolates on the basis of cell receptor utilization, its distinctive envelope and LTR sequence signatures differ from those of other FeLV-A strains (Chandhasin et al 2005b). At the amino-terminal of the envelope sequence, the surface glycoprotein, also known as gp70, encodes the receptor-binding domain, within which are 2 variable regions, A and B. These define the specificity required for binding. Further downstream, a proline-rich region encodes for the conformational changes required for viral entry (Chandhasin et al 2005b).

The 10 envelope amino acid residues synapomorphic in FeLV-Pco and FeLV-945 included 2 in variable region A and 3 in the proline-rich region (Figures 7, 9). In FeLV-945 LTR, three 21-bp repeats form 2 junctions: 1 junction is formed by the first repeat and the adjacent second repeat; the other is formed by the second and third repeats. Each junction includes a c-Myb binding site that increases the rate of viral replication through the recruitment of transcriptional coactivator binding protein (cAMP response element) (Chandhasin et al 2005). FeLV-Pco LTR sequences had 1 copy of the repeat junction (Figure 7) (Finstad et al 2004). Upstream, LTR transcriptional enhancer elements repeated in tandem have been associated with thymic lymphomas and are found only in 1 copy in non–T-cell disease (Chandhasin et al 2004). Like FeLV-945, FeLV-Pco lacks this duplication (Figure 7).

In the panthers, clinical and pathologic findings of FeLV-Pco in this outbreak consisted of FeLV-related diseases of non–T-cell origin. These findings are consistent with the pathologic changes associated with FeLV-945 in the

domestic cat. Necropsy findings of FP-115 documented interstitial pneumonia, septicemia, and suppurative lymphadenopathy. Examination of FP-109 1 month before it died found lymphadenopathy, anemia, lymphopenia, and lymphoid hyperplasia. FP-122 had similar findings 1 month before it died, including lymphadenopathy, muscle wasting, and hypercellular bone marrow with >90% hematopoietic cells. FP-132 necropsy findings included severe pallor (indicative of anemia), bronchointerstitial pneumonia, abscesses, lymphadenopathy, and hypercellular bone marrow with >90% hematopoietic cells (Cunningham et al 2008). FeLV-Pco is therefore similar to the unique and virulent domestic cat strain FeLV-945 of FeLV subgroup A, in *env* and *LTR* sequence and in non–T-cell disease outcome. In the domestic cat, FeLV-945 causes multicentric lymphoma, myeloproliferative disorder, and anemia and has never been associated with thymic lymphoma (Chandhasin et al 2004). These findings shared between FeLV-945 and FeLV-Pco implicate the 10 identified amino acid synapomorphies (Figure 9) as plausible determinants of disease. Further study of these *env* regions from T-cell and non–T-cell disease manifestations of FeLV occurring in comparative felid species is warranted and may elucidate the key sequence determinants of disease outcome in FeLV.

The role of FIV-related immune suppression, if any, in this outbreak is uncertain. Although recent studies of T-lymphocyte profiles in FIV-infected wild lions and pumas suggest that CD4 depletion occurs (Roelke et al 2006), our survey found that co-infection with FIV was present in 2 but absent in 3 FeLV-associated deaths. FIV-positive panthers could have served as a reservoir for the

spread of FeLV through the population because the earliest detected FeLV-exposed panthers (FP-96 and FP-99) were FIV positive. Furthermore, the first panther (FP-115) detected with FeLV-compatible disease in the Okaloacoochee Slough State Forest region was also positive for FIV and FeLV for at least 6 months.

An FIV serosurvey suggested an overall increase in the prevalence of FIV in Florida panthers in recent years. During 1999–2000, 3 (15%) of 20 panthers tested had FIV-positive results by Western blot. In contrast, 13 (76%) of 17 panthers tested during 2004–2005 in the FeLV-endemic Okaloacoochee Slough State Forest region (Figure 6) were FIV positive (Cunningham et al 2008). These results could support a role for FIV-mediated immune depletion in FeLV pathogenesis. In domestic cats, FIV and FeLV co-infections have resulted in conflicting interpretations (Cohen et al 1990; Ishida et al 1989; Lee et al 2002; O'Connor et al 1991). In contrast to FIV, which is found in many species of wild felids (Troyer et al 2005), FeLV in nondomestic felids has been reported only a few times, in captive cats, with documented or suspected exposure to infected domestic cats (Cunningham et al 2008). Serologic survey of free-ranging populations found an absence of FeLV in pumas in California (Paul-Murphy et al 1994), among felids in Botswana (Osofsky et al 1995), and among 38 free-ranging Florida panthers sampled during 1978–1991 (Roelke et al 1993b). However, Jessup et al. (1993) document a case of FeLV in a young adult male free-ranging puma captured from a college campus in Sacramento, California. Necropsy of this cougar found generalized lymphadenopathy and

lymphoproliferative disease. These necropsy results are consistent with and similar to the clinical findings of the FeLV-positive panthers reported here.

The outbreak of FeLV in the previously naive population of endangered Florida panthers raised questions about management of free-ranging pumas. In response, the Florida Department of Fisheries and Wildlife began a widespread vaccination program of Florida panthers; no additional FeLV cases have since been detected among them (Cunningham et al 2008).

This emerging disease outbreak was characterized by 2 factors. First, because of its unique heritage and popularity, the Florida panther has been the most intensively monitored wild felid in North America. Second, the extensive veterinary surveillance of the domestic cat has provided powerful models for studying infectious diseases relevant to understanding human health and disease (Roelke et al 1993b) including retroviruses such as FeLV. Although future cross-species transmission events among wild and domestic carnivore populations may be unavoidable, our understanding of pathogen and host genetic determinants may also be greatly enhanced by the recent release of the genome sequence of the domestic cat (Pontius et al 2007). Combining progress in biomedical genomics with intensive studies of wild species can provide insights into emerging pathogens that affect wild, domestic, and human hosts.

## CHAPTER THREE

## The recent emergence of feline immunodeficiency virus (FIV) in free-ranging Mongolian Pallas' cats

### Introduction

The Pallas' cat (*Otocolobus manul*), a small wild cat, is endemic to Central Asia and is considered threatened with extinction primarily because of habitat loss, vermin control programs, and hunting for the fur trade (Convention on International Trade in Threatened Species 2001). Taxonomically, the Pallas' cat is classified as the sole representative of its genus (*Otocolobus*) (Johnson et al 2006) and is noted for its long fur, stocky build, and flattened face (Nowell and Jackson 1996). Pallas' cats have a unique and extreme susceptibility to infectious agents, especially *toxoplasma gondii*, in comparison to other captive non-domestic cat species (Brown et al 2005). These and other cases of opportunistic infections have been associated with suspected (Ketz-Riley 2003) and confirmed (Barr et al 1995) cases of immunodeficiency due to feline immunodeficiency virus (FIV).

Feline immunodeficiency virus (FIV) causes immune dysfunction in domestic cats, resulting in depletion of CD4+ cells, increased susceptibility to opportunistic infections, and sometimes death (Pedersen et al 1987). FIV is also found in nondomestic felids; a recent serosurvey of 3055 individuals found eleven free-ranging felid species infected with FIV (Troyer et al 2005). Monophyly of FIV proviral sequence for most species suggests that FIV transfer between cat species is an infrequent event. FIV is endemic in the large African carnivores and

most of the South American felids, which maintain a lower FIV-positive level throughout their range. The free-ranging Pallas' cat is the only known species from Asia that has a species-specific strain of FIV (Troyer et al 2005). The only other known case of FIV in free-ranging non-domestic Asian cats was of a Leopard cat in Japan infected with a domestic cat FIV strain (Nishimura et al 1999) through suspected cross-species transmission.

Pallas' cat FIV, designated FIV-Oma, was first isolated from a wild-born male Pallas' cat imported into the United States from Kazakhstan (Oma-Barr) (Barr et al 1995). As in recent reports of immune depletion associated with FIV infection in lions and pumas (Roelke et al 2006, Roelke et al in preparation 2008), this Pallas' cat also exhibited a low CD4+/CD8+ T-cell ratio and was co - infected with opportunistic infections of *Trypanosoma* species and *Hepatozoon canis.* Virus from this cat was isolated and characterized *in vitro* and found to be highly cytopathic in crandell feline kidney cells in contrast to other isolates of domestic cat FIV (Barr et al 1995).

In this study, samples from wild Pallas' cats living in central Mongolia were assessed for FIV seroprevalence. Proviral DNA was amplified and FIV cloned sequences from three wild Pallas' cats was obtained and analyzed phylogenetically in relation to other known FIV-Oma and other FIV sequences. FIV-Oma was found to be monophyletic with little genetic distance among FIV isolates from disparage geographic locations, suggestive of a 20$^{th}$ century introduction of FIV into the wild Pallas' cat population.

