MOLECULAR CHARACHTERIZATION

OF A NOVEL

FIELD ISOLATE OF

INFECTIOUS LARYNGOTRACHEITIS VIRUS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Preveterinary Medicine and Animal Biosciences with Distinction

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ABSTRACT

Infectious laryngotracheitis virus is an alphaherpesvirus responsible for causing a respiratory disease of chickens. The goal of this project is to sequence a novel field isolate of ILTV, UD2011K, using DNA isolated from CAM material. CAM material has not before been used for genomic sequencing of ILTV.

After extracting the total DNA for UD2011K-infected CAM material, a DNA sequence library was constructed. Approximately 0.7% of the generated sequence was ILTV. The complete sequence of UD2011K was determined after alignment to the USDA reference strain and was found to be 151,756 base pairs long. The UD2011K sequence was found to contain 196 SNPs and 28 indels. Of note was a three nucleotide deletion of the ICP4 gene. This sequencing method may be more accurate than RFLP analysis in differentiating strains of ILTV.

Chapter 1

INTRODUCTION

1.1 Infectious Laryngotracheitis

1.1.1 The Disease

Infectious laryngotracheitis, commonly referred to as LT, is a respiratory disease of chickens that was first described in 1925 (May, 1925). The virus is specific for chickens as far as virulence, pathogenicity and susceptibility (Keeler, 2006). The chicken is the primary host; however turkeys and pheasants have low susceptibility (Tripathy, 1998). All ages of chickens are susceptible, but the disease is usually seen in birds 3-9 months old (Bagust, 1992). The disease is spread from bird to bird by respiratory or ocular routes (Fuchs, 2007).

1.1.2 Economic Impact

ILT is found in many parts of the world. It is most common in the US, Canada, Mexico, Australia, Southeast Asia, Europe, South Africa and northern parts of South America (Biggs, 1982). The disease causes a decrease in egg production and decreased growth in meat animals, although it does not affect egg quality. Mortality ranges from as high as 50% to less than 10%, depending on the strain (Bagust, 1992). Males and heavy breeds seem to be more susceptible to the disease (Jones, 2001).

1.1.3 Clinical Symptoms

Symptoms of infectious LT vary from mild to severe. This depends on the isolate since some are more virulent than others (Linars, 1994). The disease is characterized by gasping, coughing, wheezing, moist rales, bloody or cheesy exudate from the trachea, conjunctivitis and lung edema. The presence of inclusion bodies in the tracheal cells of infected chickens is a common characteristic of ILT and a good indicator of the virus (Tripathy, 1998). Symptoms can be worsened with environmental factors such as low temperatures, chemical or dust irritation of the respiratory tract or other infections (Bagust, 1992). Death often occurs from asphyxiation caused by bloody or cheesy mucoid membranes in the trachea. The severity of pathological changes, clinical symptoms and time between exposure and clinical signs varies from bird to bird (Cover, 1958). The disease can be worsened if the animal is also infected with another disease (Jones, 2011).

Alternate forms of diagnosis are necessary since clinical signs are easily confused with other diseases such as New Castle disease, avian influenza, Mycoplasma gallisepticum, infectious bronchitis, and Mycoplasma synoviae (Linars, 1994).

1.1.4 Control

The Pennsylvania Bureau of Animal Industry (BAI) has created a checklist to help farms lessen the incidence of ILT (Mallinson, 1981). To prevent the disease, it is important to increase biosecurity and keep track of all persons who come on and off of a farm (Bagust, 1992). The vaccination history of a flock and an understanding of where chickens come from, as well as what other animals are present on the premises are all important factors. The main reason that ILT is transferred from farm to farm is

fomites (Mallinson, 1981). Under the right conditions, ILTV can survive up to 80 days in the tracheal exudates of chicken carcasses. It is important to dispose of litter and carcasses properly to prevent the spread of the disease (Bagust, 1992). The virus is highly sensitive to high temperatures and disinfectants. It is important to practice sanitation practices for any trucks and crates that are used to transport chickens. Any flock that is positive for LT should be placed under quarantine.

ILT may be controlled through live-virus vaccines from either chicken embryo origin (CEO) or tissue culture origin (TCO) (Thureen, 2006). The CEO and TCO vaccines are created by passing live virus strains through either eggs or tissue culture (Spatz, 2011). CEO vaccines are more virulent than TCO vaccines and are more likely to cause disease outbreaks (Guy, 1991). However, CEO vaccines give better protective immunity than TCO vaccines. Vaccination often lowers the severity of the disease, but may not successfully prevent the disease since outbreaks often occur in vaccinated flocks (Fulton, 2000). Vaccinated birds will have higher ELISA titers and less severe symptoms than non-vaccinated birds. In order for vaccination to be effective, all birds in a flock must be vaccinated with a uniform dose.

There are different ways to administer vaccines such as eye drop, water or spray. A study by Fulton showed that each vaccination method has pros and cons (Fulton, 2000). The eye drop method was the most effective when compared to the water and spray method, however this method is more time and labor intensive. Spray vaccination did not provide protection for all of the birds. Complete coverage is often hard to guarantee for this method. Similarly, water vaccination did not provide uniform protection. It was found that birds drinking water closer to the source of the vaccine were protected better than birds which drank further from the vaccine source.

Vaccines are often administered in two doses and it is encouraged that at least the first dose is via eye drop (Mallinson, 1981).

A major issue with ILTV is that it can become latent and persist in birds who have already recovered from the disease (Tripathy, 1998). A chicken in this carrier state can shed the virus to healthy animals and cause new outbreaks. Mucosal antibodies may play a role in preventing vaccinated birds from becoming latent carriers of the disease (Fahey, 1990). Vaccinated animals may become latent carriers of the disease or get a less severe form of the disease (Jones, 2001).

It has been proposed that chickens have the potential to be bred for resistance. As breeders and researchers continue to learn the genetics behind chicken immunity, it will be possible to select animals with genes resistant to ILT to be bred (Biggs, 1982). Similar attempts have been tried with some success for Marek's disease and Leukosis in poultry.

1.1.5 Diagnosis of ILT

ILTV can be differentiated from similar diseases by a number of diagnostic methods such as enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and examination of intranuclear inclusion bodies (Bagust, 1992) (Tipathy, 1998). Clinical signs are not reliable since the symptoms of ILTV are easily confused with other diseases.

Enzyme linked immunosorbent assay, or ELISA, is a serological technique used to detect viral antibodies from a serum sample. A viral antigen is bound to a solid matrix and the serum sample is added. If the antibody is present in the sample, the antigen will bind to it and form a complex. The bound antibody is detected by an

antigen that is tagged with an enzyme. If binding occurs, a color reaction develops (MacLachlan, 2011).

Polymerase chain reaction (PCR) amplifies DNA with the use of primers. A thermocycler performs the process in three steps: denaturing, annealing and extension (Powledge, 2004). Once the DNA is amplified, it is ran on a gel against a ladder and observed under UV light. Bands will appear where the primer length should be. A band at the known primer length confirms the DNA in question is present.

Histopathology of intranuclear inclusion bodies is often used to diagnose LT. Inclusion bodies are present in the tracheal epithelial cells of ILTV infected birds. Samples of these cells are taken from sick birds and examined under a microscope for inclusion bodies (Tripathy, 1998).

1.2 Infectious Laryngotracheitis Virus

The incubation period for ILTV is usually about 5-9 days (Bagust, 1992). Neither the virus nor immunity to the virus can be passed from hen to egg. ILTV is able to survive in the environment for weeks under the right circumstances (Jones, 2011).

A characteristic of herpesviridea, including ILTV, is the ability to become latent (Bagust, 1995). During latency, there is an absence of infectious particles in cells and tissues that harbor the virus (Croen, 1991). Latency is necessary for the virus since it allows it to survive between generations (Bagust, 1992). When an animal is first infected with a virus, some cells will have the virus establish a latent state as opposed to continuing to the lytic pathway to destroy the cell (Croen, 1991). As the immunity of an animal decreases or as the animal becomes stressed, reactivation of the virus can occur. Usually, latently infected animals show no symptoms, but re-

occurrence of the disease is possible. Both field strains and vaccines can establish latent infections in birds (Jones, 2001).

