

***A Search for New Subtypes of the Feline
Immunodeficiency Virus (FIV) Through Identification
of Hawaiian Viral Haplotypes***

**By
Andrew Tompkins Bean
April 2013**



**Allegheny College
Department of Biology**

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**A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for a Bachelor of Science Degree from Allegheny College**

*I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code
and to maintain the integrity of both myself and the College community as a whole.*

Pledge

Approval by the Senior Thesis Committee:

Dr. Kristen Webb

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Abstract

The Feline Immunodeficiency Virus (FIV) is a diverse virus that affects all members of the class Felidae. There are five subgroups of the virus, A, B, C, D, and E. Several studies have analyzed the DNA sequences of viruses from different continents and found a varying distribution of the subtypes. FIV studies from island nations like Japan and New Zealand have found new subtypes however studies on other islands have not occurred. In this study, the *env* gene of the virus was sequenced from 10 FIV infected cats from Hawaii in an attempt to classify the viruses into known subgroups or identify novel haplotypes. A neighboring-joining phylogenetic tree reconstruction including previously identified sequences showed that a majority of Hawaiian samples were subtype C, most resembling sequences from Japan and New Zealand. Two sequences resembled those from California clustering with subtype A, and one sequence did not group with any known subtypes suggesting the existence of a new subgroup. The study also suggests that viral evolution is constrained to exist within the established subtypes and the novel mutants are selected against. The results indicate a need for regular molecular screening of viral populations to better predict and avoid public health crises.

Introduction

The Feline Immunodeficiency Virus (FIV) is a retrovirus characterized by a long incubation period and an RNA genome with a reverse transcriptase enzyme. The virus exclusively infects members of the class Felidae, including domestic cats, lions, leopards, and cougars (Pecon-Slaterry et al. 2008). While the virus can be transferred vertically from mother to offspring, it is mainly transmitted through saliva with the chief epidemiological explanation for transmission being a bite wound from an infected cat (Bendinelli et al. 1995). For this reason, the virus is widespread in populations of wild cats, such as cougars, where territorial and mating conflicts are common (Biek et al. 2003). It has been suggested that 38-55% of the wild cat population is infected with FIV (Biek et al. 2003). In domestic cats, however, the virus is less widespread, affecting on average only 3-4% of the population (Poss et al. 2008). Domestic cats infected with FIV experience decreased immune function and can exhibit behavioral abnormalities; however larger wild cats like cougars do not show detrimental effects, allowing the disease to exist in a higher frequency in large cat populations (Biek et al. 2003). The virus has evolved to be less virulent in populations where contact with other members of the population is less likely. This allows the host to stay alive longer, increasing the chances that the host will encounter another possible host of the disease to infect (Biek et al. 2003). Viral evolution has played key roles in virulence, transmission rates, and host adapted defenses throughout their history of infection.

In other retroviruses, like HIV, sickness and death are attributed to a failure of the immune system (AIDS) and subsequent infection. FIV acts similarly and has three stages of infection. First there is the initial stage consisting of fever, lethargy, loss of appetite, and swollen lymph nodes (Yamamoto et al. 2010). This is followed by the incubation phases in which there

are seemingly no symptoms. This phase can last multiple years and progression into the next phase can depend on the host's age, defensive viral adaptations, and the virulence of the viral subtype (Hartmann 2011). The final stage is the complete failure of the immune system, considered feline acquired immune deficiency, FAIDS (Hartmann 2011). The virus is able to live in its host for years allowing for more transmission all while constantly evolving to avoid the host's weakened immune system.

To control the frequency of the virus in domestic cat populations, a vaccine was developed in 1993 (Yamamoto et al. 1993). Studies show that the vaccine is 78-90% successful in stopping an FIV infection once a vaccinated cat has been exposed to disease after inoculation (Yamamoto et al. 1993, Yamamoto et al. 2010). The FIV vaccine contains inactive viruses of subtypes A and D which doctors claim have an acceptable level of efficacy for protection (Yamamoto et al. 2010). The vaccine has been able to protect against most strains of subtype B and C since they are relatively closely related to subtype A (Uhl et al. 2002). Rapid changes that accumulate in the viral genome account for high levels of genetic diversity between populations and can attribute to the proliferation of the disease among house cats through failure of the vaccine to continue to be effective

There are currently five internationally recognized subtypes of the FIV virus which have been named A, B, C, D, and E, and were classified based on differences in the DNA sequences of several genes (Duarte and Tavares 2006). The two main genes that have been studied are the *env* gene and the *gag* gene. The *env* gene codes for the glycoprotein on the surface of the protein shell while the *gag* gene codes for the protein capsid itself (Csaba et al. 2007). Another less common gene that is sequenced is the *pol* gene which codes for the polymerase enzyme in the virus. It mutates slowly and is not a good indicator of genetic differences (Csaba et al. 2007).

The five subtypes of the virus are genetically distinct however significant variation exists within subtypes due to the speed of viral evolution. There can be between 1-15% sequence divergence within subtypes and 16-26% divergence among the subtypes (Bachman et al. 1997). Certain hyper variable regions of the *env* gene have shown the highest amount of differences between viral strains (Sodora et al. 1994). The virus's low fidelity reverse transcriptase enzyme allows the *env* region in particular to change quickly and adapt to selective pressures created by the host's immune system or another competing viral strain (Csaba et al. 2007). These regions are known as V3, V4, and V5 and have been used in most studies to determine the subtype of viral strains (Sodora et al. 1994, Bachmann et al. 1997, Steinrigl et al. 2003). Collectively the V3-V5 regions are around 400 bp making it an ideal location to study using Polymerase Chain Reaction (PCR) (Rigby et al. 1993). Genotyping the subtypes and their worldwide distributions has allowed researchers to study viral evolution.

