DETECTING PRESENCE OR ABSENCE OF ALTERNATIVE SPLICING AT THE SUPERKDR LOCUS IN HORN FLY, *HAEMATOBIA IRRITANS*

by

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A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biology
December 2017

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ACKNOWLEDGEMENTS

This research was partially supported by the Graduate College Thesis Research Support fellowship, Texas State University and the United States Department of Agriculture, Agricultural Research Services.

I would like to thank Dr. Adalberto Perez de Leon, Dr. Felix Guerrero and his lab, Kylie Bendele, Deanna Bodine, and Dr. Luisa Domingues, for accepting me into their lab and providing insight on the methodology used in this research.

I would like to thank my colleagues from Texas State University who provided support and guided me as my time as a student. I would like to thank each of my committee members Dr. Ivan Castro-Arellano, Dr. David Rodriguez, and Dr. Dana Garcia for their support and guidance in my academic career as well as their contributions during the writing process.

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LIST OF ABBREVIATIONS

Abbreviation	Description
kdr	knockdown resistance
superkdr	super knockdown resistance
US	United States
CDC	Center for Disease Control
USDA	United States Department of Agriculture
ARS	Agriculture Research Service
DDT	dichlorodiphenyltrichloroethane
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
RNA	ribonucleic acid
SNPs	single nucleotide polymorphism
bp	base pair

ABSTRACT

Changes to pest control efforts are dependent on knowing the interaction that occurs between insecticides and the pest in question. Horn flies (*Haematobia irritans)* are agricultural pests that have had negative economic consequences due to their detrimental effects on cattle and their development of insecticide resistance. The *superkdr* locus has a single base change, from a thymine to a cytosine, distinguishing it from the *kdr* gene and resulting in replacement of methionine with threonine; this mutation has been associated with pyrethroid-resistant horn flies. In houseflies (*Musca domestica*), the *superkdr* locus was discovered to occur on mutually exclusive exons, exon C and exon D. Exon C was found in place of exon D in the cDNA from an adult horn fly head, and encodes a truncated, and presumably non-functional, sodium channel α subunit isoform. The purpose of this research is to determine if alternative splicing is occurring at the *superkdr* locus of horn flies. Two different methods were performed to determine the presence or absence of alternative splicing. The first method involved sequence analysis of the sodium channel gene containing the *superkdr* locus. cDNA and genomic DNA were cloned, sequenced, and compared using MacVector. The second method used a SNP assay to perform genotyping via real-time PCR. With the SNP assay, one can genotype the *superkdr* locus in cDNA and genomic DNA, and simultaneously detect alternative splicing if the locus was not detected in cDNA. I determined that there is no significant

difference between the genotype of the *superkdr* locus in cDNA compared to genomic DNA. If alternative splicing of the *superkdr* is occurring, it seems to be rare. Overall, this shows genomic DNA can be used to determine the prevalence of pyrethroid-resistance population. The SNP assay used to genotype is both time and cost-effective, and it can be used to determine if pyrethroid is the best choice of insecticide to use depending on the prevalence of pyrethroid-resistance in a wild population.

I. INTRODUCTION

1. Insecticide Resistance

The development of resistance to pesticides in insects is a major concern and threat to the United States (US). According to the Center for Disease Control and Prevention (CDC), insecticide resistance occurs when there is loss or reduction in the ability of the insecticide to kill the insect (Insecticide Resistance 2017). Insects such as houseflies (*Musca domestica),* fruit flies (*Drosophila melanogaster*), horn flies (*Haematobia irritans)*, mosquitoes (*Anopheles*, *Abraedes,* etc.) etc., are agricultural pests that may serve as vectors for both human and animal diseases. The use of insecticides has been the most common way of killing and managing these pests, but due to the development of insecticide resistance, it has become difficult to manage them (Barros et al. 2001). Some insecticides are effective in killing agricultural pests but are dangerous to livestock. There are safety concerns in regard to using insecticide directly applied to cattle due to toxicity to the animal and the presence of pesticide residues in the meat and milk (Bruce 1964). A decrease in insecticide production is associated with the high costs of research, development, and the time-length effectiveness of the insecticide (Feyereisen 1995). The effectiveness of most insecticides is about 3-4 years. The interaction between the insecticide and its target site (a location which insecticide binds or affects) is important to understand to find ways to prevent or slow down the process of resistance in insects as well as creating future management plans.

Insecticide resistance can result from an increase in the insect's ability to detoxify the insecticide or to modify the target site. There are three mechanisms by which insecticide resistance occurs: biochemical, physiological, and behavioral adaptation

(Sparks et al. 1985). Reduced penetration, increased metabolism, active site insensitivity, and increased excretion are biochemical and physiological factors that could lead to resistance. Resistance due to a behavioral mechanism occurs when the insect avoids contact with the insecticides by changing its behavior. An example of a behavioral mechanism is when flies move to an area of the body of the animal they are pestering and avoid the area on which the insecticide was sprayed (Oyarzun et al. 2008). An increase in an insect's natural detoxifying enzymes (multifunction oxidase, esterase and glutathione S-transferases, etc.) can block and hydrolyze the insecticide prior to reaching its target site (Li et al. 2016). Insecticides made using neurotoxins such as pyrethroid, DDT, organophosphates, and cyclodienes target different molecules in the insect's nervous system. Target-site resistance occurs when a modification to the insecticide-binding site due to alteration of amino acids cause the insecticide to be less effective (Oyarzun et al. 2008; Brogdon and McAllister 1998).

2. Voltage-Gated Sodium Channel and Target Site Insensitivity in Insects

Voltage-gated sodium channels are transmembrane proteins responsible for electrical signaling in excitable cells. Voltage-gated sodium channels play a critical role in generating action potentials in a cell. During the activation phase of an action potential, sodium channels open, allowing sodium ions to enter the cell, resulting in membrane depolarization (Davies et al. 2007). Owing to the intense study of sodium channel from diverse organisms including the electric eel, fruit fly, mammals, squid, and others, scientists have gained a greater understanding of the sodium channel structure (see Williamson et al. 1996). In the late 1980s, *DSC1* and *para* were isolated from *Drosophila melanogaster* and were thought to be sodium channel genes due to their resemblance to

mammalian sodium channel (see Dong 2007). The sodium channel of insects is similar to the α subunit of the mammalian sodium channel, having four homologous domains (domains I- IV) and each having six transmembrane segments designated S1-S6 as shown in figure 1 (Martins and Valle 2012). Insects have a single sodium channel gene (Dong 2007). The sodium channel is the target site for different types of neurotoxins such as dichlorodiphenyltrichloroethane (DDT) and pyrethroids (Catterall 1995; Dong 2007). These neurotoxins bind to the sodium channel and alter ion conductance, ion selectivity, and/or channel gating.

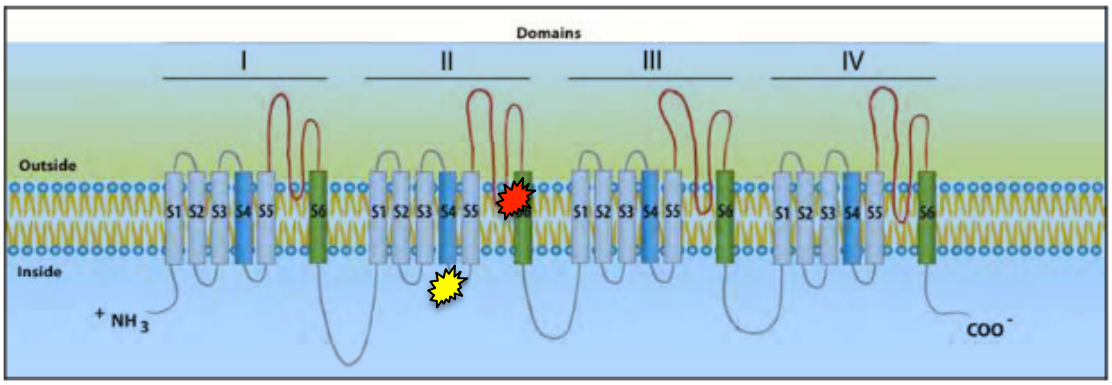


Figure 1. The Voltage-Gated Sodium Channel. The insect sodium channel contains four homologous domains (domains I- IV), each having six transmembrane segments designated S1-S6. The kdr (red) mutation occurs on IIS6 and the superkdr (yellow) mutation is located on the IIS4-S5 linkage. Figure adapted from Martins and Valle (2012).

3. The Horn Fly

The horn fly, *Haematobia irritans,* is an agricultural pest that was introduced into North America from Europe in the late 1800s (Domingues et al. 2013). Since then it has spread widely and can be found throughout the United States, Europe, North Africa, Asia Minor, and in South America, including Brazil (Foil and Hogsette 1994). The horn fly is a blood-feeding pest responsible for economic losses to the livestock industry. A typical adult horn fly is 3-5 mm in length and is gray in color. A distinct feature of horn flies is the pointed proboscis that is used to pierce the thick skin of bovines. The horn fly diet and life cycle are dependent on cattle; therefore, horn flies will remain on the cattle all day and night. Female horn flies leave the cattle only to deposit eggs on fresh cow droppings. Horn flies can also be a vector for disease-causing agents like *Stephanofilaria stilesi,* a parasitic worm that uses the horn fly as an intermediate host (Hibler 1966). Horn flies also produce sores on cattle due to feeding, and the sores can make the cattle susceptible to secondary bacterial infections from *Staphylococcus aureus* (Mohammed et al. 2016; Anderson et al. 2011).

The control and management of horn fly are of economic importance due to the drastic effect it can have on life stock. Horn flies feed on cattle multiple times per day, increasing stress on cattle, often resulting in a reduction in cattle feeding behavior, weight gain, and milk production (Sheppard and Hinkle 1985). In the US, the estimated annual loss due to horn flies is $1 billion Dollars (Oyarzun et al. 2008). In the US, horn fly control efforts are dependent on direct application of insecticides like pyrethroids. Pyrethroids are synthetic compounds derived from pyrethrum in *Chrysanthemum* flowers (Davies et al. 2007). Pyrethroid insecticides are commonly used in the agricultural industry because of the high insecticidal activity, low toxicity to mammals, and low

persistence in the environment (Scott et al. 2013). Pyrethroids were first introduced for insect control in the US in 1977. Resistance in *H. irritans* to the pyrethroid-impregnated cattle ear tags occurred after two to three years of usage (Sparks et al. 1985), and overall resistance to pyrethroid insecticide started in the 1980s (Li et al. 2009).

Pyrethroids are neurotoxins that target the sodium channel in nerve cell membranes, altering channel-gating function. Pyrethroid disrupts the normal channel, causing the channel to remain in the activation state and inhibiting deactivation and inactivation of the sodium channel (Dong et al. 2004). Thus, the sodium channel remains open, causing paralysis in the horn fly eventually leading to death (Jamroz et al. 1998). Even if the cattle owners rotate insecticide every year, the pyrethroid resistance genes would not be eliminated, because they are inherited as a complete recessive trait and remain fixed in the horn fly population (Li et al. 2009; Daives et al. 2007). Rotating insecticides is performed to slow down resistance, but depending on the resistance mechanism that arises, it can lead to cross resistance with other insecticides.

4. kdr and *superkdr*

Knockdown resistance (kdr) was first identified in domain II segment 6 of the housefly *(Musca domestica*). The *kdr* allele has a single amino acid change from leucine to phenylalanine at amino acid 1014 and is referred as L1014F (Soderlund et al. 2003). The *superkdr* has only been found in the presence of the *kdr* mutation in *M. domestica* (Williamson et al. 1996; Haddi 2012) and *H. irritans* (Guerrero et al. 1997). The *superkdr* allele has a single amino acid change from methionine to threonine at the 918th amino acid of the sodium channel. The codon for methionine is ATG, and changing the T nucleotide to a C causes a single amino acid change to threonine. Herein, I will refer the

presence of a T on the allele as a susceptible allele and a C as a resistant allele. In both the housefly and horn fly, the presence of the *superkdr* mutation was seen in higher ratios in populations with a high resistance to pyrethroids (Soderlund and Knipple 2003).

II. DIAGNOSTIC PCR

1. Introduction

The management of horn fly populations involves monitoring and studying the development of insecticide resistance, then creating control strategies. The monitoring process occurs by determining which insecticide a population is resistant to by performing a bioassay with the insecticide in question (Li et al. 2009). Polymerase chain reaction (PCR) is used to investigate the mutations that have led to the resistance of insecticides. Using a combination of bioassay and PCR has allowed for a better understanding of insecticide resistance. Guerrero et al. (1997) and Jamroz et al. (1998) developed a PCR-based assay to detect the presence of *kdr* and *superkdr* in a single reaction. The PCR-based assay is critical for resistance monitoring and management of pyrethroid-resistance in horn fly populations. This diagnostic PCR assay is an allele-specific PCR designed to detect point mutations using multiple DNA primers (Foil et al. 2005, Guerrero et al. 2002). The PCR is designed to detect the presence or absence of the *kdr* and *superkdr* on both the susceptible and resistant alleles using the genomic DNA of horn flies (Guerrero et al. 1997). There are three genotypes: homozygous susceptible (SS), the homozygous resistant genotype (RR), and heterozygous (SR). Li et al. (2009) performed a bioassay using pyrethroid to determine the relationship between the genotype and phenotype. The comparison of genotype showed RR to be the most resistant and SR to be slightly resistant compared to SS (li et al. 2009). To evaluate the diagnostic PCR assay, I tested horn flies collected between August and November to detect the *kdr* and *superkdr* sodium channel gene mutations and determine the prevalence of *kdr* and *superkdr* susceptible and resistant alleles.