1.2.1 Physical characteristics of the virus

Infectious laryngotracheitis virus, also known as *Gallid herpesvirus-1*, is characterized as an alphaherpesvirus. It has a double stranded DNA genome (Fuchs, 2007). The virus exists as one serotype with no antigenic variation (Bagust, 1992), however different strains often vary in pathogenicity (Jones, 2001). ILTV, along with *Psittacid harpesvirus-1*, is a member of the *Iltovirus* genus of *Alphaherpesvirinae* (Thureen, 2006). It is a class D virus that has inverted repeats flanking the unique short (Us) region (Garcia, 2013). The virus is composed of a core, capsid, tegument and an envelope (Mattenleiter, 2002). Its icosahedral capsid is composed of 150 hexons and 12 pentons. The envelope is composed of lipids from the host cell and viral glycoproteins.

The replication process of the virus was described by Mattenleiter (2002). It begins with the viral envelope attaching to the host cell membrane. This allows a viron to enter the host cell's cytoplasm and attach to its nuclear membrane. After the viral DNA enters the cell, the host's replication proteins are used to make viral DNA and proteins form procapsids which contain viral DNA. These procapsids form vesicles from the nuclear membrane of the cell and make stops at the endoplasmic reticulum for glycoprotein formation and golgi apparatus for packaging into secretory vesicles (Mattenleiter, 2002). These secretory vesicles join the plasma membrane of the host cell in order to release the virus outside the cell where it can go on to infect other healthy cells.

ICP4, or infected cell protein 4, is a gene that is responsible for gene regulation. It is essential for the transcription of other ILTV genes. The ICP4 gene is 1,463 amino acids long and has an overall G+C content of 59% (Johnson, 1995). There is a single copy of this gene in each of the inverted repeats of ILTV (Keeler, 2006).

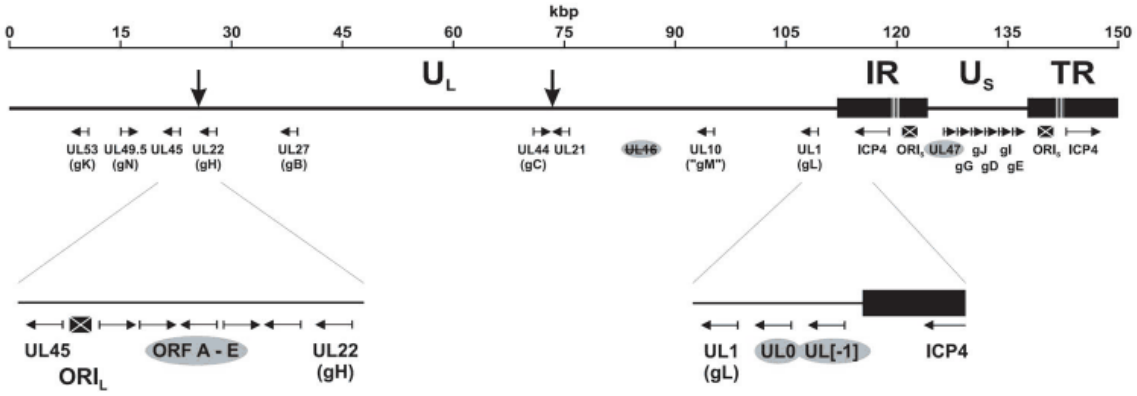

Figure 1: Genome of Infectious Laryngotracheitis Virus: The ILTV genome is composed of a unique long region (U_L) and a unique short (U_S) region. The unique short region is flanked by inverted repeats (IR_S) and a terminal repeat (TR). (Fuchs, 2007)

1.2.2 Strain differentiation

ILTV strains can be classified using a number of different techniques. One of the techniques used that was demonstrated by Keeler in 1993 was restriction endonuclease (RE) analysis. This technique depends on the RE enzymes chosen. It groups similar strains together by how similar the banding patterns are on a gel (Keeler, 1993). This technique also demonstrated that most field isolates have the same banding pattern, meaning they are similar. This is because all of the vaccines in the industry were created from field isolates.

A widely used way to differentiate strains and to group different strains is by Restriction Fragment Length Polymorphism of PCR products (PCR-RFLP), or RFLP analysis. RFLP analysis works by using different restriction enzymes to generate patterns based on their genotype by cutting at certain sites (Oldini, 2008). The samples are then run on a gel and the fragments are separated according to size. Different strains will have different patterns when compared to each other. This process requires large amounts of pure viral DNA. Oldini used RFLP analysis to differentiate different ILTV strains into 9 groups based on 11 PCR-RFLP pattern combinations (Oldini, 2007). This technique has been used to sort ILTV strains into different clades based on different genetic patterns (Garcia, 2013). Garcia also created a phylogenic tree of ILTV strains by separating them into four clades: CEO, TCO, Group V and Australian. PCR and RFLP are often able to differentiate field strains from vaccine strains (Jones, 2001). This technique has its drawbacks. Analysis of a genome is often limited because obtaining the pure viral DNA in high enough yields for an analysis is difficult (Oldini, 2007). Also, RFLP is not entirely accurate in differentiating between very similar strains of ILTV.

An important factor in differentiating strains that should also be taken into account is virulence. Different SNPs and indels can result in different amino acids that encode for virulence factors (Garcia, 2013). For example, it was found that the Glu 343 Lys polymorphism in the UL5 region increases a strain's virulence. More recently, a focus has been put on comparative genome analysis to group strains based on different properties (Thureen, 2006).

1.2.3 Efforts to sequence ILTV

In 2006, infectious laryngotracheitis virus was successfully sequenced and the entire genome was assembled for analysis (Keeler, 2006). The genome was found to be 148,665 base pairs long with a GC content of 48.16%. The unique long region is 113,039 base pairs and the unique short region is 13,232 base pairs long. The unique short region is flanked with inverted repeats. The virus has 10 structural glycoproteins responsible for host range and pathogenicity.

1.3 Objectives

UD2011K is a virulent field isolate of ILTV. It was first identified in 2011 from a farm in Delaware. The virus was taken from birds at 5 weeks of age before being transported to the University of Delaware as CAM material for research. Vaccine trials showed that the vaccines did not completely protect birds from the UD2011K virus. It also has an unusually high mortality rate and is extremely virulent.

The primary objective of this project is to use high throughput sequencing technology to sequence the DNA genome of UD2011K from relatively crude material. Although previous ILTV sequencing efforts have used relatively purified viral DNA

as a sequencing template, we propose to use DNA dropped from UD2011K-infected CAM material. This material is expected to contain predominately chicken DNA.

If successful, this experiment will be the first time that ILTV DNA will be sequenced form a crude DNA sample. In other experiments, DNA is purified in tissue culture after it is obtained from CAMs. If our procedure yields ILTV DNA, it may lay a foundation for faster sequencing. Another goal of this experiment is to learn what genetic differences there are between the USDA reference strain and the UD2011K strain of ILTV. An analysis of the SNPs should allow us to determine what amino acid differences there are and if these differences are the cause for the increased virulence of the novel field isolate. Previously, a phylogenetic tree was developed based off of the PCR-RFLP technique. In the future, whole sequencing and alignment techniques may be used instead.

Chapter 2

Materials and Methods

2.1 Viral Propagation

UD2011K is a field isolate of ILTV isolated from a farm in Delaware in 2011. UD2011K exhibits a higher mortality rate compared to other strains of ILTV, and it can cause disease in previously vaccinated birds UD2011K was propagated on chorioallantoic membranes (CAMs) as directed by Senne, 2006. Ten specific pathogen free eggs were candled and a mark was placed in the middle of the side of each egg. This mark was placed away from any veins or the embryo. Another mark was placed on the top of the egg's air sac. The eggs were then placed horizontally on their sides and a small hole was created on each mark by using a needle that had been punched through a rubber stopper. This ensures a hole is created, without going deep and puncturing the CAM. The CAM of each egg was dropped by the application of a gentle vacuum from a suction bulb, causing a new air sac to form on the side of the egg above the CAM where the second hole was punched. Using a 25-guage needle inserted just inside the eggshell on the side with the new air sac at approximately a 45º angle, 0.1mL of virus inoculum was injected into each egg. The eggs' holes were sealed with glue, sprayed with 70% ethanol, and then placed in an egg incubator for five days. The eggs are then refrigerated for one day to euthanize the embryo.