While some studies have attempted to trace the evolutionary history of different subtypes, many have found it difficult due to the widespread variance within subtypes (Steinrigl et al. 2009, Duarte and Tavares 2006). Within different subtypes exist "subclusters" (Duarte and Tavares 2006). These subclusters are a classification scheme that attempts to explain evolutionary change while linking the virus to ancestral strains. Studies have been conducted to analyze the viral DNA sequences from varying continents and identify the dominant subtypes (Bachman et al. 1997, Rigby et al. 1993, Steinrigl et al. 2009). The dominant viral subtypes present on each continent have effectively been identified (Figure 1). Subtypes A and B are the most frequent worldwide with C appearing sporadically in North America, Japan, and New Zealand. Subtypes D and E are only found in Japan and Argentina, respectively (Figure 1).

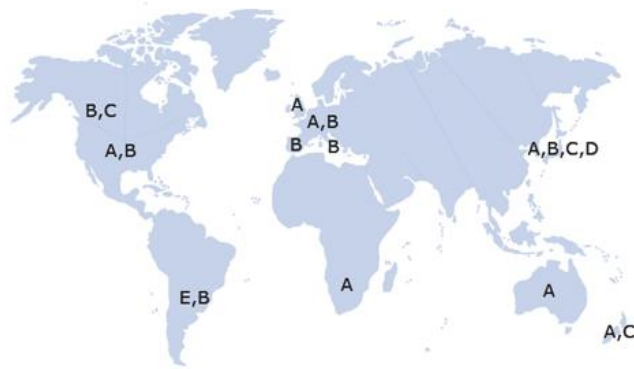


Figure 1: Geographic Distribution of Domestic Cat FIV by Continent. The FIV virus is spread worldwide with each continent having one of two main subtypes present. Subtypes A and B are most common being found on almost every continent. Subgroup D is only found on the island of Japan while subgroup E exists in South America (Horzinek 2008).

While the major continents are being assessed for genetic variability of the FIV virus, one of the major gaps in the research is assessment of the virus in more isolated island populations like Hawaii, Cuba, or the islands in the Caribbean. The virus evolves so quickly and variance within a population subtype is so high that it is possible that these islands could contain vastly different strains from those previously analyzed (Kraase et al. 2009). One study demonstrated how quickly the virus evolves by artificially infecting cats with a virus of known molecular composition (Kraase et al. 2009). After several years the DNA sequences of the viruses were shown to have significantly changed. Kakinuma et al. (1995) analyzed many sequences from Japan and found three distinctly different sequences from the three accepted subtypes at the time. This was explained by the evolutionary radiation of the virus over several hundred years and led to the acceptance of a new subtype, D. With the inception of this new subtype, the vaccine was restructured in order to include this significantly different virus and better protect domestic cats against FIV infection (Yamamoto et al. 2010). In New Zealand a distinct subtype was discovered and classified as subtype U (Hayward et al. 2007). Although this new subtype is not officially recognized, the “U” denotation meaning unknown subtype, researchers are working on

genotyping the *gag* and *pol* genes in order to definitively prove the viral strain's uniqueness (Hayward et al. 2007). These studies where new strains were found beg the question as to whether all islands could possibly have new strains of FIV. While one study has examined FIV sequences from Hawaii, the researchers only examined three samples which were all classified as subtype A, emphasizing a need for a more comprehensive examination of the virus on the Hawaiian islands (Bachman 1997).

It is important for regular analysis of various FIV populations in order to detect changes that would lead to a lower efficacy of the vaccine (Kraase et al. 2009). Since the virus has shown that it can evolve quickly and adapt to its host, comprehensive analysis of the viral populations has been suggested by several health officials (Pecon-Slattery et al. 2008). Although viruses in many regions have been analyzed and classified with the corresponding subtypes, the variation between the subtypes is very high (Steinrigl 2009, Duarte and Tavares 2006). This is significant since all of the accepted subtypes were established in the 1990s. Deviation from the subtypes occurs incredibly fast, taking only 15-20 years, resulting in 12-15% sequence differentiation (Steinrigl 2009, Duarte and Tavares 2006). These studies analyzed samples from continental locations, once again neglecting to examine the unexplored island regions. The virus evolves so quickly that ongoing research is needed to keep the information on FIV current. On several islands, analysis of the viral DNA sequences hasn't yet occurred, overlooking an opportunity to examine potentially new sequences that have been evolving for hundreds of years independently of the continental strains.

One major reason to study FIV is because it is closely related to the human virus, HIV, and therefore it allows for parallels to be drawn. Research in the field of FIV has led to breakthroughs in better understanding of HIV pathology, treatment of patients with HIV, and the

possible development of a vaccine against HIV (Bendinelli 1995; Uhl et al. 2002). Information relevant to FIV is applicable to HIV because both viruses act through the same incubation procedures and immune response mechanisms (Bendinelli 1995). The viruses are structurally similar, have a high genetic variance among subgroups, and have analogous life cycles. Both viruses attack the T-cells, effectively crippling the host's immune system (Hartmann 2011). Studying the evolution of FIV is more convenient than HIV for multiple reasons. Humans do not need to worry about contracting FIV from samples and more experimentation is allowed on animals (Pecon-Slaterry et al. 2008). Studying FIV can have greater implications to the field of HIV research and can expand the field of virology more generally.

