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Genetic variability of the stable fly, Stomoxys calcitrans (L.) (Diptera: Muscidae) assessed on a global scale using Amplified Fragment Length Polymorphism

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By

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University of Nebraska, 2011

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The stable fly, *Stomoxys calcitrans*, is a cosmopolitan pest of livestock and humans. It is a major pest in livestock facilities, where exist excellent breeding sites such as spilled feed mixed with manure. The pestiferous nature and painful bite cause stress to cattle and other animals. Cattle perform avoidance behaviors such as bunching together, standing in water, tail swishing, ear flicking and leg stamping. The stress and avoidance behaviors result in reductions in weight gain or milk production, with an estimated annual economic loss of > $1billion. Therefore, the development of more efficient control methods would benefit the global economy, as well as the animals.

Studying the population genetics of stable flies could provide information on their population dynamics, origins of outbreaks, and geographical patterns of insecticide resistance. Many studies have been conducted on a local scale, most reporting a high level of gene flow between locations. To date, few studies have been conducted on a global scale. Here I report a study of samples acquired from

4 biogeographical regions: Nearctic, Neotropical, Palearctic and Australian. No samples were acquired from the Oriental region.

The results indicate a high level of gene flow on a global scale. F_{ST} and G_{ST} values are low, and Nm values very high. The tests of neutrality suggest population expansion, and tests for genetic differentiation simply reported "no differentiation". AMOVA results show the majority of genetic diversity is within groups, and very little among groups. These results suggest that stable flies have a panmictic population, with no isolation by distance or across geographical barriers.

DEDICATION

This dissertation is dedicated to loved ones lost along this journey. To my dad, Earl Richard Kneeland, who taught me that common sense was as important as 'book learning', and truth, honesty and loyalty more treasured than gold.

To my mom, Gloria Ann Kneeland, a little lady with a huge heart, who taught me to go for what I want. I couldn't have chosen better parents, and I will love you always.

To Clayton Blaine Bomber, my free-spirited cousin. May you play your guitar for a choir of angels.

And to all my family and friends who are gone but remain in my heart.

I miss you all very much, and I am a better person for having known and loved you.

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Literature Review

INTRODUCTION

The stable fly, *Stomoxys calcitrans* (L) (Diptera: Muscidae) is a cosmopolitan ectoparasite of livestock, wildlife and humans (Brues 1913; King and Lenert 1936; Simmons 1944; Hansens 1951; Berry and Campbell 1985; Mullens and Meyer 1987; Meyer and Shultz 1990; Thomas et al. 1990; Skoda et al. 1991; Skoda and Thomas 1993; Campbell 1995; Campbell et al. 2001; Kaufman 2002; Veer et al. 2002; Jeanbourquin and Guirin 2007; Taylor and Berkebile 2008). Both sexes are hematophagous (Brain 1912; Skidmore 1985; Campbell 1995), and feed primarily on the legs of the host animals (Skoda and Thomas 1995; Campbell et al. 2001; Mullens and Peterson 2005; Mullens et al. 2006). Stable flies react to both olfactory and visual stimuli for the location of hosts (Gatehouse 1967; Allan et al. 1987; Alzogaray and Carlson 2000; Carlson et al. 2000; Birkett et al. 2004). At least one blood meal is required for reproduction (Skidmore 1985), but they will sometimes feed several times per day (Powell and Barringer 1995; Mullens et al. 2006). Oviposition occurs on decaying organic matter such as spilled hay or grain, preferably combined with feces (Berkebile et al. 1994). Stable fly parasitism has the greatest effect on the livestock industry, where animals are confined to stables or pastures, providing a pristine environment for both feeding and oviposition (Berkebile et al. 1994; Campbell 1995; Hogsette 1998; Broce et al. 2005). Their painful bite stresses confined livestock, causing them to bunch together or perform repellent behaviors, which results in significant reductions in weight gain and milk production (Campbell et al. 1977; Hall et al. 1983; Catangui et al. 1993). The stable fly is the primary pest

of cattle in the United States, causing major annual economic losses estimated to be greater than $1billion to cattle in feedlots and dairies as well as poultry farms (Suszkiw and Core 2003; Taylor and Berkebile 2006; Roeder 2007).