## Materials and Methods

### Sample collection and FIV status

Blood samples and necropsy tissues were collected from 28 free-ranging radio-collared Pallas' cats monitored in a long-term ecology study (Steve Ross, University of Bristol PhD thesis). Sample collection and animal handling was done as previously described (Brown et al 2006). Serum and buffy coat aliquots were stored at -70°C. Fifteen domestic cat serum samples from the region were also included. FIV status was determined on serum samples by enzyme-linked immunoassays (ELISA) for feline immunodeficiency virus (FIV; Petchek FIV ELISA, Idexx Laboratories, Westbrook, Maine, USA) and verified by western blot using the three-antigen detection method using FIV-Fca, Pco, and Ple (Troyer et al 2005) for samples Oma 27-Oma 38 and the FIV-Oma antigen was used for western blots run on Oma 60-Oma 122 (Cornell University Animal Health Diagnostic Center Ithaca, New York USA).

### PCR amplification of proviral DNA and Phylogenetic analysis

Genomic DNA was isolated from buffy coat samples as described previously (see chapter 2). PCR primers were designed from FIV-OmaBarr (accession number U56928; Barr et al 1997) sequence available on GenBank and PCR, cloning, phylogenetic analysis, and mean genetic distance calculations were performed as described previously (see chapter 2).

### Pathology

Opportunistic collection of necropsy specimens from free-ranging Pallas' cats at the Altanbulag study site was performed. Tissues from a deceased

Pallas' cat was cut into sections approximately 1 $cm^3$ thick and stored in 10% buffered formalin and routinely embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin (HE) (National Cancer Institute Laboratory Animal Sciences Program Frederick, Maryland, USA). Similar tissues were obtained and processed from a wild-caught captive FIV positive Pallas' cat maintained at Wildlife on Easy Street Big Cat Rescue (Tampa, Florida USA). HE slides of spleen, liver, lymph node, intestine, and kidney sections were evaluated for evidence of immune depletion by Rani Sellers, a board-certified pathologist (Department of Pathology Albert Einstein College of Medicine Yeshiva University Bronx, New York, USA).

## Results and Discussion

Seroprevalence of FIV in twenty-eight free-ranging Pallas' cats found in the central province of Mongolia (Altanbulag) sampled from 2000-2007 was 25% based on FIV ELISA and western blot results (Table 5). Additionally, 15 domestic cats found in the rocky steppe around and within the village of Altanbulag were FIV negative by ELISA. A 470 bp fragment of proviral *RT-Pol* sequence was obtained from three of the free-ranging Pallas' cats (Oma-61, Oma-118, and Oma-121) and an FIV-positive wild-born captive Pallas' cat (Oma-34) originally from the southern province of Mongolia (Gobi) which is >500 miles away from Altanbulag. PCR fragments were cloned and a total of 78 cloned sequences from these 4 cats were produced, resulting in 23 unique sequences.

FIV sequence from four additional wild-born captive Pallas' cats from Russia (Oma12, 21, 22) and Kazakhstan (Oma-Barr) were included for phylogenetic analysis. The FIV-Oma sequences from these eight cats, representing disparate geographic ranges, were monophyletic within the Pallas' cat species relative to other FIV species (Figure 10) without structure relating to geographic distribution (Figure 10) (Troyer et al 2005, Carpenter et al 1996). Of FIV isolated from other felid species, FIV-Oma is most similar to FIV-Ppa (leopard) and FIV-Aju (cheetah) (Figure 11).

**Table 5**. FIV-ELISA and FIV-western blot* results and demographic information for 28 free- ranging Pallas' cats.

| ID | Sex | Age | Sample year | FIV-ELISA | FIV-WB* | Range | Status | GenBank no. |
|---|---|---|---|---|---|---|---|---|
| Oma 27 | F | 2 yrs | 2000 | N | N | Altanbulag | Wild | |
| Oma 28 | F | 1-2yrs | 2000 | N | N | Altanbulag | Wild | |
| Oma 29 | F | 1-2yrs | 2000 | N | P | Altanbulag | Wild | reported here |
| Oma 30 | F | 1-2yrs | 2000 | N | N | Altanbulag | Wild | |
| Oma 31 | M | 2 yrs | 2000 | N | N | Altanbulag | Wild | |
| Oma 32 | M | 1-2yrs | 2000 | N | P | Altanbulag | Wild | reported here |
| Oma 35 | M | 2 yrs | 2001 | N | N | Altanbulag | Wild | |
| Oma 36 | F | 1-2 yrs | 2001 | N | N | Altanbulag | Wild | |
| Oma 37 | F | 1-2 yrs | 2001 | N | P | Altanbulag | Wild | |
| Oma 38 | F | 2 yrs | 2001 | N | N | Altanbulag | Wild | |
| Oma 60 | M | 1-2 yrs | 2004 | N | N | Altanbulag | Wild | |
| Oma 61 | M | 1-2 yrs | 2004 | P | nd | Altanbulag | Wild | reported here |
| Oma 62 | F | 3 yrs | 2005 | N | N | Altanbulag | Wild | |
| Oma 63 | F | 1-2yrs | 2005 | N | N | Altanbulag | Wild | |
| Oma 64 | M | 1-2yrs | 2005 | N | N | Altanbulag | Wild | |
| Oma 65 | M | 1-2yrs | 2005 | N | N | Altanbulag | Wild | |
| Oma 101 | F | 3-5 yrs | 2006 | N | N | Altanbulag | Wild | |
| Oma 102 | F | 3-5 yrs | 2006 | N | N | Altanbulag | Wild | |
| Oma 106 | F | 3-5 yrs | 2006 | N | N | Altanbulag | Wild | |
| Oma 107 | M | 3-5 yrs | 2006 | N | N | Altanbulag | Wild | |
| Oma 114 | F | 3-5 yrs | 2007 | N | N | Altanbulag | Wild | |
| Oma 115 | M | 8 mo | 2007 | N | N | Altanbulag | Wild | |
| Oma 117 | M | 1.5 yrs | 2007 | N | N | Altanbulag | Wild | |
| Oma 118 | M | 1.3 yrs | 2007 | P | P | Altanbulag | Wild | reported here |
| Oma 119 | M | 1.5 yrs | 2007 | P | nd | Altanbulag | Wild | reported here |
| Oma 120 | M | 10 mo | 2007 | N | N | Altanbulag | Wild | |
| Oma 121 | M | 1.5 yrs | 2007 | P | P | Altanbulag | Wild | reported here |
| Oma 122 | M | 2 yrs | 2007 | N | N | Altanbulag | Wild | |
| Oma 34 | M | 10 yrs | 2001 | P | P | South Gobi | Wild-born captive | |
| Oma 12 | M | 3-5 yrs | 1992 | nd | P | Russia | Wild-born captive | AY878239 |
| Oma 21 | ukn | 3-5 yrs | 1998 | nd | P | Russia | Wild-born captive | AY878241 |
| Oma 22 | ukn | 3-5 yrs | 1998 | nd | P | Russia | Wild-born captive | AY878238 |
| Oma Barr | M | 1-2yrs | 1992 | P | P | Kahzakstan | Wild-born captive | U31349 |

FIV positive pallas' cats are highlighted in grey. Additional isolates of FIV-Oma from wild-born captive Pallas' cats from disparate geographic regions are also listed.

F=female; M=male; Yrs=years; mo=months; N=negative; P=positive; nd=not done.

* Three-antigen detection method using FIV-Fca, Pco, and Ple (Troyer et al 2004) employed for western blots run on Oma 27-Oma 38; FIV-Oma antigen used for western blots run on Oma 60-Oma 122.