2. Methods

Sex Determination

Dr. Lane D. Foil collected horn flies from bulls in 2008 from Winnsboro, Louisiana, Louisiana State University Research Farm on August 29, September 5, September 12, October 24, and November 24, 2008. Horn flies were preserved in 100% ethanol. Individual horn flies were placed in 1.5 ml microcentrifuge tubes labeled with the corresponding collection date and fly number (males were placed in tubes 1-25 and females placed in 26-50). Before placing the flies in the 1.5 ml tubes, excess ethanol was removed from each horn fly by placing them on Kimwipes. The microcentrifuge tubes were then placed in freezer boxes labeled with the collection date and stored at -80°C. The total horn fly count for September 12 was 48 and only 23 female horn flies were collected. The overall total was 248 horn flies.

DNA Isolation

Genomic DNA was isolated from individual frozen flies following the protocol developed by Guerrero et al. (2002). Disposable pellet pestles and the 1.5 ml tubes with horn flies were placed in an ice bucket with dry ice. The horn flies were pulverized by grinding with the disposable pellet pestle for 10-15 seconds and then returned to dry ice. Next, 25 µl of DNA isolation buffer was added to each tube. DNA isolation buffer comprised 1667 µl of 3 M KCl, 600 µl of 1 M Tris-Cl (pH 8.5), 400 µl of 1 M Tris-Cl (pH 8.0), 7333 µl of water. The flies were homogenized one more time for 10-15 seconds. Afterwards, the tubes were closed and placed in a float and set in a boiling water bath for 5 minutes. A weight was placed over the tubes to prevent caps from opening and

to keep them in the boiling water. The samples were returned to the freezer boxes and stored at -20°C.

1:10 DNA Dilution

In a PCR clean area, 9 µl of PCR quality water was added to 0.5 ml microcentrifuge tubes that had been previously labeled with the collection date and fly number. The collection dates 8/29, 9/5, 9/12, 10/24, and 11/24 were represented as 1, 2, 3, 4, and 5, respectively. The 1.5 ml microcentrifuge tubes containing the horn flies were thawed on ice and centrifuged at 16,000 x g at 4°C for 5 minutes. Then, 1 µl of the supernatant was transferred to the corresponding 0.5 ml microcentrifuge tubes containing 9 µl of water to make a 1 to 10 DNA dilution. The 0.5 ml tubes containing the diluted DNA and the 1.5 ml tubes containing the individual, homogenized horn flies were stored in an -80°C freezer.

PCR Procedures

The methodology for the multiplex PCR was performed in a similar manner used in Domingues et al. (2014) and Guerrero et al. (2002; 1997). To help prevent contamination, each PCR reagent (H_2O, MgCl, PCR Buffer II, and dNTP) was aliquoted from a stock solution into five different tubes in the PCR clean area. PCR primers were resuspended in PCR quality water to a concentration of 100 µM and incubated at 55°C for 15 minutes. A 0.5 ml tube was labeled with Taq+ start and the date the mix was made. A 1 to 1 (vol: vol) mix of AmpliTaq DNA Polymerase (Perkin-Elmer, Foster City, CA) and TaqStart Antibody (Clonetech, Palo Alto, CA) was made by adding equal volumes of each to the pre-labeled 0.5 ml tube, which was mixed by inversion and then spun down using a picofuge. This process was done gently to prevent the creation of air bubbles. The

tube containing the Taq+ start mix was left at room temperature for five minutes to activate and then set on ice or back in the freezer at -20°C until needed. All reagents used in the PCR assay were stored in the freezer at -20°C until needed.

PCR amplifications were performed in 0.5 ml thin-walled (Bio-Rad) microcentrifuge tubes using 20 µl reactions with 13.6 µl of PCR quality H_2O, 1.6 µl of 25 mM $MgCl_2$, 2.0 µl of 10X PCR Buffer II, 0.4 µl of 2.5 mM dNTP mix, 0.2 µl of 1:1 vol: vol mix of AmpliTaq DNA Polymerase and TaqStart Antibody. Each horn fly sample required two amplification reactions, the susceptible reaction and the resistant reaction. The susceptible reaction (premix S) used 0.2 µl of each of the following primers: FG-130, FG-138, FG-154, FG-235, FG-234, and FG-243 (Table 1). The resistant reaction (premix R) used 0.2 µl of each of the following primers: FG-134, FG-138, FG-155, FG-235, FG-234, and FG-243 (Table 1). Amplification was performed using a DNA Engine (Bio-Rad Laboratories, Berkeley, CA) under the following thermal parameters: denaturation for 2 minutes at 95°C, followed by denaturation for 1 minutes at 94°C, annealing for 1 minute at 61°C, and extension for 1 minute at 72°C. Then the cycle returns to the denaturation step for 1 minute at 94°C, 9 times. The program continues with a denaturation for 1 minute at 92°C, annealing for 1 minute at 61°C, and extension for 1 minute at 72°C. The program returns to the denaturation step for 1 minute at 92°C, 25 times. After a final extension at 72°C for 7 minutes, the cycle remains at 8°C until PCR product is removed from thermocycler and stored at -20°C.

Table 1. Horn Fly Diagnostic PCR Primers. Primer sequences used in the Diagnostic PCR are shown including a brief description of the primer.

ID	Sequence	Primer Description
Kdr primer set (285 bp PCR Product)		
FG-130	5'-TAC-TGT-TGT-CAT-CGG-CAA-TC-3'	Susceptible, forward
FG-134	5'-TAC-TGT-TGT-CAT-CGG-CAA-TT-3'	Resistant, forward
FG-138	5'-CAA-TAT-TAC-GTT-TCA-CCC-AG-3'	Susceptible/Resistant, reverse
Superkdr primer set (74 bp PCR product)		
FG-154	5'-ACC-CAT-TGT-CCG-GCC-CA-3'	Susceptible, forward
FG-155	5'-ACC-CAT-TGT-CCG-GCC-CG-3'	Resistant, forward
FG-235	5'-CTT-CGT-GTA-TTC-AAA-TTG-GCA-3'	Susceptible/Resistant, reverse
GAPDH Control primer set: (154 bp PCR Product)		
FG-234	5'-CTT-CTT-CAT-CGG-TGT-AGC-3'	Forward
FG-243	5'-GGC-ATG-GCT-TTC-CGT-GTC-C-3'	Reverse

PCR Product Analysis

First, 2 µl of 10X gel dye was added to the 0.5 ml tubes containing the PCR product and vortexed. Electrophoresis of the PCR products was performed using 4% NuSieve gel (Reliant™ Gel System, Lonza, Rockland, ME) with TBE. The standard ladder consisted of 4 µl of ΦX 174 HaeIII DNA size markers (25 ng/µl). For gel analysis, 5 µl of each PCR products were separated using 4% NuSieve gel (Reliant™ Gel System, Lonza, Rockland, ME) at approximately 235 volts for 30-40 minutes. The gel was then stained for 30 minutes in Gel Star staining dye (Lonza) while slowly shaking. After staining, gels were rinsed in deionized water, visualized under a UV light box, and photographed with the GelDoc™ EZ Imager (Bio-Rad).

Statistical Analysis

Allelic frequency of *kdr* and *superkdr* was calculated by the following formula:

$$Allelic\ frequency = (\frac{(SR)}{2} + RR)\times 100$$

SR refers the number of individual horn flies that were identified to have a heterozygous genotype and RR refer to the individuals identified as homozygous resistant. A Fisher's exact test two-sided was used to determine if *superkdr-kdr* genotype differs between males and female using R Studio (R Core Team 2015).

3. Results

Genotype of horn flies

From the 248 horn flies, only 210 horn flies were successfully genotyped for both the *kdr* and *superkdr*. The interpretation of PCR assay using gel analysis is based on observing the presence or absence of amplified fragment bands for each mutation. For the *superkdr* locus, the susceptible allele contains a T, and the resistant allele contains a C. If the amplification of the 285 bp *kdr* diagnostic product is detected in only the S reaction it indicates a *kdr* susceptible homozygote (TT). A 285 bp product in only the R reaction indicates a *kdr* resistant homozygote (CC), and a 285 bp product in both S and R indicates *kdr* heterozygotes (TC). Detection of the *superkdr* susceptible and resistant alleles is similar to the detection of the *kdr* alleles. An individual is diagnosed as homozygous susceptible, homozygous resistant, and heterozygous by detectable amplification of the 72 bp diagnostic product in only the S reaction, only the R reaction, or both the S and R reactions, respectively. The 154 bp glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene fragment was used as a positive control to verify that genomic DNA concentration was consistent among samples. For example, in figure 2, individual (4-39) the presence of bands (285 bp *kdr* and 72 bp *superkdr*) in the S and R reaction indicates that the fly is heterozygous with susceptible and resistant alleles at each mutation site. The individual (4-41) is a *kdr* resistant homozygote (285 bp fragment in R

only) and a *superkdr* susceptible homozygote (72 bp fragment in S only). Individual 4-57 is a *kdr* resistant homozygote (285 bp fragment in R only) and a *superkdr* heterozygote (72 bp fragments in S and R). Individuals, for which the GAPDH band was absent or faint, were omitted from the study, for example, individual 4-51. The genotypes SR-SS, RR-SS, and RR-SR (*superkdr-kdr*) were not found (Table 2). Like in previous studies Guerrero et al. (2002) a resistant *superkdr* was not found in the absence of a resistant *kdr*. This means if a horn fly genotype for *kdr is* SS it must be SS for the *superkdr* genotype.

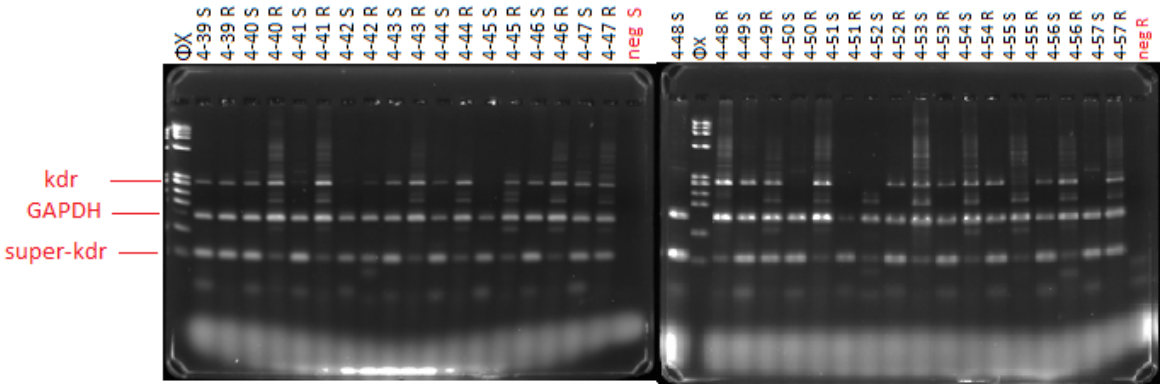


Figure 2. Gel Analysis of PCR Product. Gel analysis showing the results observed for population 4, samples 39-57. Each sample consists of one reaction to detect susceptible alleles (denoted S) and one to detect resistant alleles (denoted R). The negative controls show S and R reactions with water substituted for genomic DNA template. The molecular weight standard, ΦX 174 HaeIII DNA is shown in the far left lane denoted ΦX.

Table 2. The *superkdr – kdr* genotype of the studied horn flies.

Population	Sex	N	super-kdr			kdr			Genotype[a]					
			SS	SR	RR	SS	SR	RR	SS-SS	SS-SR	SS-RR	SR-SR	SR-RR	RR-RR
8/29	M	22	20	2	0	14	7	1	14	6	1	1	0	0
	F	22	18	3	0	13	6	2	13	4	1	2	1	0
	Total	44												
9/5	M	19	17	2	0	9	5	5	9	3	5	2	0	0
	F	19	14	4	1	10	4	5	10	1	3	3	1	1
	Total	38												
9/12	M	21	19	1	1	13	7	1	13	6	0	1	0	1
	F	14	12	2	0	5	3	6	5	3	4	0	2	0
	Total	35												
10/24	M	23	19	3	1	10	9	4	10	6	3	3	0	1
	F	22	17	5	0	4	13	5	4	8	5	5	0	0
	Total	45												
11/24	M	23	20	3	0	7	13	3	7	11	2	2	1	0
	F	25	21	3	1	4	14	7	5	12	4	1	2	1
	Total	48												

[a] S denotes a susceptible allele, R denotes a resistant allele with super-kdr genotype indicated on the left of the hyphen and kdr genotype on the right.

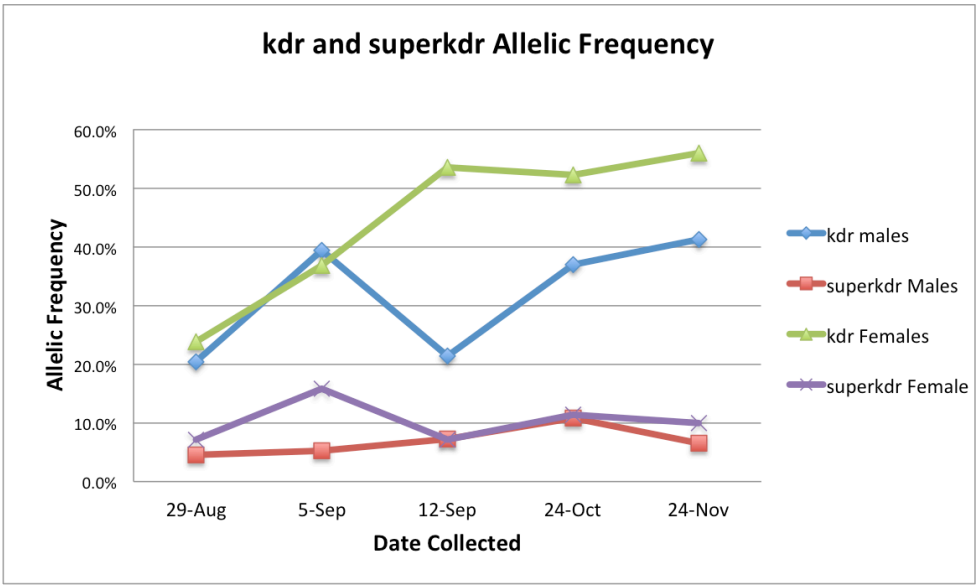


Figure 3. *kdr* and *superkdr* allelic frequency. The *kdr* and *superkdr* allelic frequency in males and females from horn flies collected.