Stable fly outbreaks also occur along beaches, causing considerable economic damage to the tourist trade (King and Lenert, 1936; Simmons and Dove 1941, 1942; Dove and Simmons, 1942; Simmons 1944; Hansens, 1951; Williams and Rogers 1976; Hogsette and Ruff 1985; Jones et al. 1991; Koehler and Kaufman 2006). In Northwest Florida they migrate on the north winds from inland livestock areas to the beaches (Fye et al. 1980), where they breed in marine grasses such as seaweed, turtle grass (*Thalassia testudium*), and manatee grass (*Halodule wrightii*) (King and Lenert 1936; Dove and Simmons 1942). They also breed in peanut litter (Simmons 1944) and waste celery (Simmons and Dove 1942). In New Jersey, stable flies were reported to breed in the marine grasses that washed onto shore, and outbreaks were concurrent with west winds (Hansens 1951).

In addition to their detrimental impact on livestock, the presence of stable flies causes legal issues between farmers and the urban population encroaching on the farmland (Meyer et al. 1990; Thomas and Skoda 1993; Campbell 1995; Suszkiw and Core 2003). Stable flies are also known to be mechanical vectors of disease (Brues 1913; Turell and Knudson 1987; Fischer et al. 2001; Veer et al. 2002; Szalanski et al. 2004; Bittencourt and De Castro 2004; Mramba et al. 2007). Due to the global distribution and adverse effects of stable fly activity, more efficient control measures are needed. Research has been carried out in areas such

as chemical, biological and mechanical control mechanisms, Integrated Pest Management (IPM) practices, dispersal and overwintering, population genetics and gene flow, physiology, and DNA analysis (Campbell and Hermanussen 1971; Bailey et al. 1973; Black and Krafsur 1985; Berkebile et al. 1994; Campbell 1995; Szalanski et al. 1996; Ratcliffe et al. 2002; Skovgard and Nachman 2004; Broce et al. 2005; De Oliveira et al. 2005; Gilles et al. 2007; Taylor et al. 2007). However, no single method of stable fly control has been successful thus far. Current control methods have had no significant success in maintaining stable fly populations below the economic injury threshold (Patterson et al. 1981; Meyer et al. 1990; Clymer 1992; Hall 1992; Pickens 1992; Seymour and Campbell 1993; Andress and Campbell 1994; Cilek and Greene 1994; Campbell 1995; Weinzierl and Jones 1998; Guglielmone et al. 2004; Macedo 2004; Skovgård and Nachman 2004; Foil and Younger 2006; Taylor and Berkebile 2006; Gilles et al. 2007; Mihok and Carlson 2007), although Integrated Pest Management (IPM) practices help to reduce stable fly populations at the local scale (Campbell and Wright 1976; Lazarus et al. 1989; Campbell 1995; Skoda et al. 1996; Thomas et al. 1996).

Further research related to stable fly populations, origins of outbreaks, and dispersal patterns could lead to the development of more effective control strategies.

LIFE HISTORY AND BIOLOGY

Stomoxys calcitrans belongs to the family Muscidae and subfamily Stomoxyinae, which includes stable flies, horn flies and buffalo flies (Zumpt

1973). There are 18 recognized species in the genus *Stomoxys* (Zumpt 1973). *S. calcitrans* is distributed worldwide and is the only species recorded in North America (Marquez et al. 2007). Stomoxyinae are characterized by their piercing proboscis and maxillary palpi. The proboscis is formed by three sclerotized parts: the labium, hypopharynx, and labrum. At rest, it is extended horizontally and can be seen beyond the head (Brain 1912). In *S. calcitrans,* the palpi are single-segmented and approximately ¼ the length of the proboscis (Brain 1912; Zumpt 1973). After puncturing the skin of the host, saliva is injected into the wound via the hypopharynx, then blood is drawn up into the pharynx via a tube composed of the hypopharynx and labrum combined (Brain 1912; Zumpt 1973). The mouthparts are alike in males and females, and both sexes are hematophagous (Brain 1912).

Stable flies may take a blood meal several times per day, and are persistent feeders (Schofield and Torr 2002). Females require at least three blood meals for ovarian development, and daily blood meals thereafter (Moobola and Cupp 1978; Chia et al. 1982; Veer et al. 2002; Schofield and Torr 2002). Anderson (1978) reported that males require a blood meal to properly inseminate the females; taking a blood meal increases their virility and the aggressiveness of their mating behavior. In addition to blood meals, stable flies also feed on nectar. Lee and Davies (1979) reported that feeding on sugar increased stable fly longevity. Moobola and Cupp (1978) report that blood feeding, not sugar, increases longevity. However, they report that sugar will increase survival rate five times more than just water if no blood meals are available. Jones et al. (1992) reported

that nectar feeding may supply energy for dispersing flies when no hosts are available to obtain a blood meal, but being fed sugars ad libitum may be detrimental to reproductive rate, even when given daily blood meals.