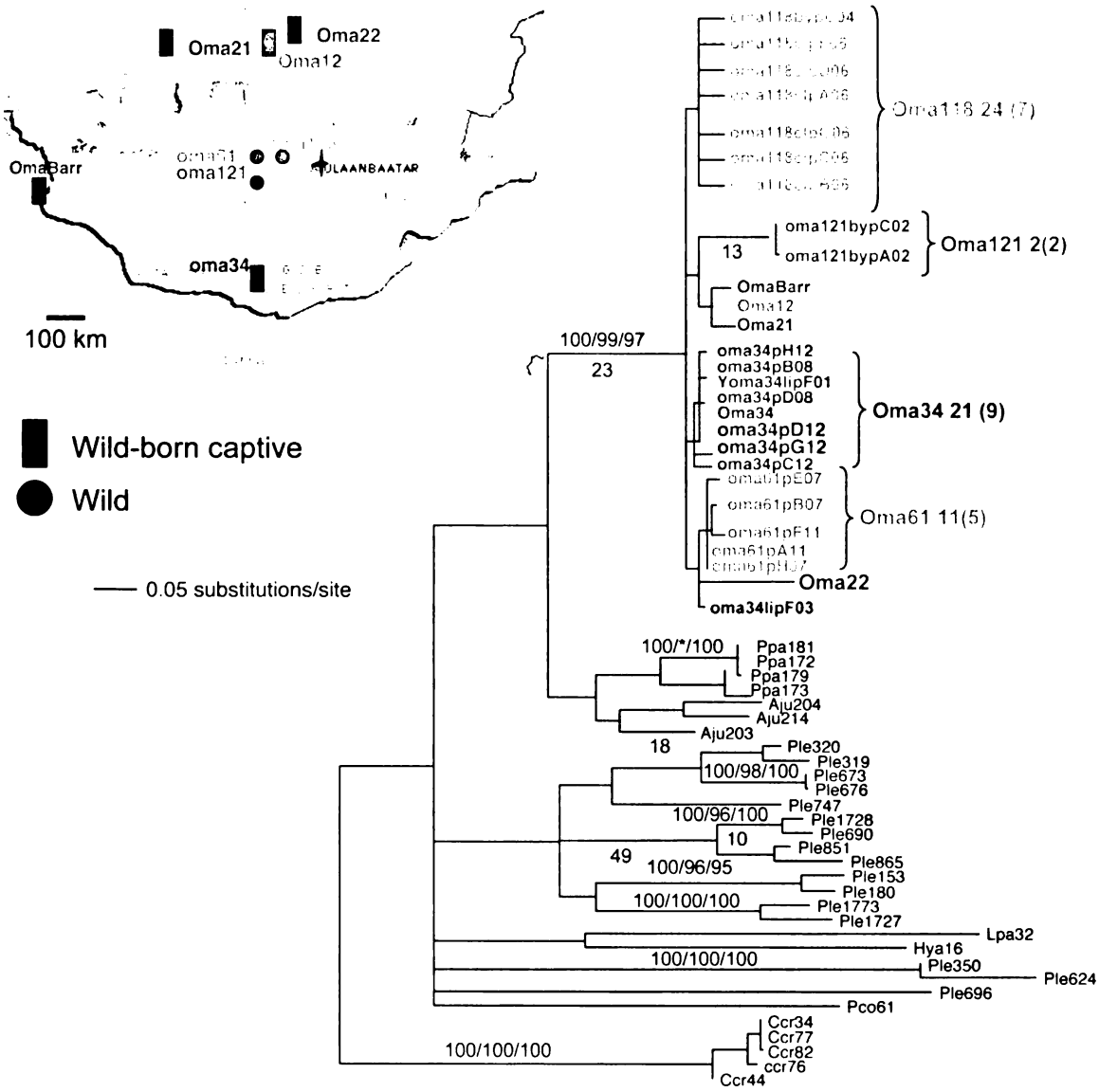



**Figure 10.** Phylogenetic tree of proviral RT-Pol (470 bp) FIV sequence highlighting the monophyletic clade of the eight FIV-Oma reported in this study. Maximum likelihood tree shown. Bootstrap values (maximum parsimony/minimum evolution/maximum likelihood) are reported when greater than 85). When maximum parsimony tree topology is concordant with maximum likelihood tree, number of steps is indicated below the branches. The score (–ln likelihood) of the best maximum-likelihood tree was 3723.037761, consistency index [CI] = 0.321, retention index [RI] = 0.701. GenBank accession numbers used in this analysis: for FIV-Ple (lion) (Ay878208-AY878222), FIV-Pco (puma) (AY878236- AY878237), FIV-Ccr (spotted hyena) (AY878196-AY878200), FIV-Aju (cheetah) (AY878201-AY87203), FIV-Ppa (leopard) (AY878204-AY878207), FIV-Lpa (AY878194) (ocelot), FIV-Hya (jaguarundi) (AY878195, FIV-Oma-22,34,12,21,Barr (pallas' cat) (AY878238-AY878241, U31349).

Mean percent genetic distances were calculated among individual Pallas' cat FIV *RT-Pol* sequences (Table 6) and among all available FIV-Oma *RT-Pol* sequences reported here and from GenBank (table 6). The low genetic variation among all FIV-Oma sequences (1.9%) is comparable to the genetic variation (2.2%) (calculated based on the same portion of *RT-Pol* sequence) of a population of 23 feral domestic barn cats, estimated to be isolated and in existence for 60 years (Carpenter et al 1998) . This is in great contrast to the genetic distances observed in FIV-Ple and FIV-Pco, which are reportedly as high as 28-34% among FIV-Ple isolated from geographically disparate lions (Troyer et al 2004) and pumas (Carpenter et al 1996, Biek et al 2006). In contrast, the monophyletic clade and low genetic distance observed here is suggestive of a 20$^{th}$ century introduction of FIV-Oma into the Asian Pallas' cat population.

**Table 6**: Mean percent nucleotide differences among individual cloned FIV-Oma isolates in the *Pol-RT* region.

| | | **no. clones** | **no. unique sequences** |
|---|---|---|---|
| **Oma 34** | 0.3 | 21 | 9 |
| **Oma 61** | 0.4 | 11 | 5 |
| **Oma 118** | 0.3 | 24 | 7 |
| **Oma 121** | 0.2 | 2 | 2 |

Based on recent full proviral genome analysis of two subtypes of FIV-Ple, FIV-Fca, FIV-Pco, and FIV-Oma revealing evidence of recombination events occurring between FIVPle from Serengetti lions and FIV-Oma, it has been proposed that FIV-Oma was first introduced to the Asian Pallas' cat population as

early as the Pleistocene era, some 1,808,000 to 11,550 years ago when lions ranged throughout Eurasia (Pecon-Slattery et al 2008). Other more recent opportunities for cross-species transmission from African felids into Asia, and more consistent with the findings reported here, would be during the reign of Kublai Khan (14$^{th}$ century some 700 years ago) when, according to the writing of Marco Polo, the Mongol ruler held one thousand captive and wild-born cheetahs and moved them throughout China and outer Mongolia on hunting expeditions (1845).

This report is limited to only eight wild or wild-born Pallas' cats. Since only one free-ranging population (Altanbulag, Mongolia) in included in this analysis, it is possible that the wild-born captive Pallas' cats from the disparate geographic regions were exposed to a Mongolian Pallas' cat FIV strain in captivity.

Veterinary surveillance of wildlife populations in Asia has revealed the emergence of FIV in the wild Pallas' cat, the only known free-ranging cat in Asia to harbor this virus. Sequence analysis of proviral *RT-Pol* from the eight available FIV-Oma isolates suggests that the current circulating virus was likely introduced into the population by an African felid during a rare cross-species transmission event sometime in the past. Whether naturally occurring FIV-Oma contributes to immune depletion, as observed in domestic cats and free-ranging lions and pumas, remains an important question to the conservation of this threatened species. Histopathological changes consistent with immune depletion (Figure 11) were observed in Oma-34, a wild-born captive Pallas' cat, which lived far beyond the lifespan of the wild Pallas' cats studied in Altanbulag (Table 5).

Further surveillance of the disease ecology in free-ranging Pallas' cats, now known to be infected by this potentially immune-debilitating virus, is warranted.

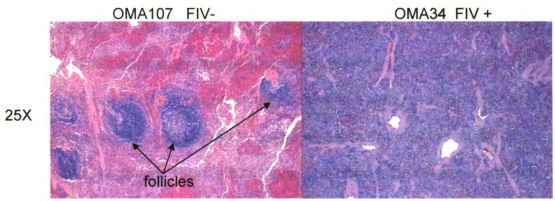



**Figure 11.** Histopathology of spleen from an FIV positive (Oma 34) versus FIV negative (Oma 107) Pallas' cat from Mongolia. Note the loss of normal tissue architecture and lack of large follicles in Oma34. HE slides shown at 25X magnification.

# CONCLUSION

In this thesis, I have genetically characterized three viral infections in naturally infected felids and discussed the viral pathogenesis and dynamics of these agents. I have interpreted the molecular viral signatures of pathogenesis in the context of host and environmental factors such as concurrent disease, host genetic make-up, and geographic and temporal association. I have recognized the conservation management implications to these findings and identified opportunities for application to veterinary population medicine. Further, I have revealed avenues for future research, based on these findings in nature, of specific molecular genetic signatures to look at in the design of experiments in the cat animal model for analogous human viral infections including HIV-AIDS (FIV, FeLV), lymphoma, leukemia, myloproliferative disorder, and anemia (FeLV), and SARS-CoV and Dengue Hemorrhagic Fever (FCoV).