After 6 days, the CAMs were observed for plaque and pock formation and areas of CAM that were infected were removed with sterile scissors and collected in a sterile vial. An equal volume of 1X Tris/Borate/EDTA (TBE) Buffer was added to the

CAM material and homogenized. The mixture was then put in the clinical centrifuge for 3-4 minutes at 1,200 rpm to remove debris. Infected CAM homogenates were stored at -80ºC until further use.

2.2 Viral DNA Isolation

Nucleic acid isolation was done by following the procedure described by Garcia and Riblet, 2001. An aliquot of infected CAM suspension (0.3mL) was thawed, refrozen, and thawed again. 150µl of lysing buffer was added to the CAM suspension and it was vortexed slightly. 22.5µl of proteinase K was added to the suspension which was then incubated at 37ºC for 45 minutes. DNA was then extracted twice with PIC (phenol/chloroform/isoamylalcohol). This was done by adding an equal volume of PIC to the CAM suspension. The tube was mixed for 1 minute and then centrifuged in a clinical centrifuge for 5 minutes. The aqueous layer was collected and the PIC extraction was then repeated. Total DNA was precipitated by the addition of 47.5µl of NaOAc and 1 ml of ethanol and frozen at -20ºC overnight. The DNA pellet was re-suspended in 100µl of distilled water and analyzed for purity using a Nanodrop.

2.3 Polymerase Chain Reaction (PCR) of the ILTV Glycoprotein E Gene

To amplify the ILTV glycoprotein E gene (Garcia, 2001), 1µL of ILTV gE Forward Primer (gE F 5'GGCTGACCAGGATAGTGAAC-3'), 1µL of Reverse Primer (gE R 5'GGTAAGATTTCCCGATTTCTC-3' and 5µL of DNA were added to 45µL of supermix. After activating the DNA polymerase for 1 minute at 95ºC, PCR was performed by 35 cycles of 94ºC for 15 seconds, 50ºC for 45 seconds and 72ºC for 1 minute. This was followed by a final extension of 72ºC for 3 minutes.

2.4 DNA Seq Library Creation

DNA was sheared on a Covaris machine according to the instructions. Five μg of DNA brought to volume of 120 μl with TE buffer and sheared for 5 minutes. A DNA seq library was created using the protocol for the NuGEN Encore NGS Multiplex Systems (Encore, 2010). End repair was performed on 200ng of fragmented DNA. End repair DNA fragments were purified using Agencourt RNA clean magnetic beads. Ligation adaptors were added to the ends of the repaired and purified DNA fragments, followed by PCR- mediated library amplification. Finally, the amplified DNA fragments were purified using magnetic beads.

2.5 Illumina Genome Analysis

A single pass of sequencing reactions was performed on the UD2011K-infected CAM DNAseq library by the Delaware Biological Institute on a highseq 2000 genome analyzer.

2.6 Sequence Analysis

The DNA sequence was initially aligned to the chicken genome, and all chicken DNA sequences were removed from further analysis. The remaining viral DNA sequences were then aligned to the USDA reference sequence (DNA Star, 2013). Comparison to the USDA reference sequence leads to the identification of nucleotide differences (SNPs) and insertions or deletions (indels).

Chapter 3

Results

3.1 ILTV Propagation and Viral DNA Extraction

A virulent field isolate of ILTV from Delaware, UD2011K, was taken from 5 week old birds and transported to UD as a CAM suspension. UD2011K was propagated on embryonated eggs via that CAM method according to the procedure stated in the Materials and Methods section. DNA was isolated from 300µL of CAM solution by treatments with lysing buffer, proteinase K and PIC (phenol/chloroform/isoamylalcohol) as described in the Materials and Methods section. A spectrophotometer measured the concentration of DNA as 243.1ng/µL with a 260/280nm ratio of 1.82. To confirm the presence of ILTV, the gE gene was amplified to see if it was present in our crude DNA sample. From the gel electrophoresis photo, (Figure 2), it can be seen that there is a band at 1847 base pairs. This band proved that ILTV DNA was present within the CAM-derived DNA.

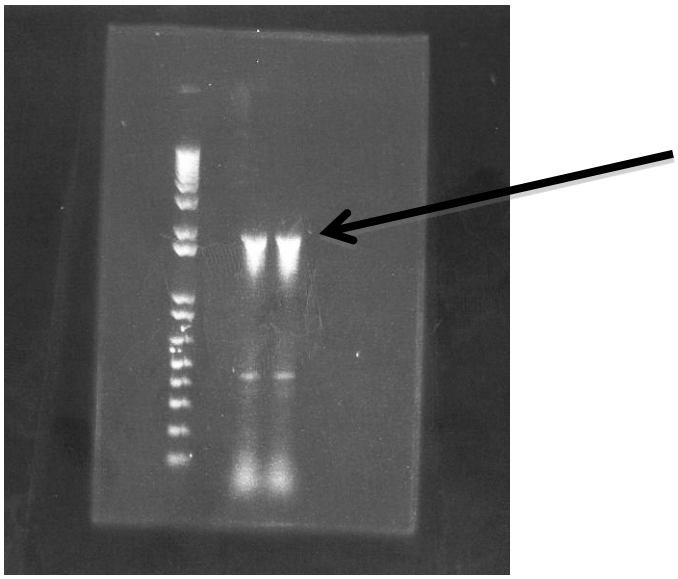

Figure 2: PCR amplification of ILTV viral DNA: Lane one has the ladder which was made from 10µ of DNA ladder and 10µL of water. Lanes 3&4 contain 5µL of the DNA from the PCR reaction. The arrow points to a DNA band at 1,847 base pairs.

3.2 Sequencing of UD2011K

Illumina sequencing technology is an efficient way to accurately assemble a genome from a library (Genome Analyzer XII Technology, 2013). There are three major steps in how the technology works: Library preparation, cluster generation and sequencing. During library preparation, DNA is fragmented and the sheared ends are repaired. Adaptors are ligated to the fragments before fragments are size selected and purified. The cluster generation step consists of single molecules being amplified. A lawn of adaptors binds to the library fragments. These fragments are then extended and copies are made and bound to a flow cell surface. Reverse strands are cleaved and washed away. Sequencing is done by a genome analyzer. DNA templates are sequenced one base at a time, with all four bases available. Since all four bases are competing, the correct base is the one that binds. The bound bases have a fluorescent tag which is recognized by a laser. The laser registers each base as a different color, and the proper base is recorded.

A DNA seq library was generated from UD2011K infected CAM DNA after extraction using the protocol for the NuGEN Encore NGS Multiplex Systems (Encore, 2010) as described in the Materials and Methods section. The library was amplified and sequenced on an Illumina Highseq 2000 genome analyzer at the Delaware Biological Institute. The average depth of coverage was 57 reads out of the 25,289,363 sequences generated by the library 0.6% were aligned to the ILTV genome (Table 1). Once aligned to the reference sequence, it was determine that the UD2011K strain of ILTV was 151,756 base pairs in length.

Unassembled	Chicken Sequences	Reads Homologous to ILTV	Reads not homologous to chicken or ILTV
25,289,363	25,100,512 99.25%	151,456 .6%	37,095 .15%

Table 1: Genomic results from Illumina and DNA Star:

This table shows the amount of reads that were discarded after being aligned to the chicken genome and what was left after being aligned to the USDA reference Strain.

3.3 UD2011K SNP and Indel Analysis

UD2011K was sequenced for the purpose of determining genetic changes that may be responsible for the strain's virulence. Differences were determined in the UD2011K sequence when compared to the USDA reference strain of ILTV.

A single nucleotide polymorphism, or SNP, is a difference of a base pair at the same location from one individual to another (Griffiths, 2008). Although many SNPs are silent and have no effect on an individual's phenotype, they are often used as markers for genetic mapping.

Figure 3A shows a TA GC SNP. The amino acid coded by ACU by messenger RNA is Threonine. The amino acid that is coded for by GCU after the SNP is Alanine. These Indels that code for different amino acids will change the protein that is coded for and this may change the function of the protein.