Female stable flies oviposit in moist, decaying organic matter such as pure manure (Brain 1912; Miller 1992; Hall 1992), silage, hay, grain or haylage mixed with manure (Berkebile et al. 1994; Campbell 2006), grass clippings, compost piles, dumpsters (Suszkiw and Core 2003), and seaweed (King and Lenert 1936). It has been shown that females are attracted to substrates with active microbial communities, because certain bacterial species, such as *Citrobacter freundii*, may aid in larval development (Romero et al. 2006). The female lays 100-400 eggs during her lifetime, at approximately 20 eggs per ovarian cycle. Two blood meals are required for each cycle (Skidmore 1985; Campbell 1997).

Stable fly development is holometabolous, consisting of the egg, 3 larval instars, pupa and adult (Zumpt 1973; Skidmore 1985). The eggs are white, about 1mm long, convex ventrally with a longitudinal groove. They hatch in 2-4 days (Brain 1912; Zumpt 1973; Skidmore 1985). Larvae grow to about 10 mm, and the larval stage lasts 2-3 weeks under favorable conditions, but unfavorable weather conditions may extend it up to 80 days (Brain 1912; Skidmore 1985). Larvae migrate to drier areas of the substrate to pupariate; pupariation lasts from 2-30 days. Puparia are brown in color and approximately 6 mm long (Brain 1912; Skidmore 1985). Adults are about 7 mm in length, with 4 black longitudinal stripes on the thorax, and a checkerboard pattern of dark spots on the abdomen

(Brain 1912; Zumpt 1973; Skidmore 1985). They can take a blood meal within hours of emergence (Skidmore 1985).

The development period from egg to adult is dependent on temperature (Melvin 1931; Simmons 1944; Kunz et al. 1977; Watson et al. 1994; Campbell 1997; Lysyk 1998; Campbell and Thomas 1999; Gilles et al. 2005a,b; Barker et al. 2007). Melvin (1931) studied the development of stable flies in the laboratory at 25ºC and 30ºC. He reported the incubation period of eggs to be 32.5-35.2 hours (mean 33.4) at 25ºC, and 25.0-28.5 hours (mean 26.5) at 30ºC. Combined larval and pupal periods were observed on 2 different rearing media. On alfalfa meal and wheat bran, mean development time at 25ºC was 377 hours (15.7 days), and 311.7 hours (13 days) at 30ºC. Mean development time at 30ºC on ground oats took 320.2 hours (13.3 days) and 326.1 hours (13.6 days) in two experiments. Melvin had difficulty rearing the flies, with only 10% adult emergence. Simmons (1944) observed stable fly development under laboratory conditions, incubating eggs at 28ºC, and larvae and pupae at 30ºC. Minimum observed time before egg hatch was 19 hr, maximum 120 hr, mean minimum 39.65 hr, mean maximum 65.1 hr., and overall mean time until hatch was 52.3 hr. Duration of larval development was recorded as overlapping instars. First instars were present from egg hatch to the 80th hr, 2nd instar from the 44th-144th hr, and third instar from the 97th hr until pupation. Minimum time until pupation was 148 hrs. For mean calculations, the larval and pupal stages were combined, with a mean developmental duration of 165.8 hrs, or 6.9 days. Separating out the pupal stage, the mean duration of this stage was 6.55 days at 28º-32ºC. The reported life cycle

from egg to adult was a minimum of 13 days, with a maximum of several months under adverse climatic conditions. Simmons (1944) reported that the duration of the larval period was longer (11.2 days) during winter months, even though the temperature was sustained at 30ºC.

Kunz *et al.* (1977) studied development at 3 different temperatures. Mean duration of development from egg to adult emergence was 400 hrs (16.6 days) at 23.9ºC, 280 hrs (11.6 days) at 29.4ºC, and 290 hrs (12.1 days) at 35.0ºC. Lysyk (1998) studied the relationship between temperature and life history, rearing stable flies at 15, 20, 25, 30, and 35ºC. The observed median immature development times ranged from 62 days at 15ºC-<12 days at 30ºC, with development at 20ºC being 29 days.

Gilles *et al.* (2005a) studied the effect of temperature on developmental time of *Stomoxys calcitrans* and *S. niger.* The mean development time observed for *S. calcitrans* from egg to adult was 70.66 days at 15ºC, 32.36 days at 20ºC, 16.65 days at 25ºC, 12.92 days at 30ºC and 13.17 days at 35ºC. Adult longevity (Gilles *et al.* 2005b) was observed to be highest at 20ºC: 23.73 days for females and 25.69 days for males.

The results of these studies show that the developmental time of *S. calcitrans* from oviposition to adult emergence ranges from 11 days to several months, depending on the ambient temperature.