In this study, a sequence analysis was performed on FIV samples taken from cats living in Hawaii. DNA sequencing of these samples allowed the Hawaiian FIV population to be compared to the previously identified subtypes. The information gathered helped to add to the understanding evolution of viruses and added new knowledge to fill the gap in the current research of the field such as viral sequences from remote island locations. New variants of the virus were found indicating a greater degree of variation in the viral gene pool. It is possible that a novel or previously unknown subgroup could exist in Hawaii, as it did in Japan in 1995 or as observed in New Zealand in 2007 (Kakinuma et al. 1995, Hayward et al. 2007). This dataset allows the origin of the Hawaiian strain to be traced by comparing it to other viral sequences from several continents. The data generated may also be applied to studying evolutionary patterns of viruses. The Hawaiian model provides an opportunity to examine a population of viruses that have been evolving mainly independently from continental strains and contribute new knowledge to the field of viral evolution and distribution.

Research Design and Methods

Sample Collection

Letters requesting blood from domestic cats infected with FIV were sent to veterinary hospitals in Hawaii in August of 2012. In addition to the blood samples, information including the age, sex, and lifestyle of each donor cat was requested. Age is important because FIV adapts to its host over time, so an older cat could have a more differentiated viral sequence. Knowing the sex of the cat is helpful for statistical purposes since the disease is most likely found in male cats. The lifestyle of the cat is referring to whether it is an indoor, outdoor, or feral cat and is important because it can explain how the disease was passed on and where the disease is most abundant in the cat population. The samples were shipped overnight to Allegheny College via FedEx and stored at -80°C until DNA extraction.

DNA Extraction

Although the genome of the virus is composed of RNA, the viral RNA is reverse transcribed and inserted into the host's nuclear genome; therefore no RNA extraction or reverse transcriptase reactions were needed. The DNA was extracted using the DNeasy Blood and Tissue Extraction Kit and the protocol was followed for nonnucleated erythrocytes (Qiagen, Valencia, California, United States). An RNase treatment was done in conjunction with the protocol and extracts were eluted in a final volume of 100µl. Extraction success was confirmed using 1-2% agarose gel electrophoresis and Nanodrop spectrophotometry, specifically looking at concentration and a 260/280 ratio indicative of purity. The DNA samples were kept at 4°C following extraction.

Amplification of Target Locus

The V3-V5 region of the *env* gene was amplified using the primers cited in Bachman et al. (1997). The forward primer has a sequence of 5'- ATACCAAATGTGGATGGTG -3' and

aligns to positions 7316 – 7335 of the *env* gene. The reverse primer has a sequence of 5'-CAAGACCAATTTCCAGCAAT -3' with alignment coordinates of 7847 - 7866 relative to the *env* gene. The primers were aligned to the FIV and domestic cat genomes using *Basic Local Alignment Search Tool (BLAST* - <http://www.ncbi.nlm.nih.gov/blast>) to check for uniqueness in order to decrease the chances of non-specific annealing. The protocol for the PCR was based on a study by Reggeti et al. (2004) and was modified to the final reaction conditions. The reaction volume of each PCR was 25 µl and included 12.5 µl Quick Load Taq 2x Master Mix (New England Biolabs, Ipswich, Massachusetts, United States) in addition to 0.5 µM of each primer and 100 ng of DNA. The Master Mix contained 1 unit of Taq, 1.5mM MgCl₂, and 0.2mM each dNTP. In some cases the reaction volume was adjusted to 50 µl and 200 ng of DNA was required. The thermal cycler conditions included an initial denaturation of 96°C for 10 minutes followed by cycling the reaction 40 times with the denaturation step for 45 seconds at 94°C, annealing step for 30 seconds at 56°C, and the elongation step at 72°C for 90 seconds. The final extension was 72°C for 7 minutes. Successful PCRs were expected to product an amplicon of about 550 bp. The success of the PCR was determined by 1% agarose gel electrophoresis using a low mass ladder (Grand Island, New York) to determine the size and concentration of the product.

DNA Sequencing and Editing

Successful amplicons were treated with Exo-SapIT (Affymetrix, Cleveland, Ohio) following the company's protocol to remove extra dNTPs and any remaining primers that could impede the sequencing process. Thermo cycler conditions for the Exo-SapIT treatment were 37°C for 15 minutes followed by 80°C for 15 minutes. Purified products were sent to Eurofins MWG Operon (Huntsville, AL) for sequencing according to the company's recommendations

(<http://www.operon.com/services/dna-sequencing/getting-started.aspx>). Samples were initially sequenced with the same forward primer as used in PCR. Some samples were also sequenced with the reverse primer as the sequencing reaction with the forward primer did not yield high enough quality data. DNA sequences were edited using FinchTV (<http://www.geospiza.com/>, Geospiza, Seattle, Washington). Editing was completed by changing any bases that were identified incorrectly by the software and trimming the excess low quality data.