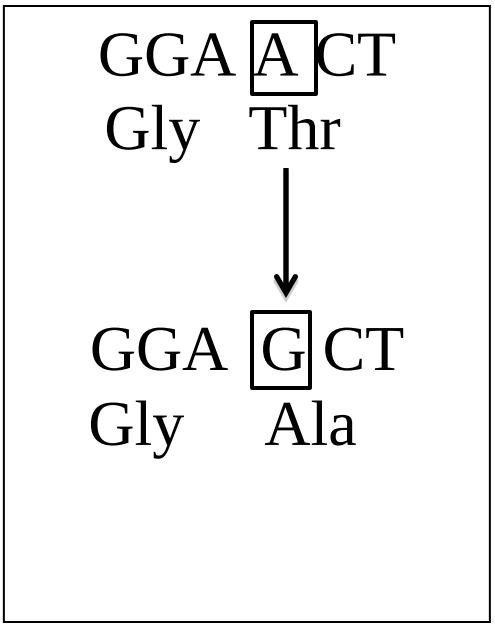

Figure 3A: An Example of a SNP

An indel is an insertion or a deletion of one or more base pairs in a genome (Griffiths, 2008). An indel will cause a frameshift mutation in a protein-coding region. An indel is likely to cause a shift because as translation occurs, the DNA will be used to produce amino acids based off of three DNA bases in a row. If the code for the amino acid is off by a nucleotide, the amino acid that is created may be different than normal. Since amino acids are the building blocks of proteins, this may cause a difference in the protein sequence.

Figure 3B shows an insertion of a TA base pair. When translation occurs, the original sequence would have ACG and GUA be translated into Threonine and Valine. After insertion, GAU would be coded for instead of GUA. GAU codes for Aspartic acid. These Indels that code for different amino acids will change the protein that is coded for and this may change the function of the protein.

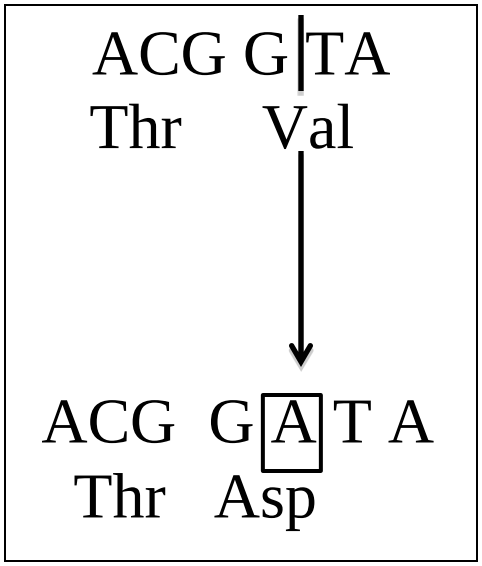

Figure 3B: An Example of an Indel

UDSA Genome Length	UD2011K Genome Length	Total Reported SNPs	SNPs at 100%	Indels	% identical
151,769 bp (Spatz, 2012)	151,756 bp	196	161	28	99.85%

Table 2: Genomic comparison of the USDA reference strain and the UD2011K strains of ILTV

Our SNP report had a cutoff of 10%. This means that any SNP between the USDA reference strain and the UD2011K strain that appeared less than 10% of the time was not documented in the SNP report. Since the average depth of coverage was 57 bp and our cut off was 10%, any SNP that appeared at least 6 (5.7) times was reported. Table 2 shows the amount of SNP's and indels. The genomic differences were counted and there were 196 SNPs and 28 indels. There were 161 SNPs that were different 100% of the time between the two strains.

Any SNPs that appeared 5 or fewer times did not meet our cutoff and were not reported. Table 3 shows a sample of the UD2011K ANP report that shows the location of SNPs and indels between the reference and consensus sequences. The reference base was from the USDA sequence and the called base was from UD2011K. The SNPs in this table are all 100% different from the USDA reference sequence. The full SNP report showing all 196 SNPs and 28 indels is located in the appendix section of this paper.

Reference Position	USDA Reference Base	UD2011K Called Base	Called Base %
2	G	C	100%
1,093	C	A	100%
4,294	C	T	100%
11,925	C	T	100%
12,594	G	A	100%
44,120	G	A	100%
63,530	C	T	100%
71,281	C	T	100%
109,846	A	C	100%
112,100	T	A	100%
116,690	A	T	100%
116,717	T	C	100%
117,942	A	G	100%
151,504	T	G	100%

Table 3: 14 SNPst: A sample of the UD2011K SNP report shows 14 differences between the USDA sequence (Reference) and the UD2011K sequence (Called Base).

3.4 The UD2011K Strain of ILTV May Have a Deletion in ICP4

ICP4 is a gene that is responsible for gene regulation. The ICP4 gene is 1,463 amino acids long. There is a single copy of this gene in each of the inverted repeats of ILTV (Keeler, 2006). Figure 4 shows the deletion of the bases ATT which corresponds to AUU in RNA and codes for the amino acid Isoleucine. This amino acid is present in the USDA strain but not in the UD2011K strain. This deletion is located between 116,886 and 116,888 base pairs in the UD2011K isolate. The other amino acid missing from the ICP4 gene in the UD2011K isolate is leucine, which is located in the inverted repeat from 144,597 and 144,599. The three bases that are missing are TTA or UUA, which code for leucine. The amino acids deleted from the ICP4 gene may have an effect on virulence and pathogenicity of the isolate.

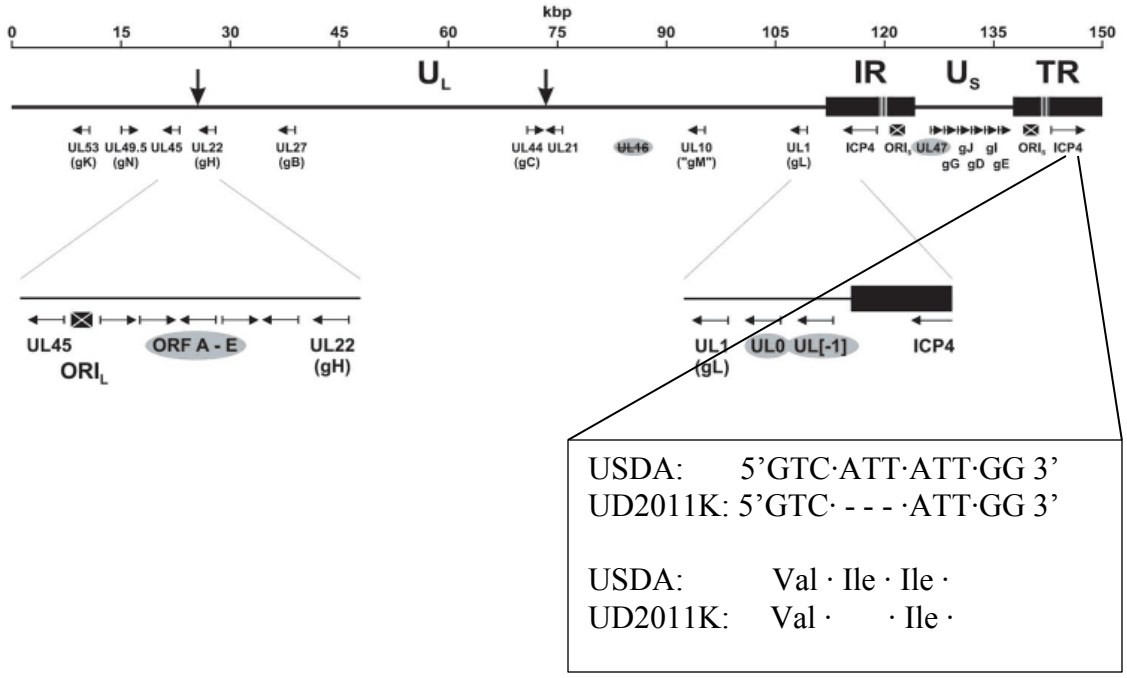

Figure 4: Location of the ICP4 gene in the USDA and UD2011K strains of ILTV: There is a single copy of the same gene in each inverted repeat. One of the copies is zoomed in on and the other is not shown. The three base pair deletion in the UD2011 ICP4 gene is shown.

Chapter 4

Discussion

4.1 Discussion

The objective of this project was to see if the sequence of a field isolate of ILTV could be sequenced from crude chorioallantoic membrane (CAM) material. The UD2011K strain was successfully sequenced and found to be 151,756 base pairs in length. This was the first time ILTV was sequenced from a crude sample. Previously, DNA took weeks to purify after the harvesting of CAM material. Since CAM material yielded DNA, swab material directly from the birds may be used to try to sequence ILTV.