Among all horn flies, both females and male had a higher allelic frequency of *kdr* compared to *superkdr.* The horn flies from August 29 had the lowest *kdr* allelic

frequency for both females and males, 23.8% and 20.5%, respectively. November 24 had the highest *kdr* allelic frequency for female and males, 56.0% and 41.3%. The range for the *kdr* allelic frequency in female ranged from 23.8-56.0%, and in males 20.5- 41.3%. The *superkdr* allelic frequency in female ranged from 7.2-15.8%, and in males 4.6-10.8%. The *superkdr –kdr* genotype were pooled by sex and compared used a Fisher's exact test two-sided, p-value = 0.1519.

4. Discussion

In this study, we used the multiplex PCR to detect the presence or absence of the *kdr* and *superkdr* mutations associated with pyrethroid resistance in horn flies. The results showed that there is higher *kdr* allelic frequency then *superkdr* allelic frequency in each of the population. Since the *superkdr* mutation has never found in the absence of a resistance *kdr* mutation, it is expected that the there would be a higher *kdr* frequency (Guerrero et al. 2002). The *superkdr* allelic frequency was higher in females compared to the males from September 5. Female horn flies have a tendency to rest on the dorsal surface of the cattle, while male horn flies prefer the abdomen area of the cattle (Bruce 1964). Since insecticide resistance is due to a combination of resistance mutations, it is thought that females are more resistant compared to males due to insecticide pressure. There has only been one article in which there has been a significant difference between *kdr* frequency of females and males (Oyarzun et al. 2011). There was no difference between *super-kdr* genotype in males to females.

The purpose of the diagnostic PCR was to determine the prevalence of *kdr* and *superkdr* in pyrethroid resistant populations. Domingues et al. (2014) created a multiplex polymerase chain reaction to detect pyrethroid, organophosphate, and cyclodienes target

site resistance simultaneously. An increased knowledge of molecular biology of insecticide and the cause of insecticide resistance will lead to better horn fly management.

III. DETECTING ALTERNATIVE SPLICING

1. Introduction

Alternative splicing is a regulatory mechanism in which variation of including or excluding exons, or coding regions, into mRNA leads to a single gene coding for multiple proteins. Alternative mRNA splicing can lead to structural and functional diversity of ion channels in the nervous system (Lee et al. 2002). Dong (2007) showed functional diversity of insect sodium channels is due to alternative splicing and mRNA editing of a single gene transcript. Recent studies of alternative exon usage in the housefly *Vssc1* sodium channel gene helped identify a region of the sodium channel protein that contains mutually exclusive exons (Williamson et al. 1996; Lee et al. 2002; Soderlund and Knipple 2003). In common houseflies (*Musca domestica*), the *superkdr* mutation occurs at a location that can be removed from the expressed transcript by alternative splicing (Lee et al., 2002). I hypothesize that alternative splicing is impacting the *superkdr* expression in horn flies, since the current method to determine the prevalence of *superkdr* mutation in wild horn fly populations is performed with genomic DNA. If alternative splicing is occurring, then using genomic DNA for *superkdr* detection is overestimating *superkdr* prevalence.

Single nucleotide polymorphisms (SNPs) are a genetic variation due to a single nucleotide difference (Park et al. 2017). The *superkdr* mutation is a single nucleotide mutation that results in a different amino acid being produced. Another method of genotyping the *superkdr* locus other than using the diagnostic PCR would be by performing an allele specific SNPs Assay via real-time PCR. In order to determine the

presence or absence of alternative splicing at the *superkdr* locus, the cDNA and genomic DNA of individual fly must be compared. Two different methods were performed to determine the presence or absence of alternative splicing. The first method involved sequence analysis of the sodium channel gene containing the *superkdr* locus. cDNA and genomic DNA were cloned, sequenced, and compared using MacVector. The second method involves performing a SNP assays to genotype the *superkdr* locus in cDNA and genomic DNA, and simultaneously detect alternative splicing if the locus was not detected in cDNA.

2. Methods

First Method

Collection of cDNA and Genomic DNA

To determine if alternative splicing was occurring at the *superkdr* locus, cDNA and genomic DNA of individual horn flies previously extracted and stored at the USDA ARS Knipling-Bushland U.S. Livestock Insects Research Laboratory was sequenced and compared. Genomic DNA was extracted and purified from the head of adult horn flies. RNA was isolated from the body of adult horn flies using ToTALLY RNA Kit (Ambion) and treated with DNase (TURBO DNA-free™ kit) to remove any residual DNA. cDNA synthesis was performed by reverse transcriptase using AMV First Strand cDNA Synthesis Kit (New England Biolabs Inc.). Dr. Luisa Domingues extracted genomic DNA from the head, and synthesized cDNA from the body of individual horn flies in December 2013. The horn flies used for sequence comparison are not related to the population used for the diagnostic PCR. The horn flies used for the sequence analysis

were from three different populations (Pullman, Georgia, or the Super Resistant population) corresponding to the location they were collected from. Pullman horn flies were collected from a ranch in Pullman Washington, US collected August 22, 2006. Georgia Saber's flies were collected from a Georgia ranch population on September 26, 1997 by Dr. Craig Shephard. Flies were collected alive, shipped on dry ice, and stored at -80°C. The Super Resistant population was from a colony established in USDA-ARS Kerrville Laboratory that received weekly treatment of permetrhrins and was tested using bioassays (Guerrero et al. 1997).

PCR Procedure

A standard PCR amplification was performed using 25 µl reaction with 12.85 µl of H_2O, 5.0 µl of 5X Q5 reaction buffer (New England BioLabs Inc., Ipswich, MA), 0.5 µl of 10 mM dNTP, 0.2 µl of 100 µM forward primer (Appendix I), 0.2 µl of 100 µM reverse primer (Appendix I), 0.25 µl of Q5®Hot Start High-Fidelity DNA polymerase (New England Biolabs Inc.), and 5.0 µl of 5X Q5 High GC Enhancer. Reaction products were analyzed using gel electrophoresis to determine the presence of the *superkdr.* Then a nested PCR was performed from the product of the previous PCR.

cDNA primers for the first reaction used are FG471 and FG474 with program HFSDR on the DNA Engine. Program HFSDR comprised denaturation for 3 minutes at 98°C, followed by 30 cycles of denaturation for 10 seconds at 98°C, annealing for 30 seconds at 61°C, and extension for 5 minutes at 72°C. Then the program continues with a final extension at 72°C for 2 minutes and remains at 4°C until PCR product is removed from thermocycler and stored at -20°C. The nested PCR uses primers FG459 and FG462 and 1.0 µl of PCR product 1 with the thermocycler program HFSKDR63. Program

HFSKDR63 is similar to program HFSDR except the annealing is for 30 seconds at 65°C.

The genomic DNA PCR primers for the first reaction used are FG459 and FG462 with program HFSKDR63 on the DNA Engine. The nested PCR uses primers FG472 and FG462 and 1.0 µL of PCR product 1 with the thermocycler program HFSKDR63. Between each PCR, gel electrophoresis is performed to examine the presence of the *superkdr* band.

Gel Extraction and Purification

The protocol from the QIAquick gel extraction kit (Qiagen, Hilden, Germany) was performed to extract the DNA and cDNA. The band fragment was cut from the agarose gel using a clean sharp razor blade. The gel slice was then weighed and placed in colorless tubes. A 3:1 volume of Buffer QG to 1 volume of gel was added. The gel slices were then incubated for 10 minutes at 50°C or until it had dissolved. Once the gel slice dissolved, isopropanol was added to the sample a 1:1 volume of isopropanol to gel slice weight. The sample was added to the QIAquick spin column and centrifuged for 1 minute at 16,000 x g. The flow-through collected in the collection tube was thrown away, and the procedure was repeated until the entire sample had gone through the spin column. Then 0.5 ml of buffer QG was added and centrifuged for 1 minute. The washing step involved the addition of 0.75 ml of buffer PE to the QIAquick column and incubation for 5 minutes prior to centrifugation for 1 minute. The flow-through was then discarded, and the QIAquick column was centrifuged for an additional minute at maximum speed. The spin column was placed in a clean 1.5 ml microcentrifuge tube, and 30 µl of elution buffer, buffer EB, was added. The column was allowed to stand for 4 minutes and then

centrifuged for 1 minute. The quality and quantity of the DNA were checked using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) prior to moving on to the addition of 3' A overhangs.

Addition of 3' A Overhangs

To add 3' overhangs a master mix with a final concentration of 0.2 mM dNTP, 2 mM MgCl, PCR Buffer II, Taq polymerase (1 unit/µl) in water was created in relation to the DNA yield in each microcentrifuge tube. The DNA yield was calculated by multiplying the DNA concentration (µg/ µl) with the estimated sample volume. The amount of master mix added to the sample was dependent on the DNA yield calculated. The samples were gently mixed and centrifuged prior to incubation at 72°C for 10 minutes. The purification was performed after the addition of 3' overhangs using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) using a microcentrifuge.

A 5:1 volume of buffer PB was added depending on the total DNA volume. A QIAquick spin column was placed in a 2.0 ml collection tube. The sample was added to the QIAquick column and centrifuged for 1 minute at 13,000 rpm for the binding step of the DNA. The flow-through was discarded and the spin column was returned to the collection tube. For the washing procedure, 750 µL of Buffer PE is added to the QIAquick column and centrifuge for 1 minute at 13,000 rpm. The flow-through was discarded and the QIAquick column was returned to the tube, and the column was centrifuged for an additional minute. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. DNA was eluted by the addition of 30 µl of Buffer EB to the center of the QIAquick membrane. To increase the DNA concentration, the column was left to stand for 1 minute at room temperature prior to centrifuging. The DNA was analyzed

using electrophoresis with Gel Star staining dye (Lonza) to detect the *superkdr* region band. The quality and quantity of the DNA were checked using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) prior to moving on to cloning the cDNA and genomic DNA.

Cloning and Sequencing of cDNA and Genomic DNA

The cDNA and genomic DNA was cloned into pCR™2.1 using the TA cloning kit (Thermo Fisher Scientific, Carlsbad, CA). Before following the TA cloning Kit, Luria-Bertani media with Mg^{2+} and Carbenicillin (100 mg/ml) plates were made. For the ligation process, the reagents and DNA sample were allowed to thaw in ice. One vial of pCR®2.1 was centrifuged to make sure the liquid is in the bottom of the tube. The 1.5 ml PCR tubes received 1.0 µl of PCR product, 1.5 µl of 5X T4 DNA ligase reaction buffer, 1.0 µl of pCR®2.1 vector (25 ng/µl), and 0.5 µl of ExpressLink™ T4 DNA ligase (5 units). The reactions were gently mixed with a pipette tip and incubated at room temperature for 60 minutes. The LB media plates were incubated at 37°C for 30 minutes. After the incubation period for the ligation reaction was completed, the vials were centrifuged and set on ice. The frozen One Shot® Competent Cells were thawed on ice. Once the competent cells thawed, 2.0 µl of each ligation reaction was added directly into the vial of competent cells and stirred gently with the pipette tip. The competent cells' vial incubated on ice for 30 minutes, and the ligation reaction was stored in the -20°C freezer. Heat shock was performed on the cells for 30 seconds at 42°C using a water bath, and then the vial containing cells was placed immediately back on ice. I added 250 µl of room temperature Super Optimal Catabolite-repression (S.O.C.) medium (provided with the TA Cloning kit) to each vial and incubated the vials horizontally at 37°C for 1 hour at

225 rpm in a shaking incubator. Two LB media plates were used per sample; in one of the plates 25 µl of S.O.C. medium was added and 25 µl from the transformation vial was added and then spread with disposable spreaders, in the other plate 100 µl from the transformation vial was added and spread with disposable spreaders. The plates were incubated overnight at 37°C.

A mixture of LB medium and carbenicillin was made prior to performing minipreps of the colonies that formed. In sterile test tubes, 2.5 ml of LB and antibiotic mix were added. For each sample, five isolated colonies were chosen at random using sterile toothpicks and dropped into corresponding test tubes and placed in a rotating wheel in the incubator. The colonies were incubated for approximately 16 hours at 37°C.

Plasmid DNA was purified using QIAprep Spin Miniprep kits and a microcentrifuge. After the incubation period was over, 1.0 ml of culture medium was added to 1.5 ml tubes and centrifuged for 3 minutes at 8,000 rpm. The supernatant was discarded and the pellet retained. The pellet was resuspended with the addition of 250 µl of buffer P1 and vortexing. Then 250 µl of Buffer P2 was added and mixed by inverting the tubes 4-6 times. Caution was taken to make sure not to vortex after the addition of Buffer P2 and Buffer N3. A white pellet formed after the addition of 350 µl of Buffer N3; tubes were mixed immediately by inverting 4-6 times. The microcentrifuge tubes were then centrifuged for 10 minutes at 13,000 rpm. Binding of plasmid DNA occurred when 800 µl of the supernatant was added to the QIAprep 2.0 spin column. The spin column was centrifuged for 60 seconds and the flow through was discarded. For the washing steps, 500 µl of Buffer PB was added and centrifuged for 60 seconds. The flow-through was discarded, and the column was returned to its collection tube. Another washing of the

QIAprep 2.0 spin column was performed by adding 750 µl of Buffer PE and centrifuging for 60 seconds. The flow-through was discarded, and the spin column was centrifuged at full speed for 1 minute to remove any residue of the wash buffer. The QIAprep 2.0 spin column was placed in new 1.5 ml microcentrifuge tubes. To elute DNA, 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) was added, left to stand for 1 minute at room temperature, and then centrifuged for 1 minute.

Digest purification of the plasmid DNA was performed in 0.5 ml microcentrifuge tubes. A pre-mix with 1.0 µl of CUTSMART Buffer, 6.5 µl of PCR water, and 0.5 µl of EcoR1 per reaction was prepared. In the 0.5 ml microcentrifuge tubes 2.0 µl of eluted DNA and 8.0 µl of pre-mix was added to each reaction tube. The samples were flicked, centrifuged, and then incubated for 1 hour at 37°C. The samples were analyzed using 1% gels with gel star solution. Samples that had a band (~1.2 kb) present were selected for sequencing, especially if some of the clones bands were smaller compared to others.