Multiple Alignment, Tree Building, and AMOVA

Multiple viral sequences from other continents as well as DNA sequences of the known subgroups A, B, C, D, E, and the unconfirmed subgroup U were downloaded from NCBI's nucleotide database (<http://www.ncbi.nlm.nih.gov/>) in order to compare the newly collected viral sequences and determine which subtype(s) the Hawaiian strains of the virus most resembles (Table 1). All previously published and newly collected sequences were aligned by MUSLCE using MEGA5 (<http://www.megasoftware.net/>). The alignment was manually inspected to confirm that homologous positions were being compared (Tamura et al 2011). A neighbor-joining phylogenetic tree of the *env* sequences and Bovine Immunodeficiency Virus (BIV) *env* sequence was reconstructed with 500 bootstrap replicates using MEGA5 towards identifying where the Hawaiian strains group based on known subgroups and sequences from the other continents. The BIV sequence was included as an outgroup and used to root the tree. Finally, the sequences were assessed using Analysis of Molecular Variance (AMOVA) via Arlequin version 3.5 (<http://cmpg.unibe.ch/software/arlequin35/>) to determine if the sequence differentiation between the island population and continental U.S. population was significant (Excoffier, L., and Lischer, H. 2010). Prior to the AMOVA, the sequences were trimmed of all missing data, leaving 272 positions for analysis.

Table 1: Genbank Accession numbers and Corresponding Studies

Genbank Accession Numbers	Study (Origin)
U02422.1- U02453.1	Sodora et al. 1994 (U.S.)
AB010406.1- AB010417.1	Nishimura et al. 1998 (Japan)
D84500.1- D84510.1	Pecoraro et al. 1996 (South America)
AY196343.1- AY196403.1	Steinrigl et al. 2003 (Europe)
EF154083.1- EF154068.1	Hayward et al. 2009 (New Zealand)
U80939.1 (BIV)	Suarez and Whetstone 1997 (BIV)

Results

Sample Collection

A total of 13 samples were collected from veterinary practices in Hawaii. The age, sex, and lifestyle of the cats was also gathered (Table 2).

Table 2: Sample Information

Sample Name	Age (years)	Sex	Lifestyle	Hospital of Origin	Island Origin
HI-01	2	M/N	I/O	VCA Family Vet Hospital	O'ahu
HI-02	1	F/S	Outdoor	Paradise Animal Clinic	Kaua'i
HI-03	8	M/N	Indoor	Paradise Animal Clinic	Kaua'i
HI-04	11	M/N	Outdoor	Ali'i Vet Hospital	Hawai'i
HI-05	6	M/N	Outdoor	Ali'i Vet Hospital	Hawai'i
HI-06	6	M/N	Outdoor	Ali'i Vet Hospital	Hawai'i
HI-07	12	M/N	Outdoor	Ali'i Vet Hospital	Hawai'i
HI-08	12	M/N	Outdoor	Ali'i Vet Hospital	Hawai'i
HI-09	7	M/N	Outdoor	Ali'i Vet Hospital	Hawai'i
HI-10	3	F/S	Outdoor	Ali'i Vet Hospital	Hawai'i
HI-11	14	F/S	Indoor	Ali'i Vet Hospital	Hawai'i
HI-12	8	F/S	Outdoor	Ali'i Vet Hospital	Hawai'i
HI-13	10	F/S	O	Ali'i Vet Hospital	Hawai'i

DNA Extraction

Genomic DNA was successfully extracted from all samples as indicated by concentration and purity (Table 3). Those extractions with a purity of 1.80 and a concentration greater than 10 ng/μl were considered to be successful. The success of the extraction of HI-13 was questionable due to the low concentration and high purity. Gel electrophoresis showed intact DNA and bright bands for all samples except HI-13. The band representing HI-13 was faint compared to the other bands.

Amplification of Target Locus

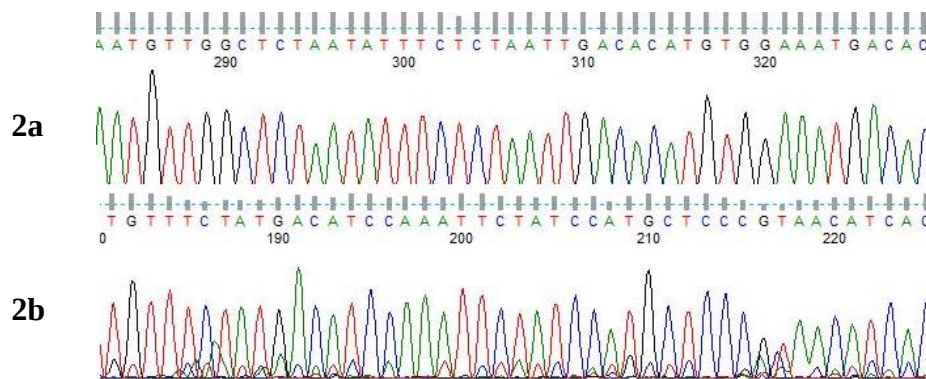
Amplicons of the *env* gene were generated for all samples except HI-08 and HI-13. PCR success was confirmed by gel electrophoresis. All successful PCR products were of the expected PCR product size of approximately 550 bp. For each product, sufficient DNA was generated for sequencing.