The genotype arrays in the viral membrane protein associated with pathogenesis in feline infectious peritonitis (FIP) reported here raise the prospects of developing a diagnostic aid in the management of feline coronavirus (FCoV). This will have widespread management implications for detecting pathogenic strains of FCoV in multi-cat households, feral cat rescue organizations, and in captive felid zoological settings. Additional sample collection of FCoV cases from diverse geographic locations for similar virus association-study will determine how generalizable these findings are and add credibility to the utility of these finding for diagnostic purposes. Based on the

findings in natural populations reported here, the exact determinants of viral pathogenesis can be further pinpointed by designing cat challenge experiments with engineered chimeric feline coronaviruses. Additionally, and in parallel, host genetic determinants of pathogenesis may be investigated for association with the pathogenesis of FIP. As is suspected here and has been revealed in HIV infection, both the viral strain and host immune genes contribute to disease progression to virus-related death, such as AIDS progression in HIV infection (Hill 2006). With the recent release of the full cat genome sequence (Pontius et al 2007) and the viral genotype arrays described here, the genomic tools are now available to proceed with both viral and host genetic association studies in the pathogenesis in feline coronavirus infection, a model for coronavirus infection in humans such as SARS-CoV.

The recent deadly outbreak of FeLV, reported here, in a genetically-impoverished relic wild population of puma, the Florida panther, was discovered, documented, treated by vaccine, and stopped. The unanticipated virulence and pathogenesis of this strain was explained by molecular phylogenetic analyses of the invading FeLV-Pco agent: a rare FeLV strain that contains genetic sequences which render it debilitating and allow it to function independent of normally requisite endogenous FeLV sequences (which are absent in pumas). A combination of unusually complete historic insight, close veterinary and demographic surveillance of free ranging Florida panthers, precise molecular biology and forensic detection led to the identification, resolution, acute vaccination, and reversal of the ongoing epidemic. Further study of the unique

*envelope* and LTR sequence elements identified here in the emergent FeLV in the naïve free-ranging Florida panther population as plausible determinants of disease will inform viral pathogenesis studies in the FeLV cat animal model.

Finally, this is the first report of FIV in a free-ranging felid population from Asia. FIV was found to occur in this wild population at a prevalence of 25%. Sequences analysed of *RT-Pol* from this population, as well as isolates from Kazakhstan, Southern Mongolia, and Russia were monophyletic without structure relating to geographic distribution such as that seen in FIV-Ple (lion) and FIV-Pco (puma) suggestive of a recent emergence of FIV into the wild Pallas' cat population. Further surveillance of the disease ecology in free-ranging Pallas' cats, now known to be infected with this suspected immune-debilitating virus, is warranted.

This thesis combines the fields of molecular viral genetics, veterinary medicine, and wildlife management. The findings advance the field of virology with significant advances and implications for both veterinary medicine and wildlife management. I have attempted to highlight the lessons learned about viral dynamics and pathogenesis in the cat family using this multi-disciplinary approach and to illustrate the promise of such pursuits in future discovery of emerging viruses at the interface of animal and human health.

# APPENDIX

**Table 7: Clinical and demographic data from 56 domestic cats sampled in Maryland from 2004-2006**

FIPV cases are shaded in grey in the cat ID column.

[α]FCAC=Frederick County Animal Shelter; NM=New Market Animal Shelter

χ Hematocrit (%) Normal range 24-45%; δ Red blood cells ($x10^6/\mu l$) Normal range 6.1-11.9; ε White blood cells ($x10^3/\mu l$) Normal range 4.9-20.0

φ Lymphocytes ($x10^3/\mu l$) Normal range 1500-7000; γ Neutrophils segmented ($x10^3/\mu l$) Normal range 3000-12000; η Neutrophils bands ($x10^3/\mu l$) Normal range 100-300; ι Total protein (g/dl) Normal range 6-8.3; φ Globulin (g/dl) Normal range 24-48; κ Total bilirubin (mg/dl) Normal range 0.0-0.2; qns=quantity of sample was insufficient for test; λ FIV status: neg=negative. Blank fields indicate test not done.

## Table 7: Cinical and demographic information 56 domestic cats.

| cat ID | Name | Hem χ | rbc δ | wbc ε | Lymph φ | Neut γ | Bands η | Pro ι | Glo ϕ | Bili κ | FIV λ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FCA-4549 | Hank | 41.1 | 8.55 | 4.2 | 210 | 3864 | 84 | | | | neg |
| FCA-4561 | PregnantF | 24.9 | 5.79 | 5.1 | 4539 | 357 | 0 | | | | neg |
| FCA-4562 | Bryan | 0.3 | 0.23 | 8.3 | 415 | 387 | 0 | 7.4 | 3.6 | 0.3 | neg |
| FCA-4563 | Dreamcylcle | 27.3 | 6.32 | 9.4 | 1222 | 7802 | 0 | 7.7 | 4.4 | 0.1 | neg |
| FCA-4564 | Michael | 7.1 | 0.24 | 6.5 | 975 | 810 | 0 | 7.2 | 3.6 | 0.1 | neg |
| FCA-4566 | Ace | 21.3 | 4.68 | 5.9 | 295 | 5192 | 0 | | | | |
| FCA-4580 | Phoebe | 33.6 | 7.61 | 11.2 | 5936 | 3920 | 0 | | | | |
| FCA-4581 | Palmer | 42.7 | 9.89 | 13.8 | 4002 | 8142 | 0 | | | | |
| FCA-4582 | Ying | 40.7 | 8.6 | 12.2 | 5490 | 4880 | 0 | | | | |
| FCA-4583 | Yang | | | | | | | | | | |
| FCA-4584 | Sydney | 28.9 | 6.12 | 8.2 | 1476 | 6150 | 0 | | | | |
| FCA-4585 | Swain | | | | | | | | | | |
| FCA-4586 | Josie | 36.6 | 8.05 | 11 | 2970 | 6050 | 0 | | | | |
| FCA-4587 | Mo jo | 41.3 | 8.33 | 11.8 | 3068 | 7080 | 0 | | | | |
| FCA-4588 | Simpson | 36.5 | 8.36 | 7.7 | 2079 | 5005 | 0 | | | | |
| FCA-4589 | Ty | 41.3 | 9.58 | 10.3 | 3193 | 5871 | 0 | | | | |
| FCA-4590 | Diesel | | | | | | | | | | neg |
| FCA-4591 | Chenille | 38 | 8.39 | 18 | 9180 | 7200 | 0 | | | | |
| FCA-4592 | Kerrigan | 39.8 | 8.85 | 19.4 | 9312 | 8730 | 0 | | | | |
| FCA-4593 | Leroy | 39.2 | 8.75 | 11.4 | 2508 | 7524 | 0 | | | | |
| FCA-4594 | Ursa | | | | | | | | | | |
| FCA-4595 | Snuffy | | | | | | | | | | |
| FCA-4596 | Teva | | | | | | | | | | |
| FCA-4597 | Bings | 27.9 | 5.95 | 14.7 | 2058 | 10731 | 0 | | | | |
| FCA-4606 | Elsa | 34.1 | 7.4 | 15.4 | 3850 | 9856 | 0 | | | | |
| FCA-4607 | BamBam | 32.3 | 7.54 | 12.4 | 3720 | 8556 | 0 | | | | |
| FCA-4608 | Layla | 32.8 | 7.68 | 14.9 | 4619 | 8940 | | | | | |
| FCA-4609 | Queenie | 33.7 | 6.61 | 6.9 | 2208 | 3933 | 0 | | | | |
| FCA-4611 | Cheeks | 35.4 | 7.61 | 14.6 | 3212 | 10366 | 0 | | | | |
| FCA-4612 | Rocket | 29.6 | 6.12 | 10.1 | 2323 | 6868 | 0 | | | | |
| FCA-4613 | Jasmine | 40 | 7.83 | 11.2 | 2240 | 7280 | 0 | | | | |
| FCA-4614 | BoBo | 37 | 9.39 | 7 | 1260 | 5180 | 0 | | | | |
| FCA-4615 | Patches | 30.4 | 6.64 | 35 | 1750 | 29750 | 1400 | | | | |
| FCA-4616 | Isis | 36.1 | 6.54 | 15.2 | 4104 | 8816 | 0 | | | | |
| FCA-4618 | Elliot | | | | | | | | | | |
| FCA-4620 | Tony | | | | | | | | | | neg |
| FCA-4623 | KofBings | | | | | | | | | | |
| FCA-4624 | Zoe | 30.5 | 6.46 | 26 | 16900 | 6760 | 260 | 6.8 | 3.4 | 0.1 | |
| FCA-4625 | Goldie | | | | | | | | | | neg |
| FCA-4626 | Saddie | | | | | | | | | | neg |
| FCA-4627 | Sassy | 31.6 | 7.93 | 8 | 6000 | 1600 | 0 | | | | |
| FCA-4628 | Rosie | qns | qns | 8 | 2560 | 4880 | 0 | 7.5 | 5.1 | 0.3 | neg |
| FCA-4629 | Lilly | 18.8 | 4.93 | 7.4 | 962 | 5994 | 0 | | | | neg |
| FCA-4630 | Rudy | 32.9 | 7.2 | 10.3 | 6901 | 2781 | 0 | 7.2 | 4.2 | 0.3 | neg |
| FCA-4631 | Boy | qns | qns | 5.6 | 3136 | 2184 | 0 | | | | |
| FCA-4653 | Zoe | 28.5 | 7.4 | 18.6 | 2046 | 15996 | 0 | 9.4 | 6.9 | 0.1 | neg |
| FCA-4654 | Thkitten | | | | | | | | | | |
| FCA-4655 | Penny | 7 | 4.62 | 6.6 | 2178 | 4158 | 0 | | | | |
| FCA-4656 | Basil | | | | | | | | | | |
| FCA-4657 | Parsley | | | | | | | | | | |
| FCA-4658 | FCAC | | | | | | | | | | |
| FCA-4659 | Concerta | | | | | | | | | | |
| FCA-4660 | Starfox | | | | | | | | | | |
| FCA-4662 | OJ male | | | | | | | | | | |
| FCA-4663 | OJ female | | | | | | | | | | |
| FCA-4664 | Buster | | | | | | | | | | |