From a total of 25,289,363 “reads”, 188,551 were found to not be chicken DNA sequence and 0.6% of the “reads” were ILTV. When aligned to the USDA reference strain of ILTV, the genome of UD2011K was found to be 151,756 nucleotides in length. The depth of coverage was 57; however there were five regions of low coverage. These five regions had too few reads to determine if the reported SNPs were real or just reading errors from the genome analyzer. PRC amplification followed by sequencing of these regions would allow us to determine what genomic differences are located in these areas. In comparison to the USDA reference strain, UD2011K had 196 reported SNPs and 28 indels. An area of interest was the ICP4 gene. The gene was located in different parts of the genome and the UD2011K strain had a three nucleotide deletion in its ICP4 gene.

This is the first report using DNA from CAM material to sequence a strain of ILTV as opposed to the tissue culture method. Previously, DNA would have been purified by taking a sample from an infected bird, inoculating eggs, collecting CAMs and then growing the virus on tissue culture. The experiment was successful in purifying DNA and took less time than if the tissue culture method was used. Future experiments may use swabs of samples directly from infected birds to obtain viral DNA. This method would be even more direct than growing the virus on CAMs and would save time.

4.2 The ICP4 gene

When the SNP report was received (see appendix), the ICP4 gene in the UD2011K strain of ILTV had a three nucleotide deletion at each of the inverted repeats. There is a deletion of the bases ATT that corresponds to AUU in RNA and codes for the amino acid isoleucine. This amino acid is present in the USDA strain but not in the UD2011K strain. This deletion is located between 116,886 and 116,888 base pairs in the UD2011K isolate. There is another three base deletion located in the inverted repeat from 144,597 and 144,599. The three bases that are missing are TTA or UUA, which codes for leucine. UD2011K is a highly pathogenic field isolate of ILTV and future research may be able to reveal if these amino acid deletions are the cause of the increased virulence of UD2011K. Other SNPs should also be analyzed to see if they give rise to changes in amino acids. A change in amino acids and protein structure could be a cause of the UD2011Ks pathogenicity. ICP4 is responsible for gene regulation in ILTV. This deletion may cause the regulation of genes to be increased, which could cause for increased pathogenicity. This deletion can also be

used to create PCR primers. This would allow PCR to be used to determine if a sequence of ILTV is the UD2011K strain or not.

Although UD2011K would have to be compared to other strains of ILTV for confirmation, the three nucleotide deletion is probably unique. PCR primers will be able to be made as a diagnostic tool based off of this three nucleotide deletion. This will allow for a rapid diagnosis of UD2011K.

4.3 Phylogeny Grouping

Previously, Oldini used RFLP analysis to differentiate different ILTV strains into 9 groups based on 11 PCR-RFLP pattern combinations (Oldini, 2007). Sequencing and aligning different strains of ILTV is an accurate way in determining how similar different strains of ILTV are to each other. Genome alignment is another method besides restriction fragment length polymorphism (RFLP) analysis to differentiate strains of ILTV. It allows us to highlight genomic differences in different genes and find what amino acids may be changed between different strains. In addition, this is able to be done accurately with crude DNA isolated from CAM material as opposed to the large quantities of pure viral DNA needed for RFLP analysis.

4.4 Conclusion

UD2011K is a virulent field isolate that was isolated from 5week old chickens in Delaware. It was successfully sequenced from crude CAM material and aligned to the USDA reference strain. It genome is 151,756 base pairs in length. It also contained a 3 base pair deletion in the ICP4 gene. This deletion may be used to create a PCR

primer for rapid diagnosis. In addition, this mutation might be one of the reasons the UD2011K strain is so virulent.

This method of crude DNA extraction from CAM material will lead to more rapid techniques in viral sequencing and genome comparison. Since CAM material yielded DNA, swab material directly from the birds may be used to try to sequence ILTV.

Genome comparison by sequencing and aligning different strains is an alternative method to differentiate strains of ILTV than PCR-RFLP analysis. Not only are large quantities of pure viral DNA no longer needed, it is able to look at each individual SNP and indel to differentiate different viral strains.

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Appendix

UD2011K SNP Report

SNP	Contig ID	Contig Pos	Ref Pos	Type	Ref Base	Called Base	SNP %	Depth	A Cnt	C Cnt	G Cnt	T Cnt	Deletion
?	JN542534USDA.seq	16	2	SNP	G	C	100.00%	2	0	2	-	0	0
?	JN542534USDA.seq	17	3	Indel	-	T	100.00%	2	0	0	0	2	0
?	JN542534USDA.seq	1004	989	SNP	A	C	10.00%	20	-	2	0	0	0
?	JN542534USDA.seq	1009	994	SNP	A	C	16.70%	18	-	3	0	0	0
?	JN542534USDA.seq	1108	1093	SNP	C	A	100.00%	19	19	-	0	0	0
?	JN542534USDA.seq	1177	1162	SNP	A	C	31.60%	19	-	5	1	0	0
?	JN542534USDA.seq	1566	1551	SNP	T	G	12.80%	39	0	0	5	-	0
?	JN542534USDA.seq	2157	2142	SNP	T	G	35.70%	14	0	0	5	-	0
?	JN542534USDA.seq	2165	2150	SNP	A	G	20.00%	10	-	0	2	0	0
?	JN542534USDA.seq	2168	2153	SNP	A	T	40.00%	5	-	0	1	1	0
?	JN542534USDA.seq	2171	2156	Indel	-	A	100.00%	10	9	0	0	1	0
?	JN542534USDA.seq	3102	3086	Indel	C	-	100.00%	53	0	-	0	0	53
?	JN542534USDA.seq	3107	3091	Indel	T	-	100.00%	54	0	0	0	-	54
?	JN542534USDA.seq	4048	4030	SNP	T	G	10.00%	30	0	0	3	-	0
?	JN542534USDA.seq	4312	4294	SNP	C	T	100.00%	11	0	-	0	11	0
?	JN542534USDA.seq	4313	4295	SNP	C	G	100.00%	9	0	-	9	0	0
?	JN542534USDA.seq	4314	4296	SNP	A	G	100.00%	10	-	0	10	0	0
?	JN542534USDA.seq	4320	4302	SNP	G	C	30.00%	20	0	6	-	0	0
?	JN542534USDA.seq	4985	4965	SNP	C	A	10.40%	48	5	-	0	0	0
?	JN542534USDA.seq	5514	5493	SNP	A	G	100.00%	62	-	0	62	0	0
?	JN542534USDA.seq	5801	5780	SNP	G	T	11.10%	45	0	0	-	5	0
?	JN542534USDA.seq	6582	6559	SNP	T	C	100.00%	13	0	13	0	-	0
?	JN542534USDA.seq	6587	6564	SNP	C	A	100.00%	7	7	-	0	0	0
?	JN542534USDA.seq	6603	6580	SNP	C	T	100.00%	14	0	-	0	14	0
?	JN542534USDA.seq	6604	6581	SNP	T	A	100.00%	17	17	0	0	-	0
?	JN542534USDA.seq	8820	8797	SNP	C	T	100.00%	63	0	-	0	63	0
?	JN542534USDA.seq	11325	11302	SNP	A	C	26.10%	46	-	12	0	0	0
?	JN542534USDA.seq	11948	11925	SNP	C	T	100.00%	46	0	-	0	46	0
?	JN542534USDA.seq	12045	12022	SNP	G	T	100.00%	60	0	0	-	60	0
?	JN542534USDA.seq	12617	12594	SNP	G	A	100.00%	76	76	0	-	0	0
?	JN542534USDA.seq	12838	12815	SNP	C	T	100.00%	62	0	-	0	62	0
?	JN542534USDA.seq	13055	13032	SNP	A	G	100.00%	69	-	0	69	0	0
?	JN542534USDA.seq	13125	13102	SNP	T	G	100.00%	53	0	0	53	-	0
?	JN542534USDA.seq	13651	13628	SNP	G	A	100.00%	48	48	0	-	0	0
?	JN542534USDA.seq	13810	13787	SNP	G	C	11.80%	34	0	4	-	0	0