Table 3: DNA Extraction Results

Sample	Concentration (ng/μl)	Purity (260/280)
HI-01	16.3	1.73
HI-02	31.9	1.63
HI-03	16.4	1.56
HI-04	12.9	1.67
HI-05	13.5	1.69
HI-06	22.5	1.96
HI-07	33.7	1.91
HI-08	21.4	1.94
HI-09	60.5	1.95
HI-10	15.6	2.11
HI-11	16.7	1.77
HI-12	30.3	1.95
HI-13	8.4	2.20

DNA Sequencing and Editing

Sequencing produced high quality data for the samples HI-02, HI-03, HI-04, HI-10, HI-11, and HI-12. Low quality, but useable, data was obtained for samples HI-05, HI-06, HI-07, and HI-09. Editing was completed by changing any bases that were identified incorrectly by the software and trimming the excess low quality data. High quality data was defined as high peaks with no smaller peaks apparent (Figure 2a). Low quality data was defined as high peaks with apparent rival peaks (Figure 2b).



Figures 2a & 2b: High Quality and Low Quality DNA Sequence Data. Figure 2a shows high quality data with defined singular peaks while Figure 2b shows low quality data with multiple peaks.

Multiple Alignment, Tree Building, and AMOVA

The alignment was assembled using 68 sequences downloaded from Genbank as well as the 10 newly collected Hawaiian sequences. After alignment, the excess positions were trimmed leaving 420 positions of nucleotide data. Partial sequences were included in the alignment with dashes added to represent insertions or deletions.

The neighbor-joining tree generated revealed that samples HI-02, HI-03, HI-04, HI-10, HI-11, and HI-12 all clustered together with sequences of known subtype C (Figure 3). All of

these samples grouped near sequences from New Zealand and one from Canada. HI-06 also clustered within the C subtype however it grouped near an isolate from Japan (Figure 3). Samples HI-07 and HI-09 clustered together with sequences of known subtype A. These samples grouped near sequences from California. Sample HI-05 seemed to cluster outside of subtype A and did not group with any other sequences (Figure 3). Samples that group with sequences of a known subtype were considered to be within that subtype.

The AMOVA comparing the Hawaiian samples to sequences from the United States produced an F_{st} value of 0.16150 with a p-value of 0.00587. An F_{st} value of 0.16150 indicates a great amount of genetic variation. The low p-value also shows the genetic differentiation between the two populations is significant. The variation between the populations is significant ($p=0.00587$) and meaningful with 16.15% variation existing between populations. The values were interpreted based on F_{st} value ranges and their corresponding amount of genetic variation (Excoffier, L., and Lischer, H. 2010).