**Figure 12**: Mid-point rooted maximum likelihood tree of unique **of *RT-Pol*** 386 bp *pol replicase* sequences (ML -ln L=1300.12586 best tree found by MP: length =125, CI=0.832, RI= 0.926), ***Spike*** ML -ln L=4122.02368 best tree found by MP: length =98, CI=0.801, RI= 0.911) and **Spike B/NSP3a-c** 1017 bp ML -ln L=2804.53198 best tree found by MP: length =280, CI=0.800, RI= 0.954 Sequence from FIPV biotypes are shown in pink; FECV biotypes in green. The number of cats is indicated in parenthesis in the key. Each sequence is labeled as follows: four-digit cat identification number, tissue source (fe=feces, af=ascites fluid, co=colon, li=liver, sp=spleen, in=intestine, je=jejunum, ln=lymph node), 2 digit year (eg. 04=2004), and finally the unique three-four digit sequence number. The number of clones for each sequence is indicated after the sequence label in parenthesis. Where maximum likelihood tree was congruent with maximum parsimony tree, branch lengths are indicated below branches and the number of homoplasies is in parenthesis. Bootstrap values are shown (maximum parsimony/minimum evolution/maximum likelihood) above branches.

**Figure 12**

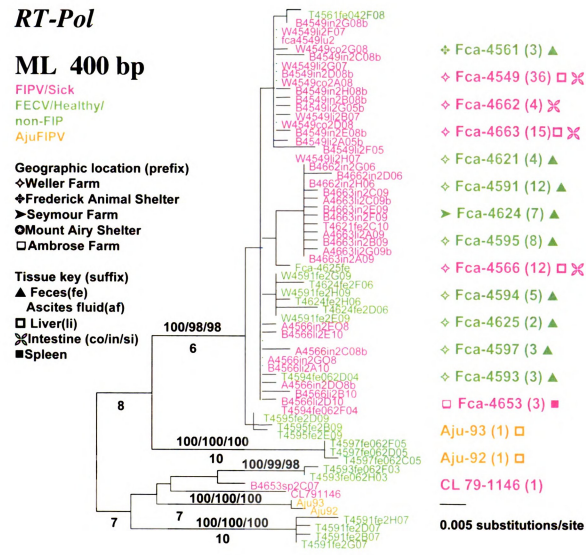

RT-Pol
ML 400 bp
FIPV/Sick
FECV/Healthy/
non-FIP
AjuFIPV
Geographic location (prefix)
✧Weller Farm
✚Frederick Animal Shelter
➤Seymour Farm
❂Mount Airy Shelter
❑Ambrose Farm
Tissue key (suffix)
▲ Feces(fe)
Ascites fluid(af)
◘ Liver(li)
Intestine (co/in/si)
■Spleen
100/98/98
6
8
100/100/100
10
100/99/98
100/100/100
7
7
100/100/100
10
T4561fe042F08
B4549in2G08b
W4549li2F07
fca4549lu2
W4549co2G08
B4549in2C08b
W4549li2G07
B4549in2D08b
W4549co2A08
B4549in2H08b
B4549in2B08b
B4549li2G05b
W4549li2B07
W4549co2D08
B4549in2E08b
B4549li2G05b
B4549li2F05
W4549li2H07
B4662inXG06
B4662in2D06
B4662in2H06
B4663in2C09
A4663li2C09b
B4663in2E09
B4663in2F09
T4621fe2C10
A4588li2A09
B4663in2B09
A4663li2G09b
B4663in2A09
Fca-4625fe
W4591fe2C09
T4624fe2F06
W4591fe2H08
T4624fe2H06
T4624fe2D06
W4591fe2E09
A4566in2E08
B4566li2E10
A4566in2C08b
A4566in2G08
B4566li2A10
T4594fe062D04
A4566in2D08b
B4566li2B10
B4566li2D10
T4594fe062F04
T4595fe2D09
T4595fe2B09
T4595fe2E09
T4597fe062F05
T4597fe062D05
T4597fe062C05
T4593fe062F03
T4593fe062H03
B4653sp2C07
CL791146
Aju93
Aju92
T4591fe2H07
T4591fe2D07
T4591fe2B07
T4591fe2G07
Fca-4561 (3) ▲
Fca-4549 (36) ◘ ✕
Fca-4662 (4) ✕
Fca-4663 (15)◘ ✕
Fca-4621 (4) ▲
Fca-4591 (12) ▲
Fca-4624 (7) ▲
Fca-4595 (8) ▲
Fca-4566 (12) ◘ ✕
Fca-4594 (5) ▲
Fca-4625 (2) ▲
Fca-4597 (3 ▲
Fca-4593 (3) ▲
❑ Fca-4653 (3) ■
Aju-93 (1) ◘
Aju-92 (1) ◘
CL 79-1146 (1)
0.005 substitutions/site