?	JN542534USDA.seq	13837	13814	SNP	A	G	100.00%	64	-	0	64	0	0
?	JN542534USDA.seq	14340	14316	SNP	C	T	100.00%	70	0	-	0	70	0
?	JN542534USDA.seq	15089	15065	SNP	T	C	100.00%	75	0	75	0	-	0
?	JN542534USDA.seq	16273	16249	SNP	A	C	11.10%	27	-	3	0	0	0
?	JN542534USDA.seq	21130	21105	SNP	A	G	100.00%	50	-	0	50	0	0
?	JN542534USDA.seq	21779	21754	SNP	G	C	16.70%	24	0	4	-	0	0
?	JN542534USDA.seq	22902	22876	SNP	C	T	100.00%	61	0	-	0	61	0
?	JN542534USDA.seq	23250	23224	SNP	A	C	100.00%	41	-	41	0	0	0
?	JN542534USDA.seq	24781	24755	Indel	-	C	10.50%	19	0	2	0	0	17
?	JN542534USDA.seq	24782	24755	Indel	-	A	10.50%	19	2	0	0	0	17
?	JN542534USDA.seq	24791	24763	SNP	T	A	33.30%	15	5	0	0	-	0
?	JN542534USDA.seq	24793	24765	SNP	T	C	28.60%	14	0	4	0	-	0
?	JN542534USDA.seq	24817	24789	SNP	T	C	19.00%	21	0	4	0	-	0
?	JN542534USDA.seq	25477	25449	SNP	A	C	23.30%	43	-	10	0	0	0
?	JN542534USDA.seq	26307	26279	SNP	C	A	100.00%	67	67	-	0	0	0
?	JN542534USDA.seq	27064	27036	SNP	G	T	18.90%	37	1	0	-	6	0
?	JN542534USDA.seq	27123	27095	SNP	C	G	100.00%	83	0	-	83	0	0
?	JN542534USDA.seq	34033	34002	SNP	C	T	100.00%	57	0	-	0	57	0
?	JN542534USDA.seq	37937	37904	SNP	C	G	27.10%	48	0	-	13	0	0
?	JN542534USDA.seq	38942	38909	SNP	G	A	100.00%	64	64	0	-	0	0
?	JN542534USDA.seq	39381	39347	SNP	C	T	100.00%	66	0	-	0	66	0
?	JN542534USDA.seq	39524	39490	SNP	A	C	33.30%	48	-	16	0	0	0
?	JN542534USDA.seq	40537	40503	SNP	C	T	100.00%	70	0	-	0	70	0
?	JN542534USDA.seq	42198	42163	SNP	A	T	100.00%	84	-	0	0	84	0
?	JN542534USDA.seq	44155	44120	SNP	G	A	100.00%	53	53	0	-	0	0
?	JN542534USDA.seq	44528	44493	SNP	G	A	100.00%	60	60	0	-	0	0
?	JN542534USDA.seq	45082	45047	SNP	C	T	100.00%	57	0	-	0	57	0
?	JN542534USDA.seq	45192	45157	SNP	T	C	100.00%	52	0	52	0	-	0
?	JN542534USDA.seq	45550	45515	SNP	C	T	100.00%	52	0	-	0	52	0
?	JN542534USDA.seq	45592	45557	SNP	A	G	100.00%	65	-	0	65	0	0
?	JN542534USDA.seq	46237	46202	SNP	T	G	30.00%	50	0	0	15	-	0
?	JN542534USDA.seq	52536	52500	SNP	G	T	22.40%	49	0	0	-	7	4
?	JN542534USDA.seq	52580	52544	SNP	T	C	100.00%	52	0	52	0	-	0
?	JN542534USDA.seq	56218	56181	SNP	C	T	100.00%	58	0	-	0	58	0
?	JN542534USDA.seq	57810	57772	SNP	G	A	100.00%	61	61	0	-	0	0
?	JN542534USDA.seq	58702	58664	SNP	C	T	100.00%	65	0	-	0	65	0
?	JN542534USDA.seq	58877	58839	SNP	C	G	18.80%	32	0	-	6	0	0
?	JN542534USDA.seq	61465	61427	SNP	C	T	100.00%	52	0	-	0	52	0
?	JN542534USDA.seq	63057	63019	SNP	A	C	14.00%	57	-	8	0	0	0
?	JN542534USDA.seq	63568	63530	SNP	C	T	100.00%	54	0	-	0	54	0
?	JN542534USDA.seq	63853	63815	SNP	G	C	24.50%	49	0	12	-	0	0
?	JN542534USDA.seq	65992	65952	SNP	T	G	100.00%	56	0	0	56	-	0
?	JN542534USDA.seq	66793	66753	SNP	G	A	98.40%	63	62	0	-	0	0
?	JN542534USDA.seq	69754	69712	SNP	A	G	100.00%	56	-	0	56	0	0
?	JN542534USDA.seq	71323	71281	SNP	C	T	100.00%	67	0	-	0	67	0
?	JN542534USDA.seq	73618	73575	SNP	A	C	16.00%	75	-	12	0	0	0
?	JN542534USDA.seq	80306	80260	SNP	A	G	97.80%	46	-	0	45	0	0

?	JN542534USDA.seq	80359	80313	SNP	C	T	100.00%	69	0	-	0	69	0
?	JN542534USDA.seq	87424	87375	SNP	C	A	22.90%	48	11	-	0	0	0
?	JN542534USDA.seq	88484	88435	SNP	C	T	100.00%	80	0	-	0	80	0
?	JN542534USDA.seq	88756	88707	SNP	A	C	12.20%	49	-	6	0	0	0
?	JN542534USDA.seq	89622	89572	SNP	G	C	18.40%	38	0	7	-	0	0
?	JN542534USDA.seq	92076	92026	SNP	A	C	100.00%	52	-	52	0	0	0
?	JN542534USDA.seq	96243	96191	SNP	A	C	22.20%	45	-	10	0	0	0
?	JN542534USDA.seq	96448	96396	SNP	T	C	100.00%	56	0	56	0	-	0
?	JN542534USDA.seq	100373	100318	SNP	G	A	100.00%	69	69	0	-	0	0
?	JN542534USDA.seq	101603	101548	SNP	T	C	17.60%	74	0	13	0	-	0
?	JN542534USDA.seq	103943	103887	SNP	G	A	100.00%	37	37	0	-	0	0
?	JN542534USDA.seq	106190	106133	SNP	T	G	100.00%	36	0	0	36	-	0
?	JN542534USDA.seq	106324	106267	SNP	C	T	100.00%	35	0	-	0	35	0
?	JN542534USDA.seq	106999	106942	SNP	A	G	100.00%	62	-	0	62	0	0
?	JN542534USDA.seq	107106	107049	SNP	G	A	100.00%	62	62	0	-	0	0
?	JN542534USDA.seq	107482	107425	SNP	C	T	100.00%	39	0	-	0	39	0
?	JN542534USDA.seq	107734	107677	SNP	C	T	100.00%	24	0	-	0	24	0
?	JN542534USDA.seq	109670	109612	Indel	G	-	89.00%	73	0	0	-	0	65
?	JN542534USDA.seq	109694	109636	Indel	-	G	60.00%	15	0	0	9	0	6
?	JN542534USDA.seq	109905	109846	SNP	A	C	100.00%	29	-	29	0	0	0
?	JN542534USDA.seq	109990	109931	SNP	A	C	100.00%	27	-	27	0	0	0
?	JN542534USDA.seq	109992	109933	SNP	C	G	100.00%	23	0	-	23	0	0
?	JN542534USDA.seq	109993	109934	SNP	C	A	100.00%	23	23	-	0	0	0
?	JN542534USDA.seq	109994	109935	SNP	C	G	100.00%	21	0	-	21	0	0
?	JN542534USDA.seq	109996	109937	SNP	G	T	100.00%	18	0	0	-	18	0
?	JN542534USDA.seq	110188	110129	SNP	G	A	100.00%	42	42	0	-	0	0
?	JN542534USDA.seq	110220	110161	SNP	C	G	10.00%	20	0	-	2	0	0
?	JN542534USDA.seq	110231	110172	Indel	-	G	100.00%	14	0	0	14	0	0
?	JN542534USDA.seq	110258	110198	SNP	C	T	100.00%	28	0	-	0	28	0
?	JN542534USDA.seq	110260	110200	SNP	C	T	96.60%	29	0	-	0	28	0
?	JN542534USDA.seq	110291	110231	SNP	C	G	100.00%	42	0	-	42	0	0
?	JN542534USDA.seq	112161	112100	SNP	T	A	100.00%	46	46	0	0	-	0
?	JN542534USDA.seq	112453	112392	SNP	C	T	100.00%	31	0	-	0	31	0
?	JN542534USDA.seq	112484	112423	SNP	C	T	100.00%	21	0	-	0	21	0
?	JN542534USDA.seq	112488	112427	SNP	C	T	100.00%	21	0	-	0	21	0
?	JN542534USDA.seq	112519	112458	SNP	A	G	100.00%	9	-	0	9	0	0
?	JN542534USDA.seq	112543	112482	SNP	A	G	100.00%	26	-	0	26	0	0
?	JN542534USDA.seq	112595	112534	SNP	G	A	100.00%	28	28	0	-	0	0
?	JN542534USDA.seq	112668	112607	SNP	C	A	100.00%	39	39	-	0	0	0
?	JN542534USDA.seq	112899	112838	SNP	A	G	100.00%	45	-	0	45	0	0
?	JN542534USDA.seq	113078	113017	SNP	G	A	97.40%	38	37	0	-	0	0
?	JN542534USDA.seq	113122	113061	SNP	G	A	100.00%	50	50	0	-	0	0
?	JN542534USDA.seq	114223	114162	SNP	G	A	100.00%	18	18	0	-	0	0
?	JN542534USDA.seq	114243	114182	SNP	C	T	100.00%	29	0	-	0	29	0
?	JN542534USDA.seq	114298	114237	SNP	G	A	100.00%	24	24	0	-	0	0
?	JN542534USDA.seq	114698	114637	SNP	T	G	10.00%	40	0	0	4	-	0
?	JN542534USDA.seq	115884	115823	SNP	C	T	100.00%	66	0	-	0	66	0