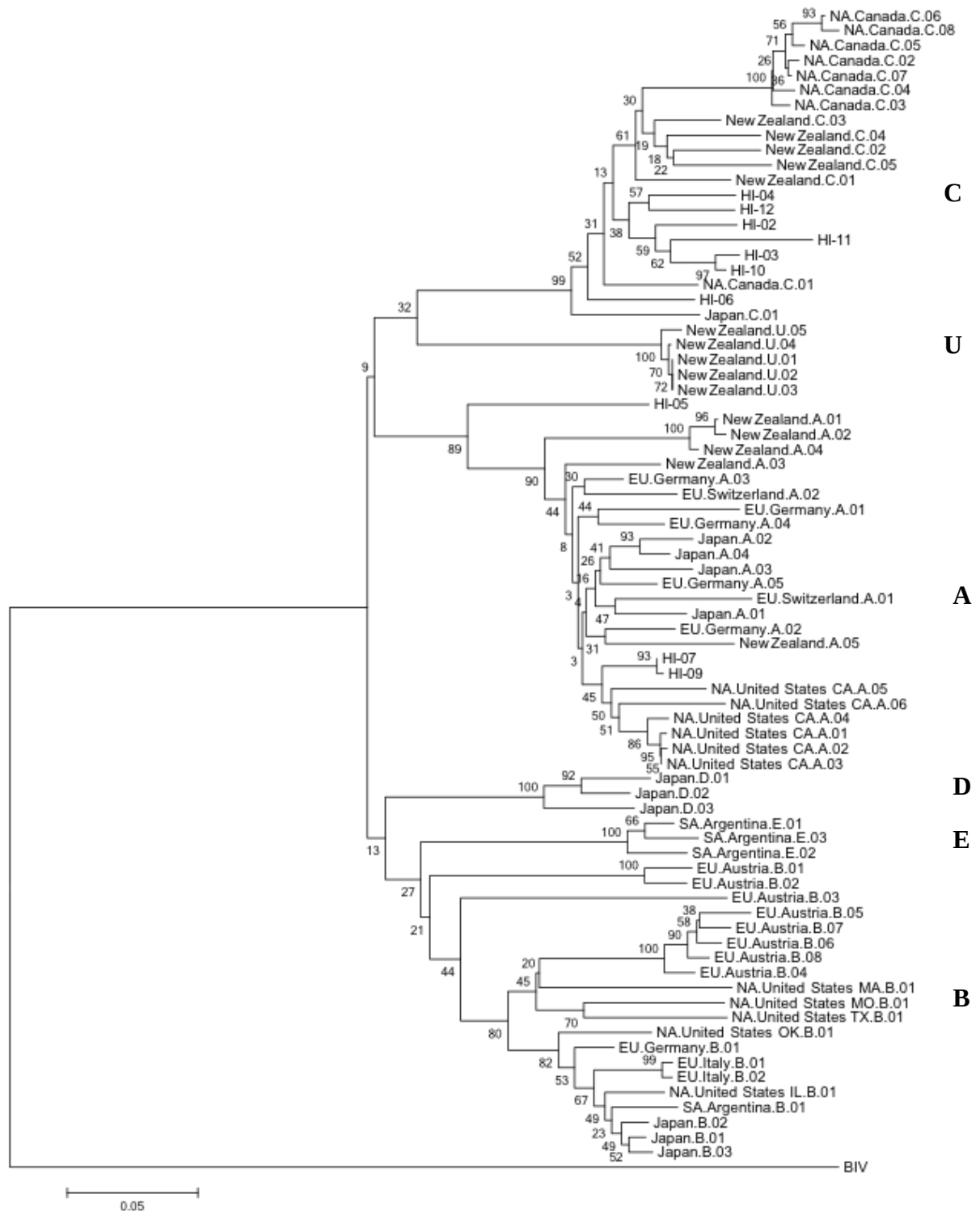


Figure 3: Neighbor Joining Tree of FIV Samples: The samples from Hawaii are marked as “HI” and a number. The samples from Genbank are labeled as their country of origin, followed by the subtype, and then number of the sample from the original paper. Bootstrap values are displayed at branch nodes.

Discussion

The Feline Immunodeficiency Virus has proven to be a virus that is particularly good at evolving and adapting quickly. Genotyping and classifying the virus worldwide has been the focus of many areas of research in an attempt to learn more about viral evolution and stay a step ahead public health concerns. Island nations like New Zealand and Japan have proposed new subtypes of the virus leading one to believe that isolated islands could foster an environment for viral evolution (Hayward et al. 2007). This experiment aimed to examine the virus on the relatively isolated island chain of Hawaii. Through classification of Hawaiian viral haplotypes this experiment expanded the knowledge of viral evolution and possibly discovered a previously unidentified subtype of the virus that could have evolved as a result of the separation from the mainland population.

The results of the phylogenetic tree reconstruction show that the newly collected samples resolve in three clades. Samples HI-07 and HI-09 group within the clade containing samples of the A subtype. Of the sequences included in the alignment, HI-07 and HI-09 most closely resemble sequences from the United States, more specifically California. The largest group of Hawaiian samples consists of samples HI-02, HI-03, HI-04, HI-06, HI-10, HI-11, and HI-12. These samples all cluster together in the C subgroup. Most of these samples are most similar to the sequences originating from New Zealand; however, HI-06 most closely resembles the sequence from Japan. This suggests that the Hawaiian FIV population contains elements of Japanese, Polynesian, and Western American strains of the virus. Similarly, this is also how the human population appears to breakdown in Hawaii (U.S. Census Bureau 2013). In Hawaii, 19.1% of the population identifies itself as Japanese, while 10.1% identifies as Pacific Islanders including New Zealand (U.S. Census Bureau 2013). The results of the tree seem to correlate with

the human population of the Hawaiian Islands. It is possible that the subtypes were introduced by the immigrants that arrived on Hawaii that brought their FIV infected cats. This relationship between the strains of FIV enhances the Hawaiian idea that their citizens have a mix of not only cultures but feline viral sequences as well.

While the relationships between the samples that grouped with other continental strains is revealing of the subtype with 12 of the 13 Hawaiian FIV samples show a close relationship to previously identified strains, sample HI-05 does not cluster within any of the subtype clades but instead resolves basal to subtype A. The bootstrap value of the node separating HI-05 from the subtype A clade is high at 89, which reinforces the idea that it is indeed separate from the A subtypes. While HI-05 does not group with any subtype, this may not mean that it is necessarily a new subtype. The established subtypes were created in the 1990s when genotyping of the FIV subtypes began. Since then many studies have found evidence of what they claim to be new subtypes in Brazil and New Zealand (Marçola et al. 2013 and Hayward et al. 2007). In this case the Hawaiian subtype HI-05 could have diverged from the A subtype, forming a new subcluster of the virus (Duarte and Tavares 2006). It has become more and more difficult to have a new subtype formally recognized and named because it usually requires examination of another gene like the *gag* or *pol* genes (Hayward 2009). Another limitation of proposing a new subtype is that many suggest that the whole gene should be sequenced instead of the highly variable V3-V5 region (Weaver et al. 2004). The sample HI-05 could be a new subtype that has evolved in Hawaii however more research is needed in order to definitively show that it is distinct from the other haplotypes.

The evolution of the virus is what concerns health officials worldwide and if the viral evolution was better understood, we would be more prepared for health crises, like preventing

the spread of drug resistant viruses (Yamamoto et al. 2010). The newly collected Hawaiian samples were mostly classified as members of known subgroups A and C with only one sample resolving outside of previously identified clades. It seems as though viral evolution is taking place on Hawaii but that has not been the case in all island studies. In Australia, 41 samples were sequenced and found to within subtypes A and B. While new subtypes are proposed in New Zealand, Japan, and now possibly Hawaii, there were no FIV anomalies on islands like Australia (Kann et al 2006). Viral evolution seems to take place faster when the population is smaller and more isolated. This is consistent with the model of allopatric speciation. This model states that a new species, or in this case a new subtype, is more likely to form within a small isolated population as a result of genetic drift. In the cases of Hawaii, Japan, and New Zealand, these are relatively small islands that have had little contact with the outside world until the mid-1900s. In contrast, Australia is the largest island in the world and has been a part of the global community since the 1800s. Therefore it is not enough to simply look at physical isolation when examining viral evolution. It is also important to consider the size of the population and any contact from neighboring communities.