Sequencing was performed by Sanger sequencing (Retrogen Inc., San Diego, CA). The sequences were then aligned using the ClustalW multiple sequence alignment with MacVector Assembler version 15.1.4 (MacVector, Inc., Apex NC). The sequences of the primers used for sequencing are located in the Appendix.

Second Method

Horn Fly Population and Preparation

The 48 horn flies used are from three populations: Super Resistant population (16), Georgia Saber (16), and Susceptible (16). The Super Resistant population was from a colony established in USDA-ARS Kerrville Laboratory that received weekly treatment

of permetrhrins and were tested using bioassays (Guerrero et al. 1997). Super Resistant males were collected on May 13, 1998 and February 4, 2002 and stored at -80°C. The Georgia Saber's flies were collected from a Georgia ranch population on September 26, 1997 by Dr. Craig Shephard. Flies were collected alive, shipped on dry ice, and store at -80°C. Two tubes were received for the Georgia Saber Population and were labeled as Georgia Saber (G) males and Georgia Saber (GS) males. The USDA-ARS Kerrville Laboratory maintained a colony of pyrethroid-susceptible adult horn flies since 1961 (Guerrero et al. 1997), from this colony, susceptible (S) males collected June 25, 1997 and susceptible (SF) females collected June 25, 1997 were used to detect for alternative splicing. The horn flies were kept in a -80°C freezer prior to cutting and extracting. Since alternative splicing has been reported to be tissue specific, genomic DNA and cDNA was collected from both the head and body of horn flies. Horn fly heads were separated from the body and then cut in half to obtain both cDNA and genomic DNA from the head and body of individual flies.

Genomic Isolation

Genomic DNA was isolated using GeneJET Genomic DNA Purification Kit (Thermo Scientific). The frozen horn fly samples were kept in a 1.5 mL microcentrifuge tube, and 180 µL of digestion solution and 20 µL of proteinase K were added. The sample was then mixed thoroughly by vortex and incubated at 56°C for four hours on an Eppendorf ThermoMixer C. After four hours of incubation, 20 µL of RNase solution was added, and the sample was set to incubate at room temperature for 10 minutes. We added 200 µL of lysis solution, vortexed for 15 seconds, and then proceeded to add 400 µL of 50% ethanol and vortex again. The sample was then transferred into the GeneJET

Genomic DNA Purification Column and centrifuged for 1 minute at 6,000 x g. The flow through and collection tubes were discarded, and the column was placed in a new collection tube. For the washing steps, 500 µL of Wash Buffer I was added, centrifuged for 1 minute at 8,000 x g, and the flow through was discarded. Then, 500 µL of Wash Buffer II was added, the samples were centrifuged for 3 minutes at 12,000 x g, and the flow through was discarded. The spin columns were centrifuged for 1 minute at 12,000 x g to remove any excess Wash Buffer. The GeneJET Genomic DNA Purification Columns were placed in sterile 1.5 mL microcentrifuge tube. The genomic DNA was eluted by the addition of 100 µL of Elution Buffer. The samples were incubated at room temperature for 2 minutes and then centrifuged for 1 minute at 8,000 x g. The elution step was repeated using the eluted DNA to increase the DNA concentration. The GeneJET Genomic DNA Purification Columns was disposed of, and the samples were stored in a freezer at -20°C till needed.

The presence or absence of genomic DNA was determined by gel electrophoresis using 1% agarose gel run at approximately 90 volts for 30 minutes. Each sample well contained 3.0 µL of loading dye mixed with gel red and 4.0 µL of genomic DNA. The ladder well contained 0.5 µL of GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific) and 3.0 µL of loading dye mixed with gel red.

RNA Extraction, DNase treatment, and cDNA Synthesis

RNA extraction from horn flies was performed using PureLink™ RNA MiniKit. Prior to starting the RNA extraction, a fresh Lysis Buffer and 2-mercaptoethanol mix needed to be prepared. For every 1000 µL of Lysis Buffer, 10 µL of 2-mercaptoethanol was added. Tissue samples remained on dry ice until 300 µL of Lysis Buffer and 2-

mercaptoethanol mix were added. The tissue sample was then crushed using an RNase-free pestle for 30 seconds. The sample was centrifuged for 2 minutes at 12,000 x g, and the supernatant was transferred to new RNase-free tubes. For the binding procedure, 300 µL of 70% ethanol made with RNase-free water was added to the samples and vortexed. The sample was then transferred into the spin cartridges and centrifuged for 15 seconds at 12,000 x g. The flow through was discarded, and the spin cartridge was placed back in the collection tube. During the washing steps, 700 µL of Wash Buffer I was added to the spin column and centrifuged at 12,000 x g for 15 seconds. The flow through and collection tube were discarded. Prior to the addition of 500 µL of Wash Buffer II with ethanol, the spin cartridge was placed in a new collection tube. The sample was centrifuged at 12,000 x g for 15 seconds, and the flow-through was discarded. The spin cartridge was centrifuged again at 12,000 x g for 1 minute to remove excess Wash Buffer. The collection tube was discarded, and the spin cartridge was placed in a recovery tube. The RNA was eluted from the spin cartridge by the addition of 50 µL of RNase-free water. The sample was set to incubate at room temperature for 1 minute and then centrifuged for 2 minutes at 12,000 x g.

Following the RNA extraction, the RNA was treated with TURBO DNA-free™ Kit to remove any contaminating DNA. The DNase treatment starts with the addition of 5.0 µL of 10X TURBO DNase Buffer and 1.0 µL of TURBO DNase to the RNA sample. The samples were then gently mixed. The samples were then placed on the Eppendorf ThermoMixer C and set to incubate at 37°C for 30 minutes. The DNase Inactivation Reagent was vortexed, and 5 µL was added to the samples. The samples were mixed occasionally and set to incubate at room temperature for 5 minutes. The sample was then

centrifuged at 10,000 x g for 1.5 minutes, and the supernatant was transferred to a sterile microcentrifuge tube.

First strand cDNA was synthesized using Cloned AMV First Strand cDNA Synthesis Kit (Invitrogen). A 20.0 µL aliquot reaction was made with 4.5 µL of RNA, 7.5 µL of master mix 1, and 8.0 µL of master mix 2. Master mix 1 contained 1.0 µL of oligo$(dT)_{20}$ primers (50 µM), 2.0 µL of 10 mM dNTP mix, and 4.5 µL of DEPC-treated water per reaction. For master mix 2, 4.0 µL of cDNA synthesis buffer, 1.0 µL of 0.1 M DTT, 1.0 µL of RNase Out™ (40 U/µL), 1.0 µL of DEPC-treated water, and 1.0 µL of cloned AMV RT (15 U/µL) per reaction. To 0.5 ml tubes, 7.5 µl of master mix 1 and 4.5 µL of RNA was added. Denaturation of RNA and primers was accomplished by incubating at 65°C for 5 minutes. The samples were then placed on ice immediately. The cDNA synthesis buffer was vortexed prior to making master mix 2 on ice. While samples were on ice, 8.0 µL of master mix 2 was added to each sample. The samples were then placed on the Eppendorf ThermoMixer C and set to incubate for 60 minutes at 50°C. The reaction was terminated by increasing the incubation temperature to 85°C for 5 minutes. The cDNA was then stored in the -20°C freezer.

To determine the presence of cDNA, 3.0 µl of cDNA was analyzed by gel electrophoresis with 1% agarose gel run at approximately 90 volts for 30 minutes. Each sample well contained 3.0 µl of loading dye mixed with gel red (400 µl of 6X Blue dye, 600 µl 50% sucrose, and 2 µl gel red) and 3.0 µl of cDNA. The ladder well contained 0.5 µL of GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific) and 3.0 µl of loading dye mixed with gel red.

SNP Assay

A Custom Taqman® SNP Genotyping Assay (Applied Biosystem, Foster City, CA) was designed with sequence-specific forward and reverse primers to genotype the *superkdr* locus. The forward primer and reverse primer designed for the SNP assay were 5'-GCCCACCCTGAACTTACTCATTT-3' and 5'TGTTAGATTACCCAATGCACCCATT-3', respectively. The Custom Taqman® SNP Genotyping Assay (Applied Biosystem, Foster City, CA) also consists of two reporter dye probes. VIC is reporter dye 1 with the sequence 5'-CGGCCCATAATTG-3', and FAM is reporter dye 2 with the sequence 5'-CGGCCCGTAATTG-3'. The master mix reaction included 5.0 µL of Taqman® Fast Advanced Master Mix, 0.5 µL of 20X Custom Taqman® SNP Genotyping Assay (Applied Biosystem, Foster City, CA), and 4.0 µL of Ultra Pure Water. The reagents were vortexed prior to creating the master mix. Each individual reaction was composed of 9.5 µL of the master mix and 1.0 µL of the genomic DNA or cDNA template. The well plate was then vortexed and centrifuged before placing in the QuantStudio™ 3 Real-Time PCR System. The Genotyping Fast Program compromised 1 cycle at 60.0°C for 30 seconds, 95.0°C for 20 seconds, followed by 40 cycles of 95.0°C for 1 second and 60°C for 20 seconds, and a final extension at 60°C for 30 seconds.

Statistical Analyses

The comparison of the *superkdr* genotypes of flies in the cDNA and genomic DNA was made using a Chi-Square tests (R Core Team 2015).

3. Results

Genomic DNA and cDNA Sequence Comparison

We successfully collected both genomic DNA and cDNA sequences for five horn flies: Georgia Fly 4, Pullman Fly 10, Pullman Fly 10, Super Resistant Fly 1, and Super Resistant fly 4. The genomic DNA of each individual fly is located in the APPENDIX. A matrix was created to compare the genomic DNA of the flies using MacVector. Genomic DNA sequences ranged in length from 828 bp to 1280 bp. The differences between the genomic DNA occurred in the intron region. Genomic DNA sequences were aligned to one another to compute a consensus sequence. The genomic DNA consensus sequence was used as to create a diagram with the location of the primers and to determine the major splice sites using the cDNA consensus sequence (APPENDIX B).

Multiple sequence alignment

15 Sequences Aligned Alignment Score = 194283
Gaps Inserted = 0 Conserved Identities = 292

** Identity Scores (%) **

	Georgia Fly 4 genomic 9A	Georgia Fly 4 genomic 9D	Georgia Fly 4 genomic 9G	Georgia Fly 4 genomic 9H	Georgia Fly 4 genomic 9J	Georgia Fly 4 genomic 9K	Pullman Fly 10 genomi c 2A	Pullman Fly 10 genomi c 2C	Pullman Fly 10 genomi c 2G	Pullman Fly 11 genomi c 1E	Pullman Fly 11 genomi c 1I	Pullman Fly 11 genomi c 1L	Super R esistan t Fly 1 genomi c 27C	Super R esistan t Fly 1 genomi c 27D	Super R esistan t Fly 4 genomi c 28B
Georgia Fly 4 genomic 9A	100.0	72.3	72.3	72.3	99.9	100.0	67.5	70.1	36.9	37.0	80.6	69.2	100.0	59.1	69.2
Georgia Fly 4 genomic 9D	72.3	100.0	99.9	100.0	72.3	72.3	68.0	69.6	35.4	36.6	69.2	70.8	72.3	59.5	72.3
Georgia Fly 4 genomic 9G	72.3	99.9	100.0	99.9	72.3	72.3	68.0	69.6	35.4	36.5	69.3	70.8	72.3	59.5	72.3
Georgia Fly 4 genomic 9H	72.3	100.0	99.9	100.0	72.3	72.3	68.0	69.6	35.4	36.6	69.2	70.8	72.3	59.5	72.3
Georgia Fly 4 genomic 9J	99.9	72.3	72.3	72.3	100.0	99.9	67.5	70.1	36.9	36.9	80.7	69.3	99.9	59.1	69.3
Georgia Fly 4 genomic 9K	100.0	72.3	72.3	72.3	99.9	100.0	67.5	70.1	36.9	37.0	80.6	69.2	100.0	59.1	69.2
Pullman Fly 10 genomic 2A	67.5	68.0	68.0	68.0	67.5	67.5	100.0	84.0	35.8	34.9	68.0	72.5	67.5	63.7	67.9
Pullman Fly 10 genomic 2C	70.1	69.6	69.6	69.6	70.1	70.1	84.0	100.0	37.5	38.3	70.3	70.9	70.1	58.7	70.4
Pullman Fly 10 genomic 2G	36.9	35.4	35.4	35.4	36.9	36.9	35.8	37.5	100.0	68.8	36.6	37.1	36.9	25.5	35.0
Pullman Fly 11 genomic 1E	37.0	36.6	36.5	36.6	36.9	37.0	34.9	38.3	68.8	100.0	36.9	37.1	37.0	26.1	37.1
Pullman Fly 11 genomic 1I	80.6	69.2	69.3	69.2	80.7	80.6	68.0	70.3	36.6	36.9	100.0	72.0	80.6	58.8	70.9
Pullman Fly 11 genomic 1L	69.2	70.8	70.8	70.8	69.3	69.2	72.5	70.9	37.1	37.1	72.0	100.0	69.2	59.0	80.8
Super Resistant Fly 1 genomic 27C	100.0	72.3	72.3	72.3	99.9	100.0	67.5	70.1	36.9	37.0	80.6	69.2	100.0	59.1	69.2
Super Resistant Fly 1 genomic 27D	59.1	59.5	59.5	59.5	59.1	59.1	63.7	58.7	25.5	26.1	58.8	59.0	59.1	100.0	64.7
Super Resistant Fly 4 genomic 28B	69.2	72.3	72.3	72.3	69.3	69.2	67.9	70.4	35.0	37.1	70.9	80.8	69.2	64.7	100.0

** Similarity Scores (%) **

Figure 4. Matrix of Genomic DNA. Genomic DNA clones of the five flies were compared using a matrix to determine their similarity to one another. No gaps were inserted in the sequences for the matrix of the sequences.