**Figure 12 cont.**

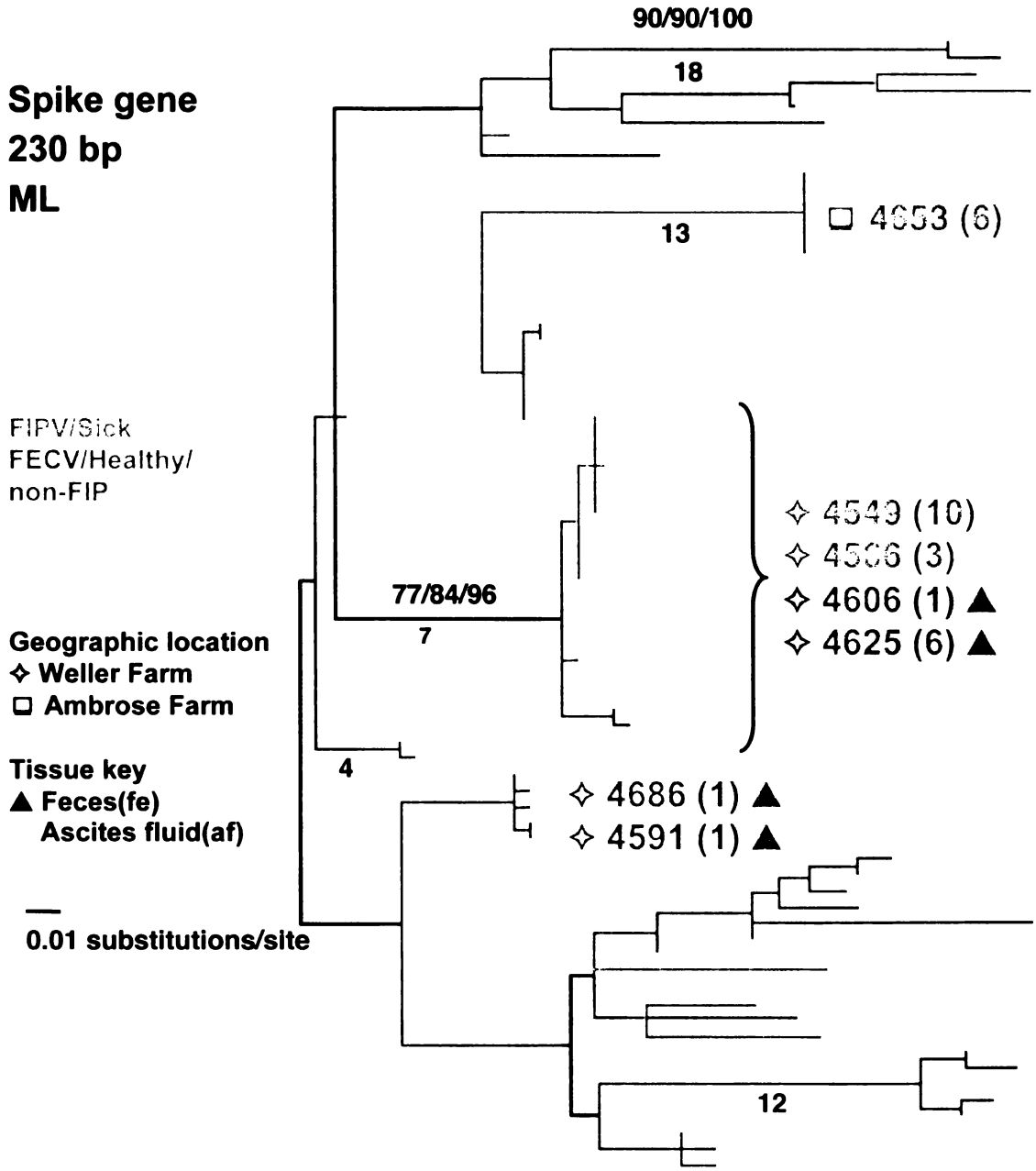


**Figure 12 cont.**

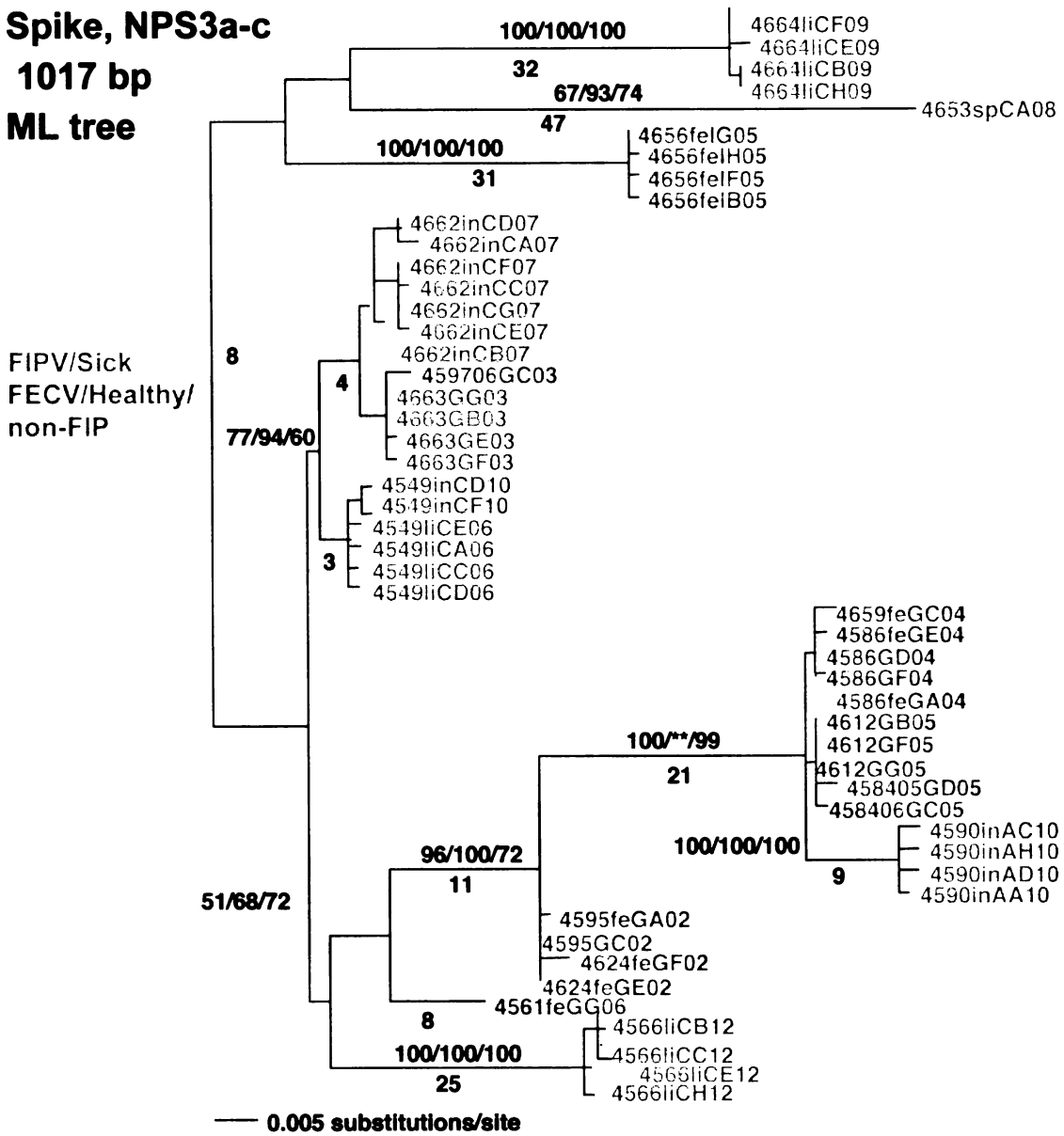


**Figure 13**: Alignment of variable sites of unique amino acid sequences of *membrane* and *NSP7b* genes of FIPV (grey shaded), FECV, FCoV-Aju, and reference sequences for SARS-CoV, MHV-1, IBV-Beu, BVC-K, HcoV-229E, TGEV-Purdue, and FCoV 79-1146 (GenBank accession numbers P59596, AB587268, P69602, BAF75636, P15422, PO4135, and P25878, respectively). FCoV reference sequences for FECVUCD, FIPVUCD1, FIPV791146, and FIPVUCD3 are also included. Diagnostic sites are highlighted in the *membrane*. For membrane, cat ID and two digit year of sampling is listed and the number of original clones is in parenthesis; the frequency of unique amino acid sequences is reported in column 2. No diagnostic sites were found correlating with FIPV and FECV biotype in NSP7b.