The genetic variation contained within Hawaiian strains of the virus is significant given the fixation index generated by the AMOVA. With an F_{st} of 0.1651 the Hawaiian population of the virus is considered to have a great amount of genetic differentiation when compared with the population that exists in the continental United States. The results of the AMOVA are shown to be statistically significant with a p-value of .00587. This genetic differentiation can be accounted for due to the isolation of Hawaii has from the other populations that could have created by a bottleneck in the founding population of viruses. The separation from other populations could result in genetic drift, resulting in a relatively quick division between the populations of viruses.

The genetic differences present in the Hawaiian viral samples prove that it is indeed a separate population. It is important to draw the distinction between the populations of Hawaii and the United States in particular. It is easier to transport animals within the country and this possibly would result in a high amount of gene flow between the two populations. It could confound the results if the Hawaiian population was identical to the United States viral population because it would imply that there is little isolation. The AMOVA shows that the Hawaiian viruses do constitute a separate population from the mainland. The isolation of the population has accounted for the accumulation of genetic variation and possibly a new subtype of the virus.

While it seems that there has been some viral evolution leading to the differentiation of the Hawaiian and continental U. S. strains, the majority of the viral sequences identified with known subtypes. The samples that were sequenced were different from other samples; however, they were similar enough to group within the subtype. FIV adapts to its host by evolving while incubating in the cat, however the virus does not appear to mutate out of its subtype (Kraase et al. 2009). Perhaps there is a selective advantage for not evolving into a new subtype because the nucleotides essential to coding for a functional envelope protein are unique to different subtypes. It is possible that the best and most effective glycoproteins on the viral surface are made from the combination of bases already found in known subtypes. If there was a selective advantage to having subtype A, B, C, D, or E then it would be difficult for new subtypes to survive and viruses with these novel types would quickly be lost from the population or found at low frequencies. In many of the cases, the proposed new subtypes were only found in very low numbers and were not found in a convincing amount of samples to define a new subtype (Marçola et al. 2013 and Hayward et al. 2007). Divergence from a subtype has been hypothesized to take 15 years (Duarte and Tavares 2006). The established subtypes were

classified in the mid-1990s therefore making it currently around 15 years since their inception. However the viral strains extracted in 2013 continue to cluster with strains from the 1990s. Although the virus evolves very quickly, divergence of the major subtypes has not occurred therefore it is possible that there is a selective advantage to staying within the molecular constraints of the established subtypes.

The Human Immunodeficiency Virus (HIV) has many things in common with FIV therefore many of the conclusions about FIV can be related to its human counterpart. The most prevalent type of HIV has 14 different subtypes most of which are only found in Africa (Sharp and Hahn 2011). The distribution of these subtypes is better understood relative to the FIV subtypes since many HIV samples are collected every year. HIV subtype B is dominant in North America, South America, and Europe while subtype C is mostly found in Africa and Asia. The remaining 12 subtypes are distributed throughout Africa and are not usually seen outside of the continent. The differences between the subtypes have health officials concerned that the virus could mutate into even more subtypes and create larger problems such as drug resistance strains (Bártolo et al. 2009). However it is possible that the subtypes of HIV have encountered a similar phenomenon of the FIV subtypes where the dominant subtypes are selectively advantageous when compared to new variants. This would actually be good news to public health officials since the subtypes appear to be static. Many of the 14 subtypes have only been seen in 1 or 2 patients since the 1990s leading one to believe that novel subtypes are ineffective at competing with established subtypes. While genetic diversity within subtypes is high in FIV and HIV, it seems that these mutations do not constitute a change in the subtypes.

There are many directions that future research can go in order to learn more about FIV and viral evolution. A larger study should be launched to collect more FIV samples from Hawaii

to see if the unknown subtype exists in other patients. Another gene locus like the *gag* or *pol* genes could be sequenced from Hawaiian samples to help determine whether a novel subtype exists. Another possible experiment would be to examine the subtype frequencies of other islands like Cuba or Ireland. It would perhaps be even more worthwhile to examine a smaller more isolated island like Mauritius or Iceland, although collected samples may be difficult. This would go further in demonstrating the broader ideas about viral evolution on islands when compared with the mainland. The field of FIV research is ongoing and recently researchers have been examining isolated continental populations like in Latin America and South Africa, where no studies have yet to be completed. FIV may be constantly evolving or may be static with respect to the subtypes; future research will be needed to confirm this hypothesis. Public health officials have recommended regular screening of FIV populations to study the change over time of the virus (Yamamoto et al. 2010). The FIV research today is only taking a snapshot of the populations; most studies find and report the subtype frequencies however none give the full story of how viral evolution occurs over many years. Screening FIV populations over time would be beneficial to learning more about the mechanisms that drive viral evolution. Regular screening of FIV subtype distribution is needed for both public health concerns and to further our understanding of viral evolution.

The Feline Immunodeficiency Virus is a disease that affects thousands of cats a year and serves as a great model to study viral evolution. The virus adapts to its host quickly and can undergo rapid differentiation. Scientists have classified the virus into five subtypes and it is using these subtypes that they structured a vaccine to combat the virus and researchers hope to one day use this same logic to find a vaccine for the Human Immunodeficiency Virus. This project has shown conditions under which viral populations can mutate and diversify from parental strains.