Genomic DNA sequences ranged in length from 828 bp to 1280 bp. The differences between the genomic DNA occurred in the intron region. Genomic DNA sequences were aligned to one another to compute a consensus sequence. The genomic DNA consensus

sequence was used as to create a diagram with the location of the primers and to determine the major splice sites using the cDNA consensus sequence (APPENDIX B).

Multiple sequence alignment

11 Sequences Aligned Alignment Score = 29484
Gaps Inserted = 0 Conserved Identities = 185

** Identity Scores (%) **

	Genomic Fly 4- cDNA 3C	Genomic Fly4- cDNA 3E	Pullman Fly 11 - cDNA LD4D	Pullman Fly 10 - cDNA 8B	Pullman Fly 10 - cDNA 8D	Super Resistant Fly 4 - cDNA 32A	Super Resistant fly 4 - cDNA 32B	Super Resistant Fly 4 - cDNA 32D	Super Resistant Fly 4 - cDNA 32E	Super Resistant Fly 1 - cDNA 33A	Super Resistant Fly 1 -cDNA 33E
Genomic Fly 4- cDNA 3C	100.0	99.5	99.5	99.5	99.5	100.0	100.0	100.0	94.4	100.0	100.0
Genomic Fly4- cDNA 3E	99.5	100.0	100.0	100.0	100.0	99.5	99.5	99.5	93.9	99.5	99.5
Pullman Fly 11- cDNA LD4D	99.5	100.0	100.0	100.0	100.0	99.5	99.5	99.5	93.9	99.5	99.5
Pullman Fly 10- cDNA 8B	99.5	100.0	100.0	100.0	100.0	99.5	99.5	99.5	93.9	99.5	99.5
Pullman Fly 10- cDNA 8D	99.5	100.0	100.0	100.0	100.0	99.5	99.5	99.5	93.9	99.5	99.5
Super Resistant Fly 4- cDNA 32A	100.0	99.5	99.5	99.5	99.5	100.0	100.0	100.0	94.4	100.0	100.0
Super Resistant fly 4- cDNA 32B	100.0	99.5	99.5	99.5	99.5	100.0	100.0	100.0	94.4	100.0	100.0
Super Resistant Fly 4- cDNA 32D	100.0	99.5	99.5	99.5	99.5	100.0	100.0	100.0	94.4	100.0	100.0
Super Resistant Fly 4- cDNA 32E	94.4	93.9	93.9	93.9	93.9	94.4	94.4	94.4	100.0	94.4	94.4
Super Resistant Fly 1- cDNA 33A	100.0	99.5	99.5	99.5	99.5	100.0	100.0	100.0	94.4	100.0	100.0
Super Resistant Fly 1-cDNA 33E	100.0	99.5	99.5	99.5	99.5	100.0	100.0	100.0	94.4	100.0	100.0

** Similarity Scores (%) **

Similarity Scores(s) are shown below the diagonal (x) with Identity Scores(I) above

	a	b	c	d	e
a	x	i	i	i	i
b	s	x	i	i	i
c	s	s	x	i	i
d	s	s	s	x	i
e	s	s	s	s	x

Figure 5. Matrix of cDNA Sequences. All but one of the cDNA sequences were ~197 bp in length. Super resistant fly 4 clone 32 E was truncated at the 3' making it ~186 bp. No gaps were inserted in the sequence.

The cDNA of Georgia fly 4 clone 3C, Super Resistant fly 4 clones 32A, 32B, 32 D, and Super Resistant fly 1 clones 33A, 33E are 100% identical and show the signature sequence for resistance at the *superkdr* locus. Georgia fly 4 clone 3E, Pullman Fly 11 clone 4D, and Pullman Fly 10 clone 8B, 8D had susceptible alleles and were 100% similar. Super Resistant fly 4 clone 32E is 94.4% similar to the others due to the 3' truncation. Super Resistant fly 4 clone 32E contains a cytosine at the *superkdr* locus.

Georgia Fly 4

The genotype of the superkdr locus of Georgia Fly 4 is SR. There was a 1 bp difference between the genomic DNA sequence clones of Georgia Fly 4 each ~1277 bp

or ~1278 bp in length. The one nucleotide addition was a thymine following a chain of thymines in clone 9D, clone 9G, and clone 9H. Clones 9D and 9H of Georgia Fly 4 were 100% similar to one another with a C at the *superkdr* locus, making them resistant alleles. Clones 9A and 9K were 100% similar to each other and also identified as being resistant alleles. Clones 9G and 9J were susceptible alleles with a T at the *superkdr* locus. As for the cDNA of Georgia Fly 4, only two of the clones of the cDNA were sent to be sequenced. Clone 3C was identified as a resistant allele and clone 3E as a susceptible allele.

Pullman Fly 10

The superkdr genotype of the genomic DNA is SR. The genomic DNA included clones 2A, 2C, and 2G. Clone 2A was 1175 bp, clone 2C was 1274 bp, and clone 2G 1271 bp in length. Clone 2C was a resistant allele, and clone 2G was a susceptible allele. Pullman fly 10 clone 2A sequence was short and did not include the superkdr locus. As for the cDNA, clone 8B and 8D were susceptible alleles.

Pullman Fly 11

The superkdr genotype of the genomic DNA is SR. The genomic DNA included clones 1E, 1I, and 1L. Clone 1L and 1I differ from each other due to an extra T following a chain of thymines, but both are susceptible alleles for the *superkdr*. Clone 1E is a resistant allele. For the cDNA of Pullman Fly 1, only one of the cloned cDNAs was successfully sequenced. cDNA clone 4D is susceptible at the *superkdr* locus.

Super Resistant Fly 1

The genomic DNA superkdr genotype is RR. For the genomic DNA clone 27C is a resistant allele with 1277 bp. Clone 27D is 828 bp and was not long enough to contain the *superkdr* locus. cDNA clone 33A and 33E are both resistant alleles and 100% similar to each other.

Super Resistant Fly 4

The genomic DNA *superkdr* genotype is RR. For Super Resistant Fly 4, we were able to successfully sequence clone 28B. Clone 28B is ~1279 bp and is a resistant allele. cDNA clones 32A, 32B, 32D, and 32 E are all resistant alleles. The length of clone 32E is shorter than the others because it is truncated at the 3' end.

The genomic DNA of the horn flies was compared to the corresponding cloned cDNA sequence using Mac Vector. cDNA successfully aligned with the genomic DNA that contained the *superkdr* locus. The splice site from the cDNA was located at the ~39 bp and the rest of the cDNA is the exon that includes the *superkdr*.

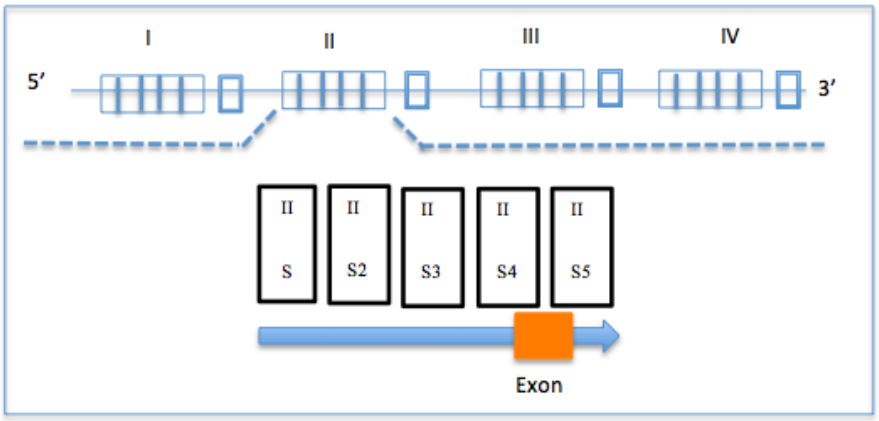


Figure 6. cDNA Exon Location. This diagram demonstrate the location of the exon compared to the sodium channel.

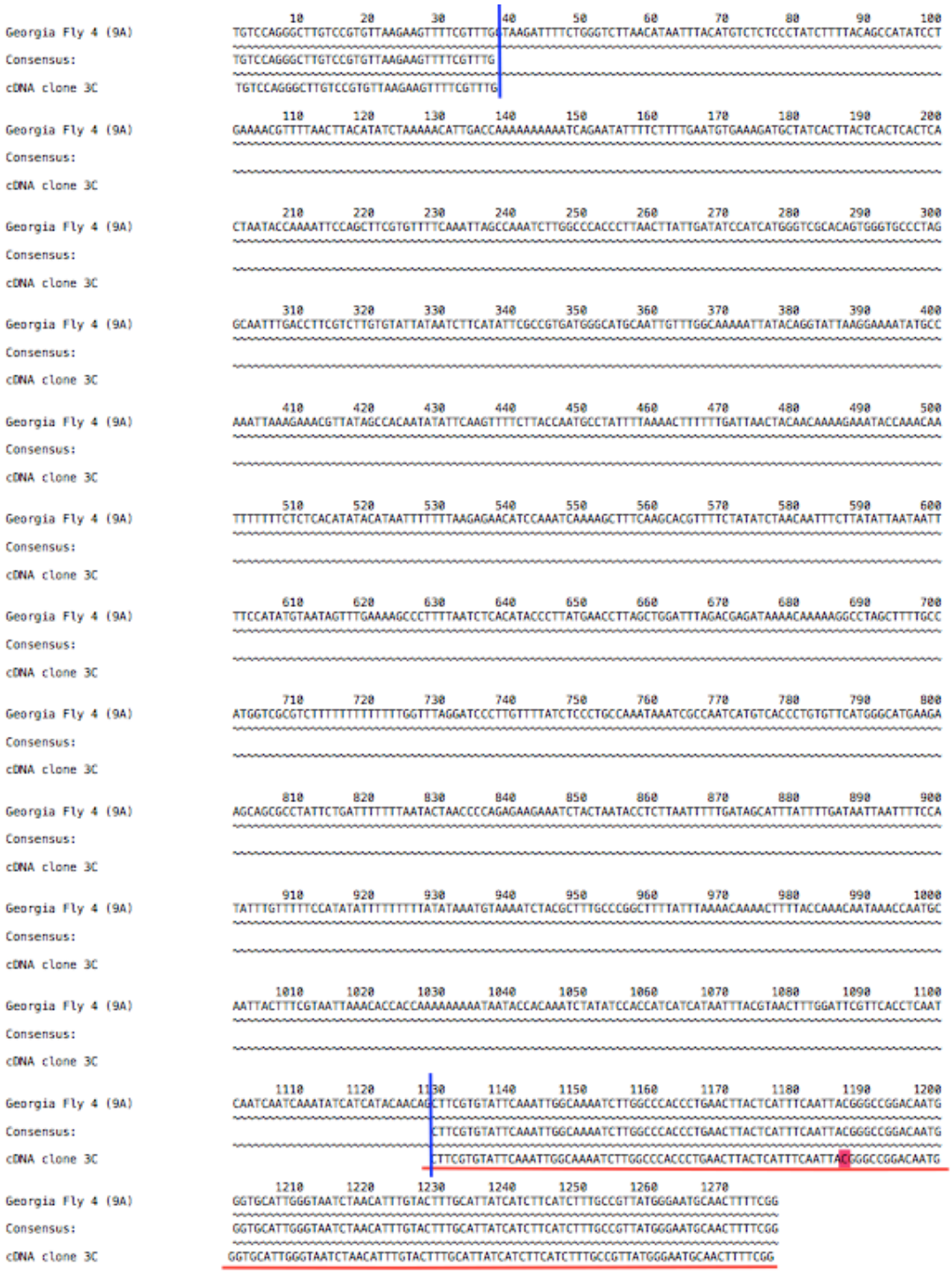


Figure 7. cDNA and Genomic DNA Comparison. The figure shows the comparison of Georgia fly 4 genomic DNA with one of the cloned cDNA. The blue vertical lines indicate the intronic section of the genomic DNA. The highlighted C is the location of the superkdr mutation. The red underline denotes the exon section.

Using BLAST, I compared the exon obtained to the exon D in the housefly, *Musca domestica, Vssc1* sodium channel gene (Lee et al. 2002). The nucleic acid sequence from this horn fly was 95% similar to the house fly matching 187 out of 197 bp.

SNP Assay Genotype and Detection of Alternative Splicing

The results were analyzed by two reporters, VIC and FAM. VIC is a reporter dye attached to a probe with a short specific sequence meant to bind at the *superkdr* locus of the susceptible allele (allele 1) that have a T on the template DNA. FAM (reporter dye 2) is attached to a probe with a short specific sequence meant to bind to a resistant allele (allele 2), an allele expressing a C at the *superkdr* locus. Real time PCR records and monitors the increase of fluoresces after each cycle, depending on the intensity of the signal it will genotype the superkdr locus. In figure 8, the x-axis corresponds to VIC and the y-axis refers to FAM, if equal signaling of VIC and FAM is reported then the individual genotype is heterozygous (allele1/allele2). If most of the signal being reported is FAM, then the individual genotype is homozygous C (resistant). The homozygous C individual is reported as allele1/allele 1 and is represented by a red dot. If most of the signal being reported is VIC, then the individual genotype is homozygous T (susceptible). A homozygous T individual is reported as allele 2/allele 2 and is represented by a blue dot.