?	JN542534USDA.seq	116044	115983	SNP	C	T	100.00%	42	0	-	0	42	0
?	JN542534USDA.seq	116207	116146	SNP	G	T	11.10%	9	0	0	-	1	0
?	JN542534USDA.seq	116210	116149	SNP	G	A	55.60%	9	5	0	-	0	0
?	JN542534USDA.seq	116212	116151	SNP	G	A	36.40%	11	4	0	-	0	0
?	JN542534USDA.seq	116535	116473	SNP	C	T	98.20%	55	0	-	0	54	0
?	JN542534USDA.seq	116540	116478	SNP	G	T	100.00%	49	0	0	-	49	0
?	JN542534USDA.seq	116612	116550	Indel	A	-	89.40%	47	-	0	0	0	42
?	JN542534USDA.seq	116652	116590	SNP	C	A	100.00%	20	19	-	1	0	0
?	JN542534USDA.seq	116663	116601	SNP	G	T	100.00%	38	0	0	-	38	0
?	JN542534USDA.seq	116664	116602	SNP	T	A	100.00%	38	38	0	0	-	0
?	JN542534USDA.seq	116713	116651	SNP	G	A	100.00%	54	54	0	-	0	0
?	JN542534USDA.seq	116732	116670	SNP	A	G	100.00%	38	-	0	38	0	0
?	JN542534USDA.seq	116752	116690	SNP	A	T	100.00%	44	-	0	0	44	0
?	JN542534USDA.seq	116766	116704	SNP	C	A	100.00%	76	76	-	0	0	0
?	JN542534USDA.seq	116779	116717	SNP	T	C	100.00%	99	0	99	0	-	0
?	JN542534USDA.seq	116792	116730	SNP	C	A	100.00%	103	103	-	0	0	0
?	JN542534USDA.seq	116854	116792	SNP	C	A	100.00%	49	49	-	0	0	0
?	JN542534USDA.seq	116886	116824	Indel	A	-	83.90%	31	-	0	0	0	26
?	JN542534USDA.seq	116887	116825	Indel	A	-	86.70%	30	-	0	0	0	26
?	JN542534USDA.seq	116888	116826	Indel	T	-	86.70%	30	0	0	0	-	26
?	JN542534USDA.seq	117090	117027	SNP	A	T	100.00%	35	-	0	0	35	0
?	JN542534USDA.seq	117148	117085	SNP	A	G	100.00%	20	-	0	20	0	0
?	JN542534USDA.seq	117385	117322	SNP	G	A	11.10%	9	1	0	-	0	0
?	JN542534USDA.seq	117857	117794	SNP	G	A	100.00%	1	1	0	-	0	0
?	JN542534USDA.seq	117947	117884	SNP	A	T	100.00%	28	-	0	0	28	0
?	JN542534USDA.seq	118005	117942	SNP	A	G	100.00%	18	-	0	18	0	0
?	JN542534USDA.seq	119483	119420	SNP	C	A	18.80%	32	6	-	0	0	0
?	JN542534USDA.seq	119613	119550	SNP	A	C	100.00%	47	-	47	0	0	0
?	JN542534USDA.seq	119831	119768	SNP	A	G	33.30%	12	-	0	4	0	0
?	JN542534USDA.seq	119864	119801	SNP	G	A	100.00%	10	10	0	-	0	0
?	JN542534USDA.seq	121038	120975	SNP	C	T	100.00%	8	0	-	0	8	0
?	JN542534USDA.seq	121051	120988	SNP	G	A	100.00%	1	1	0	-	0	0
?	JN542534USDA.seq	121072	121009	SNP	G	A	94.40%	18	17	0	-	0	0
?	JN542534USDA.seq	121073	121010	SNP	T	C	100.00%	21	0	21	0	-	0
?	JN542534USDA.seq	121103	121040	SNP	T	G	100.00%	44	0	1	43	-	0
?	JN542534USDA.seq	121766	121703	SNP	C	G	11.10%	9	0	-	1	0	0
?	JN542534USDA.seq	121862	121799	SNP	G	A	11.10%	9	1	0	-	0	0
?	JN542534USDA.seq	121875	121812	Indel	C	-	75.00%	8	0	-	0	0	6
?	JN542534USDA.seq	121876	121813	Indel	C	-	87.50%	8	0	-	0	0	7
?	JN542534USDA.seq	122221	122158	SNP	G	A	100.00%	24	24	0	-	0	0
?	JN542534USDA.seq	122903	122840	Indel	C	-	11.10%	9	0	-	0	0	1
?	JN542534USDA.seq	124001	123938	SNP	A	C	10.00%	20	-	2	0	0	0
?	JN542534USDA.seq	124249	124186	Indel	A	-	97.00%	33	-	0	0	0	32
?	JN542534USDA.seq	124921	124858	SNP	A	G	100.00%	64	-	0	64	0	0
?	JN542534USDA.seq	125270	125207	SNP	A	G	100.00%	51	-	0	51	0	0
?	JN542534USDA.seq	126175	126112	SNP	A	G	100.00%	36	-	0	36	0	0
?	JN542534USDA.seq	126449	126386	SNP	T	A	100.00%	65	64	1	0	-	0