Most of the viruses in this study, however, have not differentiated into new subtype suggesting that evolution of the virus is constrained to exist as the establish subtypes. These results can have beneficial implications for both animal and human health systems. Regular screening of viral populations can yield more results on the mechanisms of viral evolution. In a century where both veterinary and human medicine has made great strides, it is possible that retroviral detection and treatment may be on the edge of a breakthrough as well, which would have far reaching implications for both human and animals populations.

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Appendix

Appendix I: Sequences Collected from Hawaiian Samples. The sequences collected from the samples are included in fasta format. The sample names are consistent with the neighbor-joining tree and results section.

>HI-02

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GCTAATGTAACATTTCAATGTCAAAGAACACAAAGTCAACCAGGAACTTG
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AGGAGCATGGATAGAATTTGGATGTCATAGAAATCCAACACGAACTCATA
CAGCTGCAAGATTTAGAATAAGATGTAGATGGAATGTGGGCTCTAATACT
ACTCTAATTGACACATGTGGAAAGAACAACATTACAGGAGCTAATCCTGT
GGATTGTACTATGATGGCAAATATTTTGTACAATTGCTCATTACAAGAGG
GATTTACTATGAAAATAGAAGATCTTATAATGCATTTTAACATGACAAA
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>HI-03

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GATTAGGGCAATGTTCGTCATGGAGGATAAGGAATAGATGGGAATGGAGGC
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AGAACTTAACTTTTGCAATGAGAAGCTCAGCTGATTTTGGTGATGTTGT
AGGAGCATGGATAGAATTTGGGTGTCATAGAAACCGAACACGAACTCATA
CAGATGCAAGATTTAGAATAAGATGTAGATGGAATGTTGGCTCTAATATT
TCTCTAATTGACACATGTGGAAATGACACTATTACAGGAGCTAATCCTGT
AGGTTGTACCATGACAGCAAATACTCTGTACAATTGCTCGTTACAAGAGG
GATTTACTATGAAAATAGAAGATCTTATAATGCATTTTAACATGACAAAG
GCTGTAGAAATGTATAATATTGCTGGAAATTGGTCTT
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>HI-04

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AGGTAAAAATATCATTACAATGTAACAGTACAAAAGACTTAACTTTTGCA
ATGAGAAGCTCAGCTGATTTTGGTGATGTTGTAGGAGCATGGATAGAATT
TGGATGTCATAGAAACCAACAAAATTTTCATACAGAGGCAAGATTTAGAA
TAAGATGTAGATGGAATGTTGGCTCTAATACTTCTCTAATTGACACATGT
GGAAATGAAGGCAATATTACAGGAGCTAATCCTGTAAATTGTACTATGAA
AGCAAAGACTCTGTACAATTGCTCATTACAAGAGGGATTTACTATGAAAA
TAGAAGATCTTATAATGCATTTTAACATGACAAAGGCTGTAGAAATGTAT
AATATTGCTGAAA
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>HI-05

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TGGATGGTGGAATCMAGCGGCTTACTATWATAGTTGTAAATGGGAAAAAA
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AGATTTTGAAAGTGAAAAGGTGAAAGTATCTCTAAAGTGTAAATACCACAA
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GGAGCTTGGATAGAGTTTGGATGTCATAGAAATAAATCAAAAACTCATAC
TGATGCAAGATTTAGAATAAGATGTAGATGGAATGTTGGTTCTAATAGTT
CTYTAATTGACACATGTGGAAGAAAGAAAACAATATTTTCAGGAGCTAATCCY
GTAGATTGTACCATGACTGCAAATAAAATGTACAATTGTACTTTACAAAA
TGGGTTTACTATGAAGGTAGATG

>HI-06

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TGGATTAGAGCAAWATCGTCATGGAGGGCAAGGAATAGATGGGAATGGAG
GCCAGATTTTGAAAGTGAAAAAGTAAAAATATCAATACAATGTAACAGTA
CAAAAAATTTAACTTTTGCAATGAGAAGCTCATCTGATTTTGGTGATGTT
ACGGGAGCATGGATAGAATTTGGATGTCATAGAAACAAAACAAAATTACA
TACAGAGGCCAAAATTTAGAATAAGATGTAGATGGAATGTTGGCTCTAATA
CTTCTCTAATTGACACATGTGGAAATGAAAAAAATATTTCAAGAGCTAAT
CCTGTAGATTGTACGATGAMAGCAAATACTCTGTACAATTGCTCATTACA
AGAGGGATTACTATGA

>HI-07

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TTCATACTGAAGCAAGGTTTAGAATTAGATGTAGATGGAATACAGGG

>HI-09

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CAATGAGAAGTTCAGGAGATTATGGAGAAGTAACGGGAGCTTGGATAGAG
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GGTAGATG

>HI-10

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AGCTCAGCTGATTTTGGTGATGTTGTAGGAGCATGGATAGAATTTGGATG
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GGAAATTGGTCTTGA

>HI-11

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>HI-12

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TCAGCTGATTTTGGTGATGTTGTRRGAGCATGGATAGAATTTGGATGTCA
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TTATAATGCATTTTAACATGACAAAAGCTGTAGAAATGTATAATATTGCT
GGAAATTGGTCTT

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