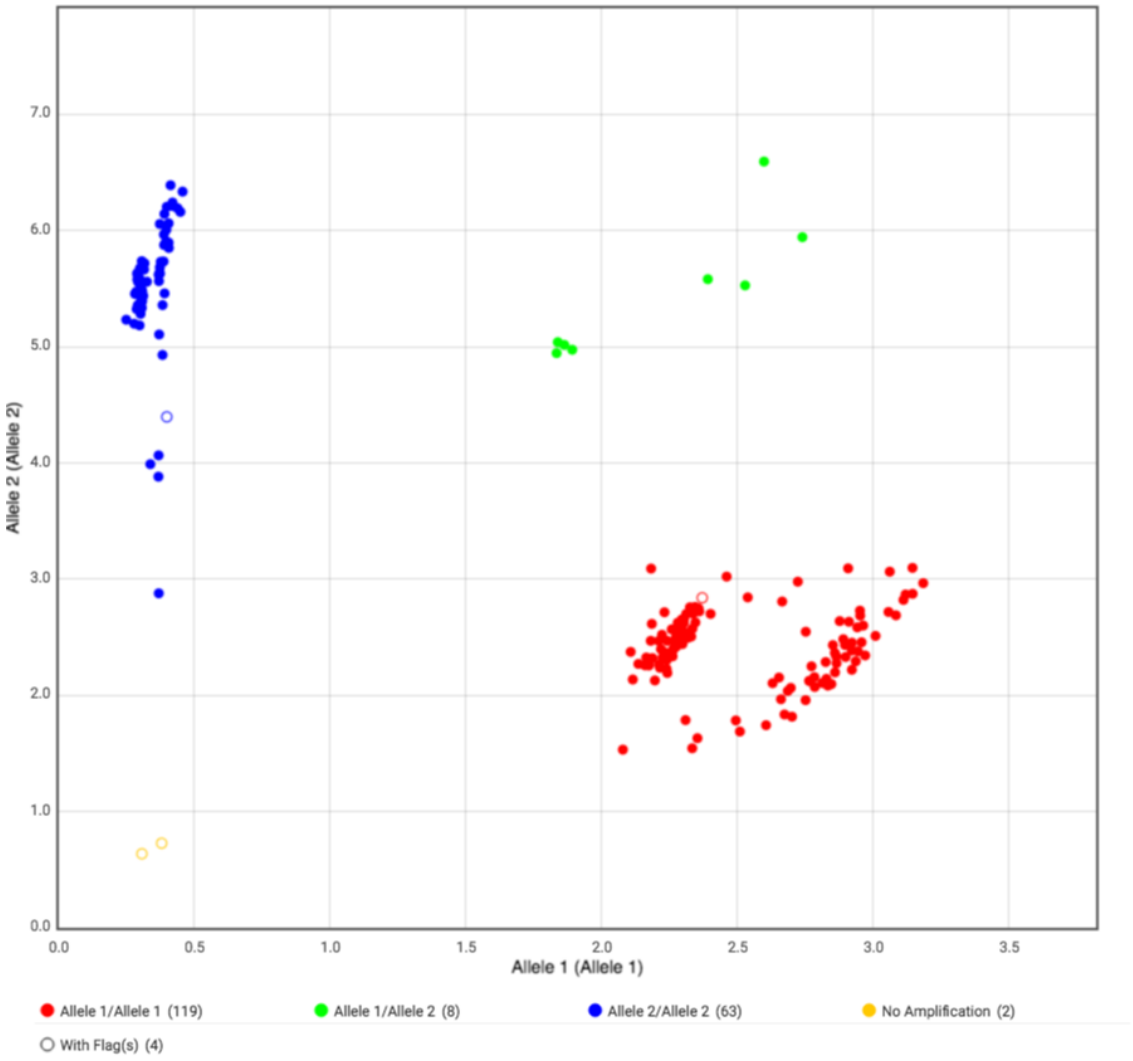


Figure 8. SNPs Assay Genotyping.

Allele 1/Allele 1 (represented by the red color, TT) sample are homozygous for allele 1. Allele 1/ Allele 2 (represented by the green color, CT) sample are heterozygous allele 1 and allele 2. Allele 2/Allele 2 (represented by the blue color, CC) samples are homozygous for allele 2.

Table 3. cDNA and Genomic DNA Genotype.

Population	Type	*Superkdr* *superkdr* allelic *Frequency*	Genotype		
			TT	TC	CC
Georgia Saber	cDNA	6.67%	28	4	
Georgia Saber	Genomic DNA	6.67%	28	4	
Susceptible	cDNA	0.0%%	32	0	0
Susceptible	Genomic DNA	0.0%	32	0	0
Super Resistant	cDNA	100%		0	32
Super Resistant	Genomic DNA	100%		0	31

The genotype of the cDNA and genomic DNA were compared using a Chi-Square test. The results indicated that there was no difference between the cDNA and genomic DNA with a Chi-squared = 0.010638, df = 2, p-value = 0.9947.

Alternative splicing would be detected by the SNP assay by the absence of *superkdr* genotype in cDNA but present in the genomic DNA. Referring to Figure 8, there are two yellow dots indicating that two of the samples did not amplify. The yellow dot refers to genomic DNA samples Super Resistant 6 Body 2002 and Susceptible Female fly 7 head. The SNP assay was performed again for Susceptible Female fly 7 head with three different volume of cDNA and genotyping (TT) was successful. Each cDNA sample resulted with amplification and was successfully genotyped by the SNP assay.

4. Discussion

Alternative splicing is a mechanism in which a single gene can generate a diversity of protein isoform. The housefly and horn fly have been identified as having pyrethroid resistance-associated mutations, *kdr* and *superkdr.* Alternative splicing has

been reported to be tissue and stage specific (Dong et al. 2014; Lee et al. 2002; Liu et al. 2001; Martins and Valle 2012); therefore, I probed RNA from both the head and body of adult horn flies. Williamson et al. (1996) investigated the housefly sodium channel gene and sequenced cDNA to determine if alternative exon sites would be identified like in the *para* mutant; they confirmed alternative splicing was absent through direct RT-PCR. Only one clear sequence for the entire sodium channel coding for both the *kdr* and *superkdr* was obtained (Williamson et al. 1996). Lee et al. (2002) compared the genomic DNA and cDNA sequence of houseflies' *Vssc1* gene. The *superkdr* locus was reported to occur on mutually exclusive exons C and D. The clones from larvae, pupae, and adult head and body all had exon D except for one adult housefly head which had exon C in the location in which exon D was suppose to be (Lee et al. 2002). Exon C is truncated by a premature stop codon, which would be expected to make that sodium channel isoform non-functional (Lee et al. 2002; Dong 2007). A sodium channel with exon C would encode domain II S4 and part of S5 (Williamson et al. 1996); the premature codon would result in eliminating more than half of the sodium channel.

The current method to determine the prevalence of *superkdr* in wild populations of horn flies is performed using the genomic DNA (Guerrero et al. 1997). The presence of alternative splicing would result in an overestimation of the *superkdr* locus in a wild population using genomic DNA. Therefore, I sampled cDNA and genomic DNA to compare and determine whether alternative splicing occurred at the *superkdr* locus. If alternative splicing is occurring, then using the cDNA to determine *superkdr* prevalence should be preferred over using genomic DNA.

cDNA and genomic DNA sequences comparison

When cDNA and genomic DNA were compared from individual horn flies, I observed that they all had the major spliceosome splice site at the same locations separating exon from intron. When comparing all the cDNA clones sequenced, it was observed that most of the cDNA sequences were identical except for at the *superkdr* locus where either a cytosine or thymine was present. Super Resistant Fly 4 clone 32 E was the only cDNA that was different from other resistant cDNA clones due to 3' truncation; it still contained the *superkdr* locus. The genomic DNA and cDNA clones of Super Resistant Fly 4 were not truncated like cDNA clone 32 E. This result could imply 1) alternative splicing, 2) PCR primer binding error, or 3) incorrect insertion of ECOR1. Further tests may be performed using the cDNA of Super Resistant Fly 4 to examine and compare the protein isoform predicted.

SNP Assay

The SNP assay resulted in genotype for most samples, except for the genomic DNA sample in which the body was missing from the test tube. Since genomic DNA is the same it can be alleged the genotype for Super Resistant Fly 6 Body is the same as Super Resistant Fly 6 Head (CC). This would indicate all the genotypes from the head match the body of the corresponding horn flies. As for alternative splicing, all the cDNAs were amplified meaning from my results the presence of alternative splicing was not detected.

Overall from the results of both methods, it was not possible to determine the presence or absence of alternative splicing and further work is needed to investigate this phenomenon. From the data collected, we can say if alternative splicing is occurring at the *superkdr* locus, then it appears to be rare as it is in the housefly. Therefore using the

genomic DNA to survey wild population for pyrethroid-resistance is a better approach than using cDNA.

Method Pros and Cons

The first method involved using diagnostic PCR and then analyzing the results with electrophoresis to detect the presence or absence of the susceptible and resistant allele. The diagnostic PCR is an inexpensive technique that allows amplification of the target size, but gel electrophoresis must be performed to analyze results. The genotype is dependent on presence or absence of expected band fragments; the interpretation of the presence or absence of a band may be subjective. In detecting the presence or absence of alternative splicing, sequencing DNA and cDNA provides a direct sequence comparison, which allowed for the truncation to be observed. The process to extract, purify, clone genomic DNA is a tedious and costly process. The second method leveraging a SNP assay allowed for both genotyping and detection of alternative splicing at the *superkdr* locus. The SNP assay is time efficient and, depending on sample size, can be considered cost-effective. For this experiment, it took approximately three months for the first method performed and took about three weeks to prepare and perform the SNP Assay.

IV. FUTURE IMPLICATIONS

In my research, I concentrated on the sodium channel gene expressed by adult horn flies at the *superkdr* locus. An extensive study on alternative splicing of sodium channel gene transcript has been done for the housefly, fruit fly, and German cockroach (Dong 2007). In future research, a complete comparison between the genomic DNA and cDNA of the horn fly sodium channel should be performed to compare the extent of similarities and differences among other insect sodium channel exons. Moreover, such a study should also consider different life stages of horn flies as research in houseflies has shown differential frequencies of splice variants generated by the exons among different life stages in this species (Lee et al. 2002). Creating an overview of the exons of the sodium channel of horn flies for different life stages may lead to a better understanding of mutations in the sodium channels caused by insecticides, thus leading to better horn fly management. The SNP assay is a time effective and cost-effective way to genotype single nucleotide mutations associated with insecticide-resistance compared to running a diagnostic PCR and then checking the PCR product through gel electrophoresis. Research in the molecular biology of insecticide resistance should allow cattle owners to know which insecticide will be most effective in their susceptible herd.

Horn Fly Management

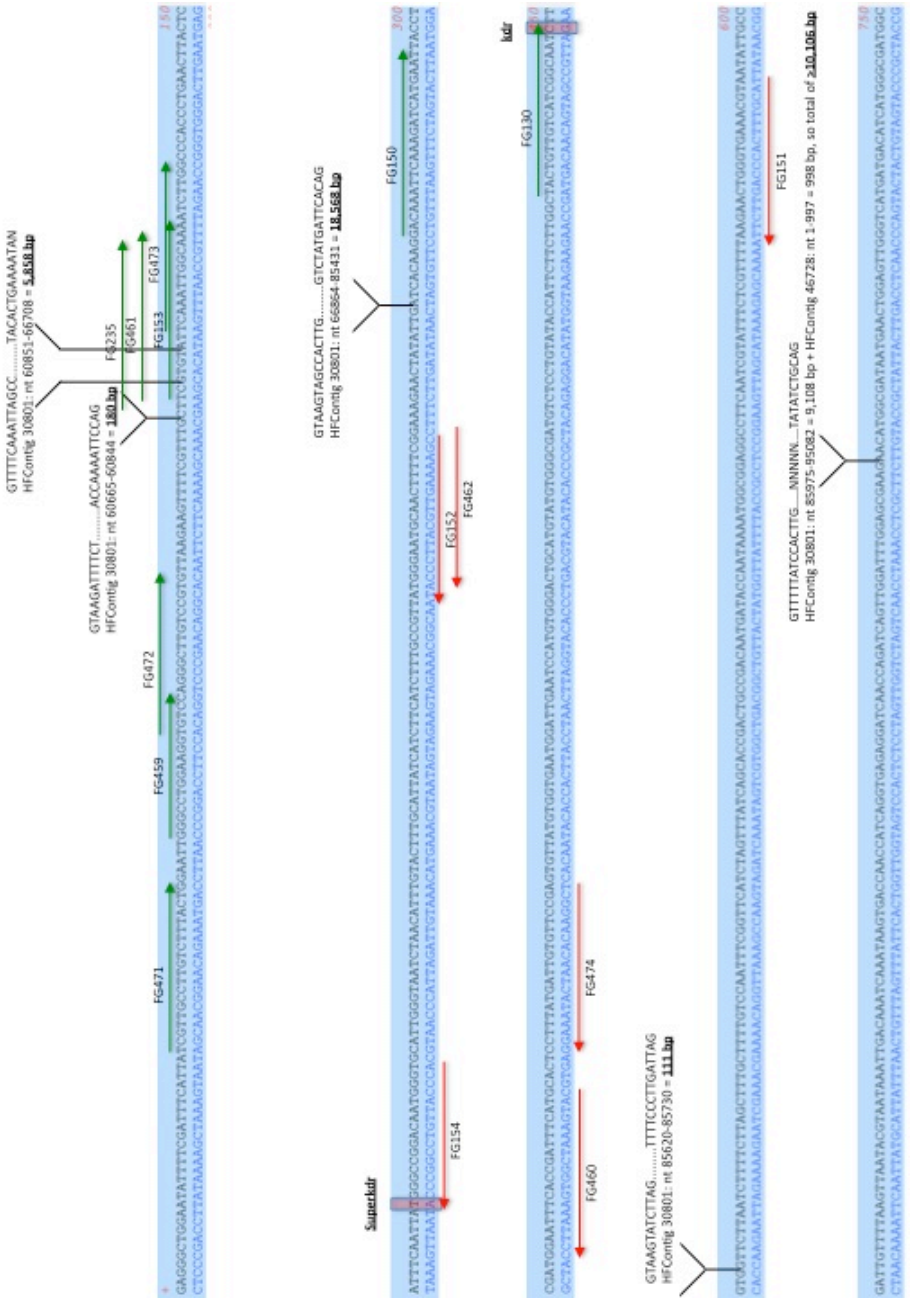
Due to increased insecticide resistance and cross-resistance with multiple insecticides, the use of insecticides as a management tool should be evaluated with caution. First of all, insecticides such as pyrethroids target the adult horn flies, but should only be used when the threshold of more than 200 horn flies per cattle has been reached (Foil and Hogsette 1994) since small amounts of horn flies do not present an economic

problem. If cattle owners are dealing with endoparasites and ectoparasites (ticks, horn flies, stable flies), they should use broad-spectrum parasiticides like Macrocyclic lactone or vaccines (Oyarzun et al. 2008). Another potential management strategy would consist of testing a sample of horn fly populations at each site to determine which of insecticide would work best against them on specific pastures. By knowing if horn flies are resistant to an insecticide at a given site, cattle owners can save money by using alternative insecticides that are effective and thus reduce insecticide runoff. One potential disadvantage of this management practice is the accessibility of these tests for farmers and ranchers. If the use of insecticide and vaccines are not an option for some owners, there are other methods to mitigate horn flies like walk-through traps and vacuum traps that can help reduce the number of adult horn flies on cattle (Foil and Hogsette 1994).