**Figure 13 membrane**

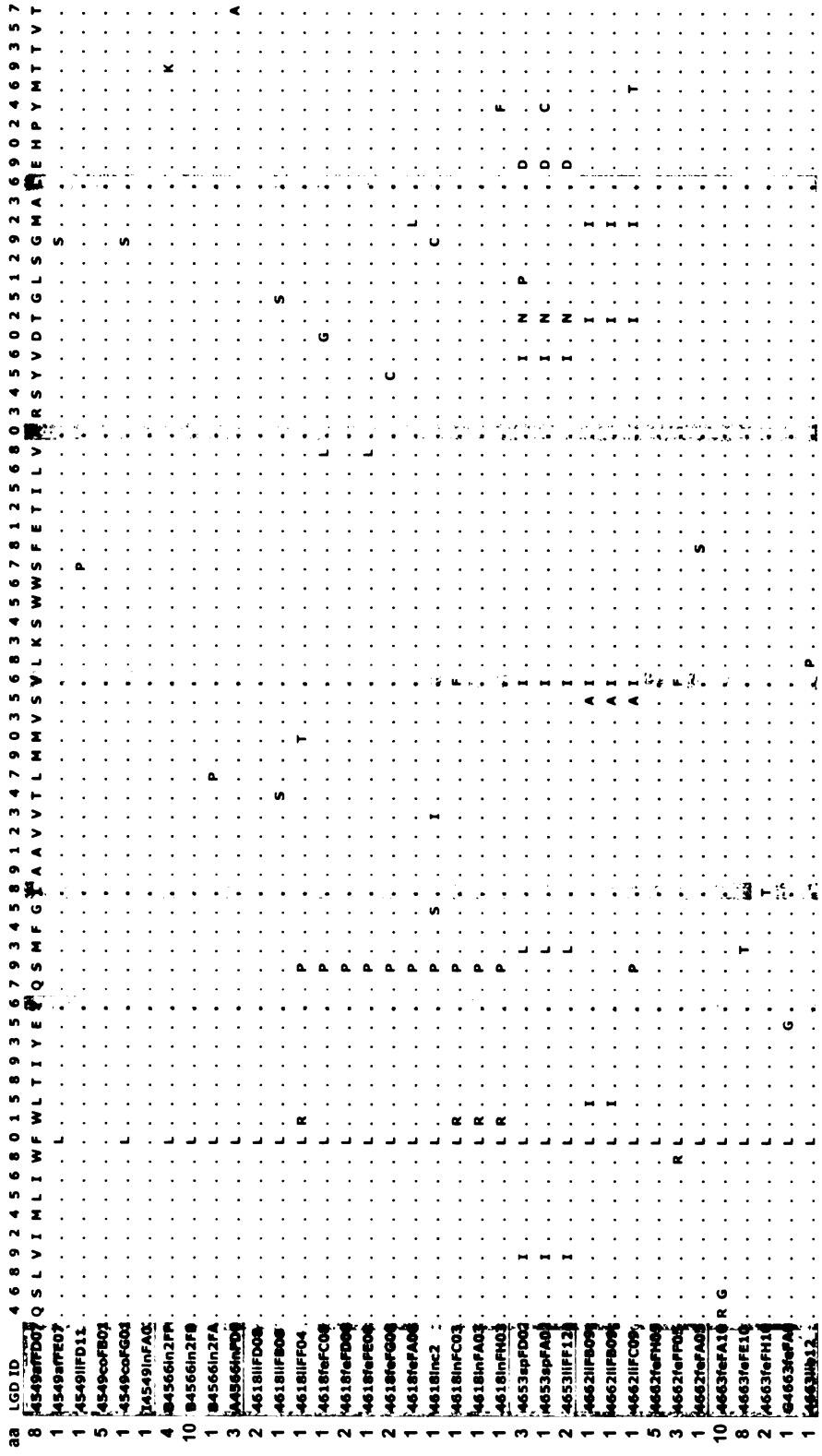


**Figure 13 membrane cont.**

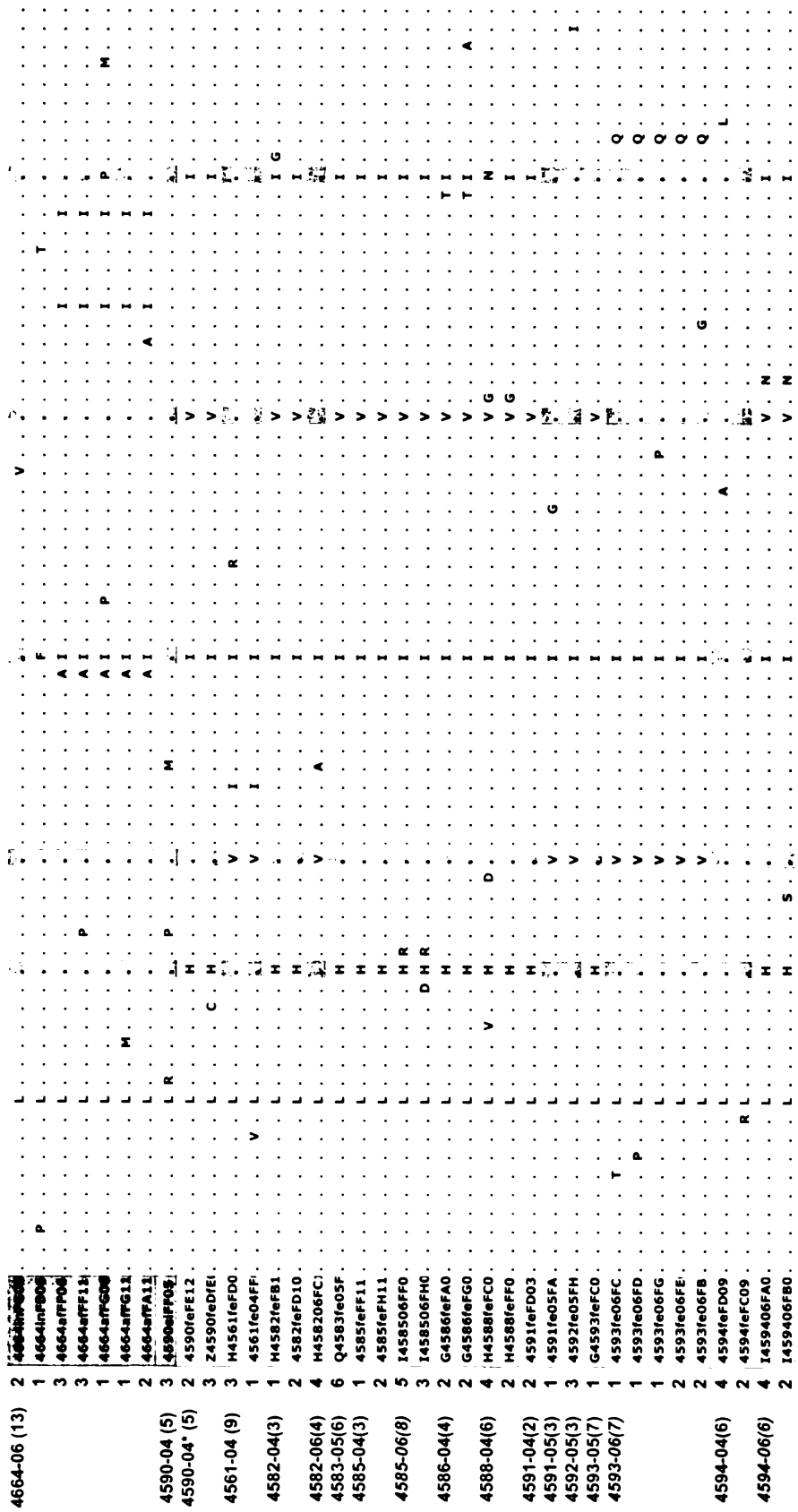


**Figure 13 membrane cont.**

**Figure 13 NSP 7b cont.**

**Figure 13 NSP 7b cont.**

**Figure 13 NSP 7b cont.**

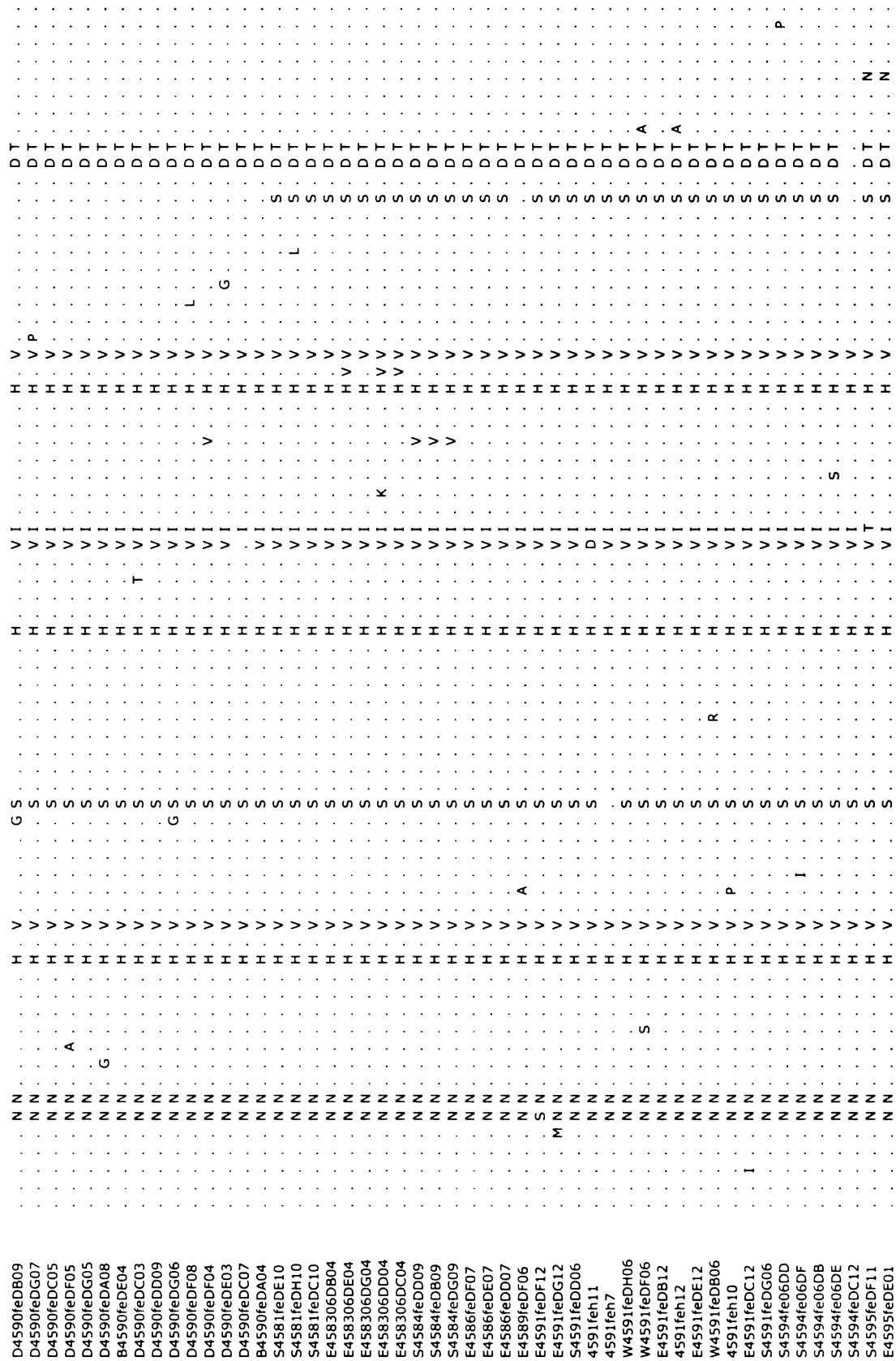


**Figure 13 NSP 7b cont.**

**Table 8**: Proviral PCR screening, 61 puma samples, 1988–2006*

*The 376-bp amplification of *env* was confirmed by sequencing.
†FP ID, Florida panther identification.
‡LGD ID, Laboratory of Genomic Diversity identification.
§Date, month/day/year format.
¶Range, E, Everglades National Park; F, Florida Panther National Wildlife Refuge; O, Okaloacoochee Slough State Forest; P, private lands; S, Big Cypress Seminole Indian Reservation; BC-N, Big Cypress North, BC-S, Big Cypress South.
#Source of DNA isolation: WBC, white blood cell; BY, buffy coat; CT, clot; BM, bone marrow; SP, spleen; LN, lymph node; nvPBMCs, nonviable peripheral blood mononuclear cells.
**Neg, negative; Pos, positive.
††ND, not detemined. Antibody titer >0.25 was considered positive (Cunningham et al 2008).

**Table 8**: Proviral PCR screening, 61 puma samples, 1988–2006*

| | LGD ID‡ | Date§ | Range¶ | Source# | PCR** | Antigen**†† | Antibody titer** |
|---|---|---|---|---|---|---|---|
| FP25 | Pco-116 | 2/16/88 | F | WBC | Neg | ND | ND |
| FP18 | Pco-68 | 1/23/89 | F | BY | Neg | ND | ND |
| FP28 | Pco-154 | 3/11/89 | S | BY | Neg | ND | ND |
| FP21 | Pco-75 | 5/23/89 | E | CT | Neg | ND | ND |
| FP29 | Pco-155 | 1/10/91 | S | BY | Neg | ND | ND |
| | Pco-409 | 8/17/91 | Peru | CT | Neg | ND | ND |
| FP54 | Pco-487 | 4/7/92 | F | BY | Neg | ND | ND |
| FP12 | Pco-20 | 1/4/93 | F | BY | Neg | ND | ND |
| FP31 | Pco-157 | 1/6/93 | F | BY | Neg | ND | ND |
| | Pco-554 | 1/23/93 | Nicaragua | WBC | Neg | ND | ND |
| | Pco-579 | 5/15/93 | Argentina | WBC | Neg | ND | ND |
| | Pco-581 | 5/19/93 | Paraguay | WBC | Neg | ND | ND |
| | Pco-582 | 5/19/93 | Uruguay | WBC | Neg | ND | ND |
| TX33 | Pco-741 | 1/4/96 | Texas | WBC | Neg | ND | ND |
| WC-0 | Pco-742 | 4/14/97 | S | BY | Neg | ND | ND |
| FP78 | Pco-908 | 2/16/99 | F | BY | Neg | ND | ND |
| WC-06 | Pco-926 | 7/19/99 | S | BY | Neg | ND | ND |
| WC-03 | Pco-923 | 1/13/00 | S | BY | Neg | ND | ND |
| FP61 | Pco-898 | 2/17/00 | E | BY | Neg | ND | ND |
| FP89 | Pco-969 | 3/2/00 | BC-S | BY | Neg | ND | ND |
| FP92 | Pco-916 | 4/6/00 | F | BY | Neg | ND | ND |
| TX107 | Pco-736 | 4/19/00 | F | BY | Neg | ND | ND |
| TX105 | Pco-739 | 12/1/00 | E | BY | Neg | ND | ND |
| FP96 | Pco-972 | 1/8/01 | F | BY | Pos | Neg | 0.337 |
| FP99 | Pco-990 | 1/27/01 | F | BY | Neg | ND | 0.296 |
| FP100 | Pco-991 | 2/1/01 | BC-S | BY | Pos | Neg | 0.3 |