?	JN542534USDA.seq	130100	130035	SNP	T	C	100.00%	47	0	47	0	-	0
?	JN542534USDA.seq	130494	130429	SNP	C	T	100.00%	40	0	-	0	40	0
?	JN542534USDA.seq	132090	132025	SNP	C	G	11.50%	26	0	-	3	0	0
?	JN542534USDA.seq	136232	136166	SNP	G	A	100.00%	43	43	0	-	0	0
?	JN542534USDA.seq	136825	136758	SNP	C	T	100.00%	41	0	-	0	41	0
?	JN542534USDA.seq	138970	138899	SNP	T	G	15.00%	40	0	1	5	-	0
?	JN542534USDA.seq	139263	139192	SNP	C	T	100.00%	31	1	-	0	30	0
?	JN542534USDA.seq	139598	139527	SNP	A	G	12.50%	8	-	0	1	0	0
?	JN542534USDA.seq	139600	139529	Indel	G	-	37.50%	8	0	0	-	0	3
?	JN542534USDA.seq	139601	139530	Indel	G	-	37.50%	8	0	0	-	0	3
?	JN542534USDA.seq	139608	139537	Indel	-	C	33.30%	9	0	3	0	0	6
?	JN542534USDA.seq	139614	139542	Indel	C	-	12.50%	8	0	-	0	0	1
?	JN542534USDA.seq	140382	140310	SNP	A	C	100.00%	27	-	27	0	0	0
?	JN542534USDA.seq	140394	140322	SNP	C	T	11.10%	18	0	-	0	2	0
?	JN542534USDA.seq	140412	140340	SNP	A	G	100.00%	4	-	0	4	0	0
?	JN542534USDA.seq	140413	140341	SNP	C	T	100.00%	4	0	-	0	4	0
?	JN542534USDA.seq	140434	140362	SNP	C	T	100.00%	12	0	-	0	12	0
?	JN542534USDA.seq	140447	140375	SNP	G	A	100.00%	31	31	0	-	0	0
?	JN542534USDA.seq	141621	141549	SNP	C	T	100.00%	9	0	-	0	9	0
?	JN542534USDA.seq	141622	141550	SNP	A	G	10.00%	10	-	0	1	0	0
?	JN542534USDA.seq	141650	141578	SNP	T	C	30.80%	13	0	4	0	-	0
?	JN542534USDA.seq	141872	141800	SNP	T	G	100.00%	46	0	0	46	-	0
?	JN542534USDA.seq	143482	143408	SNP	T	C	100.00%	28	0	28	0	-	0
?	JN542534USDA.seq	143540	143466	SNP	T	A	100.00%	29	29	0	0	-	0
?	JN542534USDA.seq	144118	144044	SNP	C	T	100.00%	2	0	-	0	2	0
?	JN542534USDA.seq	144134	144060	Indel	A	-	100.00%	2	-	0	0	0	2
?	JN542534USDA.seq	144135	144061	Indel	C	-	100.00%	2	0	-	0	0	2
?	JN542534USDA.seq	144140	144066	SNP	G	T	33.30%	3	0	0	-	1	0
?	JN542534USDA.seq	144142	144068	SNP	G	A	25.00%	4	1	0	-	0	0
?	JN542534USDA.seq	144149	144075	SNP	A	T	100.00%	4	-	0	0	4	0
?	JN542534USDA.seq	144176	144102	SNP	C	T	50.00%	4	0	-	0	2	0
?	JN542534USDA.seq	144187	144113	SNP	T	C	100.00%	1	0	1	0	-	0
?	JN542534USDA.seq	144339	144265	SNP	T	C	94.70%	19	0	18	0	-	0
?	JN542534USDA.seq	144397	144323	SNP	T	A	100.00%	25	25	0	0	-	0
?	JN542534USDA.seq	144568	144494	SNP	T	A	11.10%	36	4	0	0	-	0
?	JN542534USDA.seq	144572	144498	Indel	-	T	15.20%	33	0	0	0	5	28
?	JN542534USDA.seq	144597	144522	Indel	T	-	90.60%	32	0	0	0	-	29
?	JN542534USDA.seq	144598	144523	Indel	T	-	90.60%	32	0	0	0	-	29
?	JN542534USDA.seq	144599	144524	Indel	A	-	100.00%	30	-	0	0	0	30
?	JN542534USDA.seq	144633	144558	SNP	G	T	100.00%	54	0	0	-	54	0
?	JN542534USDA.seq	144695	144620	SNP	G	T	100.00%	23	0	1	-	22	0
?	JN542534USDA.seq	144708	144633	SNP	A	G	100.00%	9	-	0	9	0	0
?	JN542534USDA.seq	144721	144646	SNP	G	T	100.00%	20	0	0	-	20	0
?	JN542534USDA.seq	144735	144660	SNP	T	A	100.00%	39	39	0	0	-	0
?	JN542534USDA.seq	144755	144680	SNP	T	C	100.00%	53	0	53	0	-	0
?	JN542534USDA.seq	144774	144699	SNP	C	T	100.00%	55	0	-	0	55	0
?	JN542534USDA.seq	144823	144748	SNP	A	T	100.00%	17	-	0	0	17	0

?	JN542534USDA.seq	144824	144749	SNP	C	A	100.00%	11	11	-	0	0	0
?	JN542534USDA.seq	144835	144760	SNP	G	T	100.00%	21	0	0	-	21	0
?	JN542534USDA.seq	144870	144795	Indel	T	-	87.30%	63	0	0	0	-	55
?	JN542534USDA.seq	144947	144872	SNP	C	A	100.00%	59	59	-	0	0	0
?	JN542534USDA.seq	144952	144877	SNP	G	A	100.00%	60	60	0	-	0	0
?	JN542534USDA.seq	145274	145199	SNP	C	A	70.00%	10	7	-	0	0	0
?	JN542534USDA.seq	145275	145200	Indel	-	T	87.50%	8	0	0	0	7	1
?	JN542534USDA.seq	145277	145201	SNP	C	T	10.00%	10	0	-	0	1	0
?	JN542534USDA.seq	145443	145367	SNP	G	A	100.00%	35	35	0	-	0	0
?	JN542534USDA.seq	145600	145524	SNP	C	G	100.00%	10	0	-	10	0	0
?	JN542534USDA.seq	145603	145527	SNP	G	A	100.00%	10	10	0	-	0	0
?	JN542534USDA.seq	147189	147113	SNP	C	T	100.00%	30	0	-	0	30	0
?	JN542534USDA.seq	147244	147168	SNP	G	A	100.00%	13	13	0	-	0	0
?	JN542534USDA.seq	147264	147188	SNP	C	T	100.00%	22	0	-	0	22	0
?	JN542534USDA.seq	148365	148289	SNP	C	T	100.00%	47	0	-	0	47	0
?	JN542534USDA.seq	148409	148333	SNP	C	T	100.00%	52	0	-	0	52	0
?	JN542534USDA.seq	148588	148512	SNP	T	C	100.00%	37	0	37	0	-	0
?	JN542534USDA.seq	148819	148743	SNP	G	T	100.00%	37	1	0	-	36	0
?	JN542534USDA.seq	148892	148816	SNP	C	T	100.00%	32	0	-	0	32	0
?	JN542534USDA.seq	148944	148868	SNP	T	C	100.00%	42	0	42	0	-	0
?	JN542534USDA.seq	148968	148892	SNP	T	C	100.00%	34	0	34	0	-	0
?	JN542534USDA.seq	148999	148923	SNP	G	A	100.00%	16	16	0	-	0	0
?	JN542534USDA.seq	149003	148927	SNP	G	A	100.00%	17	17	0	-	0	0
?	JN542534USDA.seq	149034	148958	SNP	G	A	100.00%	24	24	0	-	0	0
?	JN542534USDA.seq	149326	149250	SNP	A	T	100.00%	42	-	0	0	42	0
?	JN542534USDA.seq	151196	151119	SNP	G	C	100.00%	39	0	39	-	0	0
?	JN542534USDA.seq	151227	151150	SNP	G	A	100.00%	25	25	0	-	0	0
?	JN542534USDA.seq	151229	151152	SNP	G	A	100.00%	25	25	0	-	0	0
?	JN542534USDA.seq	151266	151189	Indel	-	C	100.00%	16	0	16	0	0	0
?	JN542534USDA.seq	151267	151189	Indel	-	C	20.00%	15	0	3	0	0	12
?	JN542534USDA.seq	151300	151221	SNP	C	T	100.00%	48	0	-	1	47	0
?	JN542534USDA.seq	151490	151411	SNP	T	A	33.30%	3	1	0	0	-	0
?	JN542534USDA.seq	151492	151413	SNP	C	A	100.00%	1	1	-	0	0	0
?	JN542534USDA.seq	151496	151417	SNP	G	C	100.00%	1	0	1	-	0	0
?	JN542534USDA.seq	151498	151419	SNP	T	G	100.00%	1	0	0	1	-	0
?	JN542534USDA.seq	151583	151504	SNP	T	G	100.00%	31	0	0	31	-	0
?	JN542534USDA.seq	151802	151723	Indel	-	C	66.70%	9	0	6	0	0	3
?	JN542534USDA.seq	151811	151731	Indel	C	-	72.70%	11	0	-	0	0	8
?	JN542534USDA.seq	151813	151733	SNP	C	A	10.00%	10	1	-	0	0	0
?	JN542534USDA.seq	151830	151750	SNP	C	T	12.50%	8	0	-	0	1	0