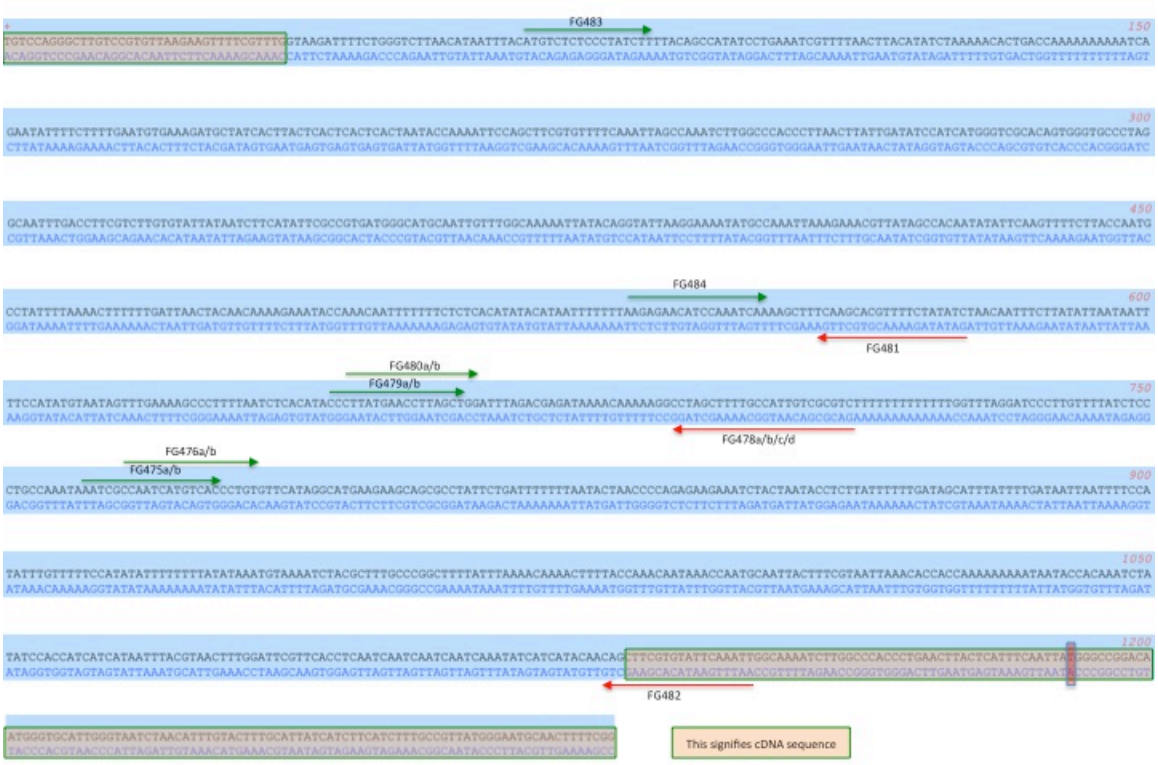
Adult horn flies are normally the main target of sprayed and ear tag insecticides. But to manage horn flies in a more holistic way, other life stages will need to be targeted. Since the egg, larvae, and pupae stages all occur in cattle manure, destroying manure pads will likely result in a reduction of the adult population. The destruction of cattle droppings is indeed an effective method to diminish horn fly populations (Bruce 1964). Also, owners can integrate strategies like pasture rotation and cattle manure destruction to reduce horn fly populations. Further options like pasture flooding, in which cattle manure is dispersed and thinned (Foil and Hogsette 1994), or controlled burning of pastures (Scasta et al. 2002) can also be used as management tools. Pyric-herbivory reduces 41% of horn fly populations, and benefits owners with brush control and enhance forage quality (Scasta et al. 2002) while at the same time keeping horn fly counts below the 200 flies per cattle head thresholds. Depending on which method works best for the

cattle owner, the goal should be the integration of these strategies so different pest life stages and pastures can be targeted with the most economically and practically feasible methods. In sum, correctly managing horn flies will lead to an increase of weight gain, milk production, and less stress (Byford et al. 1992), thus increasing the productivity of the farm.

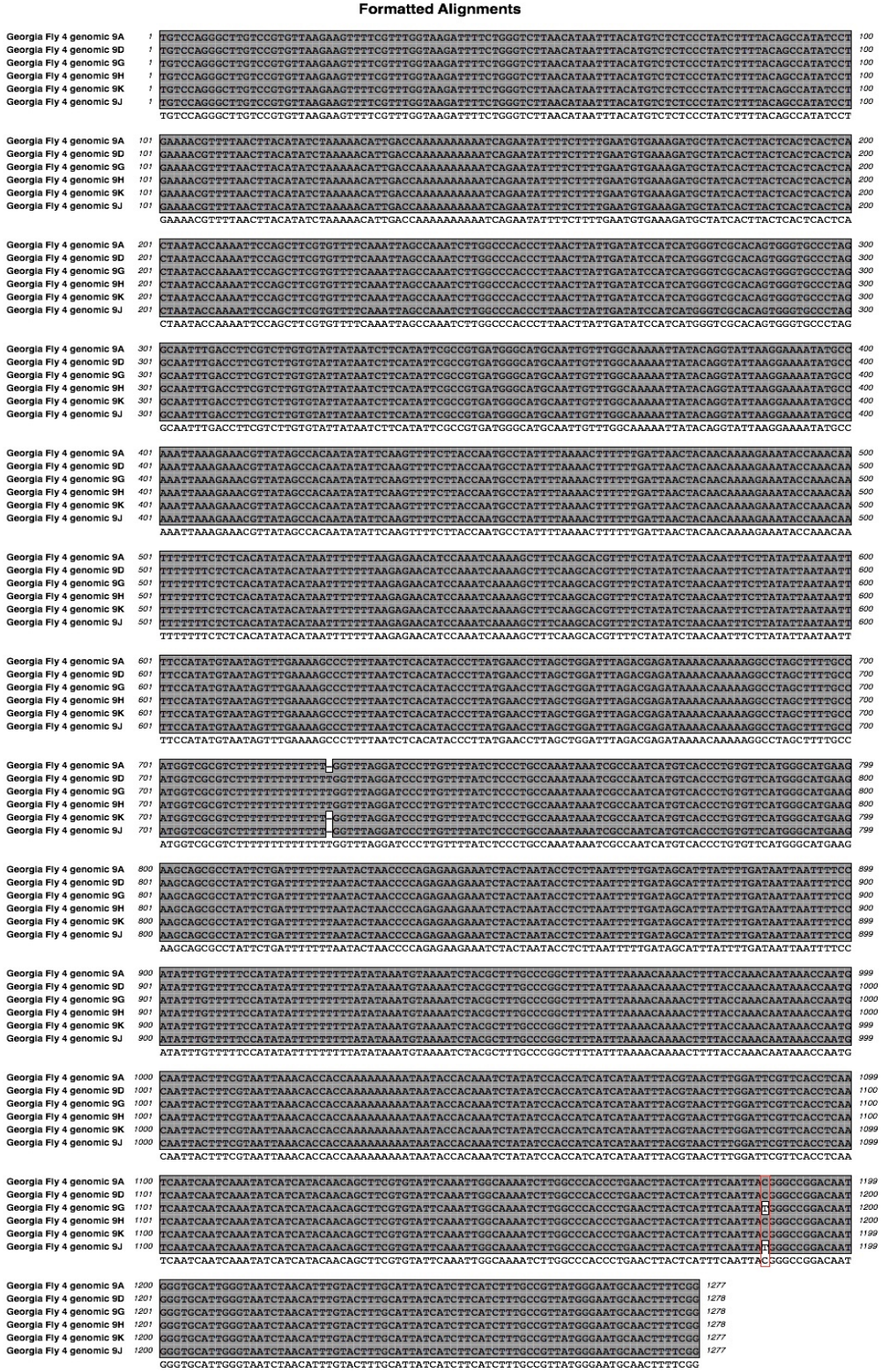
APPENDIX SECTION



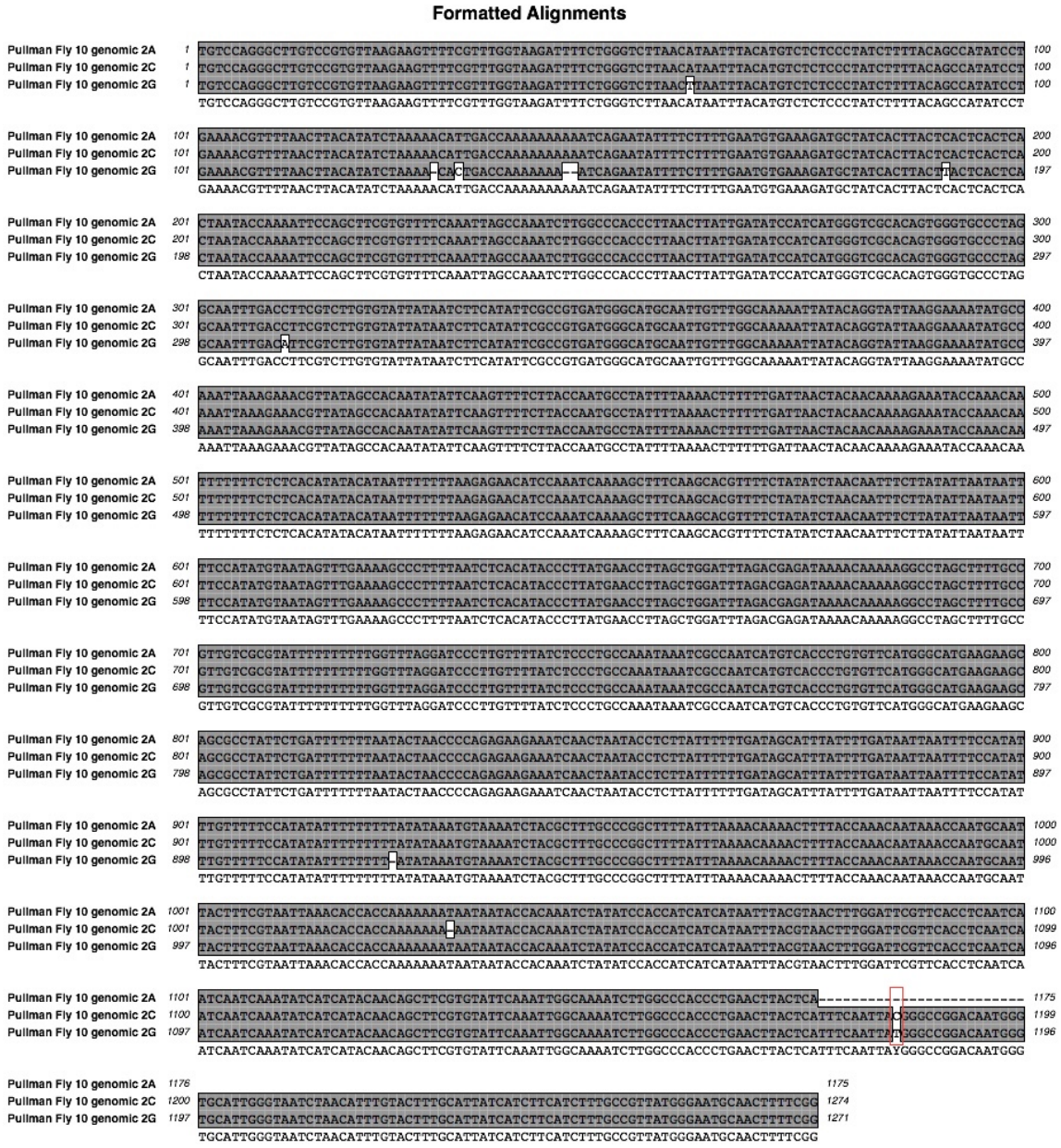
APPENDIX A. Primers used for sequencing of the genomic and cDNA. The diagram corresponds to the information gathered about the genomic DNA regions that surrounds the superkdr and kdr locus by Dr. Guerrero and Dr. Jamroz in 1997 with corresponding primers. The 'Y" refers to introns' location and an estimate of length.