| FP101 | Pco-992 | 2/5/01 | BC-S | BY | Neg | ND | ND |
| FP102 | Pco-996 | 2/20/01 | BC-S | BY | Neg | ND | ND |
| FP104 | Pco-1000 | 4/3/01 | BC-S | BY | Neg | Neg | 0.292 |
| UCFP39 | Pco-1004 | 5/7/01 | BC-S | LN | Neg | Neg | ND |
| FP107 | Pco-971 | 11/1/01 | F | BY | Neg | Neg | 0.324 |
| FP96 | Pco-972 | 11/3/01 | F | BY | Pos | Neg | ND |
| FP108 | Pco-994 | 11/6/01 | BC-S | BY | Neg | Neg | 0.273 |
| FP78 | Pco-908 | 12/14/01 | F | BY | Neg | Neg | 0.454 |
| FP96 | Pco-972 | 1/18/02 | F | SP | Pos | Neg | ND |
| FP110 | Pco-984 | 2/13/02 | O | BY | Neg | Neg | ND |
| FP111 | Pco-1023 | 2/14/02 | O | nvPBL | Neg | Neg | 0.224 |

**Table 8 continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| FP112 | Pco-1024 | 2/25/02 | BC-S | BY | Neg | Neg | ND |
| K109FP73 | Pco-1025 | 3/3/02 | BC-S | BY | Neg | Neg | ND |
| UCFP46 | Pco-1029 | 4/10/02 | BC-S | SP | Neg | ND | ND |
| K12FP78 | Pco-1038 | 10/23/02 | BC-S | BY | Neg | ND | 0.242 |
| FP110 | Pco-984 | 11/25/02 | O | BY | Pos | Neg | ND |
| FP115 | Pco-1058 | 11/26/02 | O | BY | Pos | Pos | 0.499 |
| FP82 | Pco-962 | 12/6/02 | O | BY | Neg | ND | 0.262 |
| TX106 | Pco-733 | 1/9/03 | F | BY | Neg | ND | ND |
| FP109 | Pco-1022 | 1/24/03 | O | BM | Pos | Pos | 0.546 |
| FP118 | Pco-1060 | 3/6/03 | S | BY | Pos | Neg | 0.157 |
| FP119 | Pco-1064 | 4/4/03 | BC-N | BY | Pos | Neg | 0.125 |
| FP115 | Pco-1058 | 5/27/03 | O | BM | Pos | Pos | ND |
| FP118 | Pco-1060 | 5/27/03 | S | SP | Pos | Neg | ND |
| K151 | Pco-1073 | 6/12/03 | O | BY | Neg | ND | ND |
| UCFP57 | Pco-1076 | 6/17/03 | BC-N | LN | Neg | ND | ND |
| UCFP58 | Pco-1084 | 6/30/03 | S | SP | Neg | ND | ND |
| FP121 | Pco-1085 | 12/2/03 | S | BY | Neg | ND | ND |
| FP117 | Pco-1059 | 12/3/03 | BC-N | BY | Neg | Neg | 0.17 |
| FP100 | Pco-991 | 1/6/04 | BC-N | BY | Pos | Neg | ND |
| FP122 | Pco-1087 | 2/2/04 | O | BY | Pos | Pos | ND |
| FP123 | Pco-1088 | 2/2/04 | O | BY | Pos | Pos | ND |
| FP124 | Pco-1091 | 2/13/04 | B | BY | Neg | ND | ND |
| FP127 | Pco-1094 | 2/16/04 | B | BY | Neg | ND | ND |
| FP71 | Pco-1095 | 2/17/04 | S | BY | Neg | ND | ND |
| FP131 | Pco-1097 | 3/10/04 | F | BY | Neg | ND | ND |
| FP132 | Pco-1098 | 3/18/04 | O | BY | Pos | Neg | ND |
| FP78FP83 | Pco-914 | 3/31/04 | BC-S | BY | Neg | ND | ND |
| UCFP65 | Pco-1103 | 4/6/04 | F | SP | Neg | ND | ND |
| FP113 | Pco-1037 | 4/7/04 | BC-N | BY | Neg | ND | ND |
| FP117 | Pco-1059 | 7/29/04 | BC-N | BM | Neg | ND | ND |
| FP132 | Pco-1098 | 8/1/04 | O | SP | Pos | Pos | ND |
| FP119 | Pco-1064 | 11/17/04 | BC-N | BY | Neg | Neg | ND |
| UCFP43 | Pco-1016 | 8/30/05 | P | BY | Neg | Neg | 0.277 |
| FP67 | Pco-722 | 4/23/06 | P | SP | Neg | Neg | 0.26 |

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