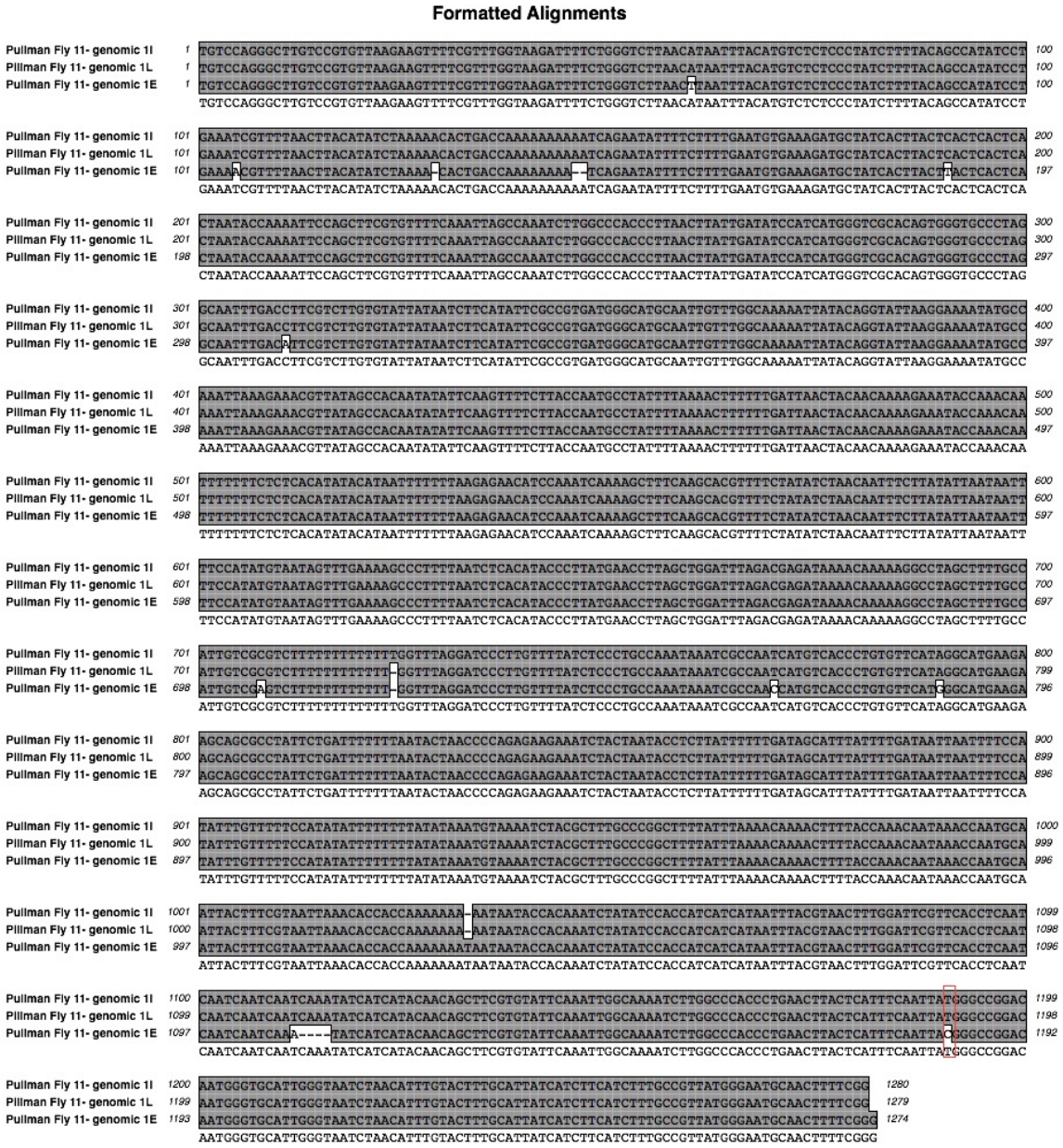
APPENDIX B. Genomic DNA with cDNA sections highlighted. Primers shown were created and used for Sanger sequencing to determine the sequence of the genomic and cDNA. Green arrows represent forward primers, and red arrows represent reverse primers. The sequence not included in the orange box represents the intro of the genomic DNA.



APPENDIX C. Genomic DNA sequence of Georgia Fly 4. An alignment of the clones for Georgia Fly 4 was performed, and gaps were inserted in areas in which extra nucleotide was inserted. The grey area represents identical nucleotide among the clones. *Superkdr* locus is identified in a red box.

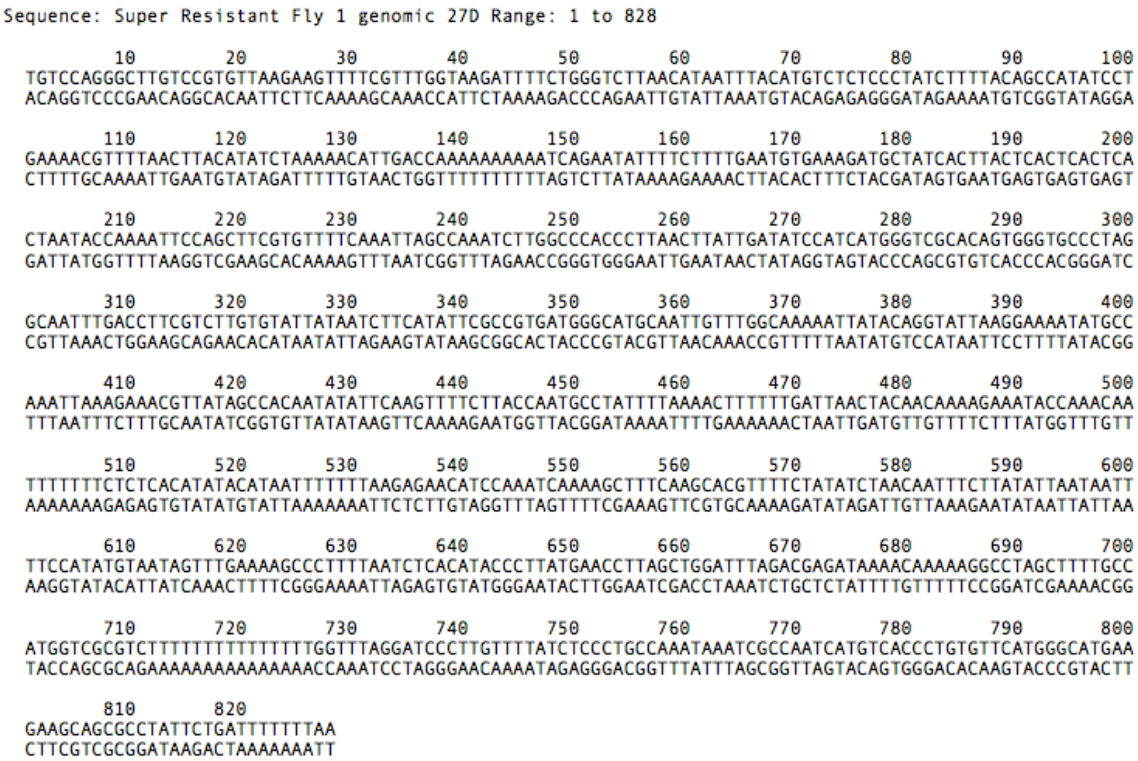


APPENDIX D. Genomic DNA sequence of Pullman Fly 10. An alignment of the clones for Pullman Fly 10 was performed, and gaps were inserted in areas in which extra nucleotide was inserted. The grey area represents identical nucleotide among the clones. Genomic DNA for clone 2A is shorter. *Superkdr* locus is identified in a red box.



APPENDIX E. Genomic DNA of Pullman Fly 11. An alignment of the clones for Pullman Fly 11 was performed, and gaps were inserted in areas in which extra nucleotide was inserted. The grey area represents identical nucleotide among the clones. *Superkdr* locus is identified in a red box.

APPENDIX F. Super Resistant Fly 1 clone 27C. After receiving the sequences corresponding to the primer site from Sanger sequencing, MacVector was used to construct the genomic DNA. This represents the genomic sequence of Super resistant Fly 1 clone 27C with *superkdr* locus expressed in the red box. The complementary strand is also being shown.



APPENDIX G. Genomic Sequence of Super Resistant Fly 1 clone 27 D. After receiving the sequences corresponding to the primer site from Sanger sequencing, MacVector was used to construct the genomic DNA. This represents the genomic sequence of Super resistant Fly 1 clone 27D. The complementary strand is also being shown.

Sequence: SR4H-28BConsensus Range: 1 to 1279

```
         10        20        30        40        50        60        70        80        90       100
TGTCCAGGGCTTGTCCGTGTTAAGAAGTTTTCGTTTGGTAAGATTTTCTGGGTCTTAACATAATTTACATGTCTCTCCCTATCTTTTACAGCCATATCCT
ACAGGTCCCGAACAGGCACAATTCTTCAAAAGCAAACCATTCTAAAAGACCCAGAATTGTATTAAATGTACAGAGAGGGATAGAAAATGTCGGTATAGGA

        110       120       130       140       150       160       170       180       190       200
GAAAACGTTTTAACTTACATATCTAAAAACATTGACCAAAAAAAAAATCAGAATATTTTCTTTTGAATGTGAAAGATGCTATCACTTACTCACTCACTCA
CTTTTGCAAAATTGAATGTATAGATTTTTGTAACTGGTTTTTTTTTTAGTCTTATAAAAGAAAACTTACACTTTCTACGATAGTGAATGAGTGAGTGAGT

        210       220       230       240       250       260       270       280       290       300
CTAATACCAAAATTCCAGCTTCGTGTTTTCAAATTAGCCAAATCTTGGCCCACCCTTAACTTATTGATATCCATCATGGGTCGCACAGTGGGTGCCCTAG
GATTATGGTTTTAAGGTCGAAGCACAAAAGTTTAATCGGTTTAGAACCGGGTGGGAATTGAATAACTATAGGTAGTACCCAGCGTGTCACCCACGGGATC

        310       320       330       340       350       360       370       380       390       400
GCAATTTGACCTTCGTCTTGTGTATTATAATCTTCATATTCGCCGTGATGGGCATGCAATTGTTTGGCAAAAATTATACAGGTATTAAGGAAAATATGCC
CGTTAAACTGGAAGCAGAACACATAATATTAGAAGTATAAGCGGCACTACCCGTACGTTAACAAACCGTTTTTAATATGTCCATAATTCCTTTTATACGG

        410       420       430       440       450       460       470       480       490       500
AAATTAAAGAAACGTTATAGCCACAATATATTCAAGTTTTCTTACCAATGCCTATTTTAAAACTTTTTTGATTAACTACAACAAAAGAAATACCAAACAA
TTTAATTTCTTTGCAATATCGGTGTTATATAAGTTCAAAAGAATGGTTACGGATAAAATTTTGAAAAAACTAATTGATGTTGTTTTCTTTATGGTTTGTT

        510       520       530       540       550       560       570       580       590       600
TTTTTTTCTCTCACATATACATAATTTTTTTAAGAGAACATCCAAATCAAAAGCTTTCAAGCACGTTTTCTATATCTAACAATTTCTTATATTAATAATT
AAAAAAAGAGAGTGTATATGTATTAAAAAAATTCTCTTGTAGGTTTAGTTTTCGAAAGTTCGTGCAAAAGATATAGATTGTTAAAGAATATAATTATTAA

        610       620       630       640       650       660       670       680       690       700
TTCCATATGTAATAGTTTGAAAAGCCCTTTTAATCTCACATACCCTTATGAACCTTAGCTGGATTTAGACGAGATAAAACAAAAAGGCCTAGCTTTTGCC
AAGGTATACATTATCAAACTTTTCGGGAAAATTAGAGTGTATGGGAATACTTGGAATCGACCTAAATCTGCTCTATTTTGTTTTTCCGGATCGAAAACGG

        710       720       730       740       750       760       770       780       790       800
ATGGTCGCGTCTTTTTTTTTTTTTTTGGTTTAGGATCCCTTGTTTTATCTCCCTGCCAAATAAATCGCCAATCATGTCACCCTGTGTTCATGGGCATGAA
TACCAGCGCAGAAAAAAAAAAAAAAAACCAAATCCTAGGGAACAAAATAGAGGGACGGTTTATTTAGCGGTTAGTACAGTGGGACACAAGTACCCGTACTT

        810       820       830       840       850       860       870       880       890       900
GAAGCAGCGCCTATTCTGATTTTTTTAATACTAACCCCAGAGAAGAAATCTACTAATACCTCTTAATTTTTGATAGCATTTATTTTGATAATTAATTTTC
CTTCGTCGCGGATAAGACTAAAAAAATTATGATTGGGGTCTCTTCTTTAGATGATTATGGAGAATTAAAAACTATCGTAAATAAAACTATTAATTAAAAG

        910       920       930       940       950       960       970       980       990      1000
CATATTTGTTTTTCCATATATTTTTTTTTATATAAATGTAAAATCTACGCTTTGCCCGGCTTTTATTTAAAACAAAACTTTTACCAAACAATAAACCAAT
GTATAAACAAAAAGGTATATAAAAAAAAATATATTTACATTTTAGATGCGAAACGGGCCGAAAATAAATTTTGTTTTGAAAATGGTTTGTTATTTGGTTA

       1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
GCAATTACTTTCGTAATTAAACACCACCAAAAAAAAATAATACCACAAATCTATATCCACCATCATCATAATTTACGTAACTTTGGATTCGTTCACCTCA
CGTTAATGAAAGCATTAATTTGTGGTGGTTTTTTTTTATTATGGTGTTTAGATATAGGTGGTAGTAGTATTAAATGCATTGAAACCTAAGCAAGTGGAGT

       1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
ATCAATCAATCAAATATCATCATACAACAGCTTCGTGTATTCAAATTGGCAAAATCTTGGCCCACCCTGAACTTACTCATTTCAATTACGGGCCGGACAA
TAGTTAGTTAGTTTATAGTAGTATGTTGTCGAAGCACATAAGTTTAACCGTTTTAGAACCGGGTGGGACTTGAATGAGTAAAGTTAATGCCCGGCCTGTT

       1210      1220      1230      1240      1250      1260      1270
TGGGTGCATTGGGTAATCTAACATTTGTACTTTGCATTATCATCTTCATCTTTGCCGTTATGGGAATGCAACTTTTCGG
ACCCACGTAACCCATTAGATTGTAAACATGAAACGTAATAGTAGAAGTAGAAACGGCAATACCCTTACGTTGAAAAGCC
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APPENDIX H. Genomic sequence of Super Resistant fly 4. After receiving the sequences corresponding to the primer site from Sanger sequencing, MacVector was used to construct the genomic DNA. This represents the genomic sequence of Super resistant Fly 4 clone 28B with *superkdr* locus expressed in the red box. The complementary strand is also being shown. This was the only clone of the genomic DNA from Super Resistant Fly 4 sent for sequencing.

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