DISTRIBUTION AND CLIMATIC VARIABLES

Stomoxys calcitrans is native to Palaearctic regions of the Old World, and is now distributed worldwide, where it is most abundant in temperate regions

(Brues 1913; Zumpt 1973; Skidmore 1985; Szalanski et al. 1996). It likely arrived in North America with the immigrants from Europe, and is reported to have been abundant in Philadelphia as early as 1776 (Brues 1913). Distribution patterns vary with climate, with precipitation and temperature having significant effects on population dynamics (Cruz-Vazquez et al. 2004; Mullens and Peterson 2005; Rodriguez-Batista et al. 2005; Taylor et al. 2007). In the Midwestern United States, stable fly populations follow a bimodal pattern of seasonal activity. They begin to appear in late March or early April and increase in numbers until they peak at the end of June. During the warmest part of summer the numbers recede, then peak again in mid-September (Mullens and Peterson 2005; Taylor et al. 2007). In California, the population peaks only once, in the late spring, but an active population remains throughout the year (Mullens and Peterson 2005). A study in Brazil showed stable fly activity during the spring and summer, (the time of year with the most precipitation) with a peak during November and December, and a smaller peak at the beginning of fall; there was no activity during the winter months. The results of this study suggested that stable fly population increases were related to rainfall (Rodriguez-Batista et al. 2005). A study in an arid region of Mexico, however, found no correlation between rainfall and stable fly populations. Instead, their results showed that the increase in population was correlated with relative humidity, and temperature was the primary factor in decreasing populations (Cruz-Vazquez et al. 2004).

The results of these studies suggest that climatic factors such as rainfall, relative humidity, and temperature all have an effect on stable fly populations. In

the warmer climates populations declined during midsummer, showing sensitivity to high temperatures (Mullens and Peterson 2005). The correlation between population increases and rainfall or relative humidity indicates that stable flies require sufficient moisture to survive. Therefore climatic variables need to be considered in any population studies.

STABLE FLY PARASITISM OF LIVESTOCK

Stable flies have a pestiferous nature and their bite is extremely painful. Brain (1912) described the bite as having "a decided stab after the first puncture had been completed". The pain and annoyance to cattle results in economic losses due to reductions in weight gain, feed efficiency and milk production, as well as the expense of control measures (Hall et al. 1983; Campbell 2006; Mullens et al. 2006; Roeder 2007).

Many studies have been performed on the effect of stable flies on the weight gain of cattle (Campbell et al. 1977; Catangui et al. 1993, 1995, 1997; Campbell et al. 2001; Broce et al. 2005), and the economic injury level on cattle in feed lots is estimated to be an average of five flies per front leg (Campbell et al. 2001). Cattle have developed repellent behaviors to dislodge the flies, such as leg stamps, tail flicking, skin twitches and head throws (Campbell 1997; Schofield and Torr 2002; Mullens et al. 2006). They will also bunch together or stand in water in an attempt to escape the fly annoyance (Campbell 1995; Campbell and Thomas 1999). The bunching behavior may cause heat stress, which adds to the overall discomfort of the animal (Wieman et al. 1992; Campbell 1995; Catangui et al. 1995, 1997). Being stressed by the flies and engaging in repellent and

avoidance behaviors, the cattle do not feed, which results in decreased weight gain from .1 pound to .48 pound per head per day, and a decrease in milk production of up to 40 percent (Campbell 1995).

Stable flies were originally considered pests of cattle confined to feed lots, but they are now recognized as a pest of pastured cattle as well (Campbell et al. 2001; Mullens et al. 2006). There are numerous breeding sites on feed lots, such as drainage areas, or areas where manure and soil or spilled feed can accumulate, such as along fences, in corners of pens, and at the edge of feed handling and storage areas (Campbell 1997). In pasture environments immatures have been found under large round bales or where the round bales are distributed and a portion of the hay is wasted. The wasted hay mixes with manure and urine, and if the bales are placed in the same area consistently, the substrate becomes attractive as a breeding site for the stable flies (Hall et al. 1982; Berkebile et al. 1994; Broce et al. 2005; Talley et al. 2009). Detritus from large round bales also provides a competent site for overwintering (Berkebile et al. 1994).

CONTROL METHODS

Many types of control methods for the stable fly have been researched, including insecticides, baits, biological control, and sterile insect release methods (Campbell and Hermanussen 1971; Campbell and Wright 1976; Campbell and Doane 1977; LaBrecque et al. 1981; Patterson et al. 1981; Williams et al. 1981; Gersabeck et al. 1982; Black and Krasfsur 1985; Andress and Campbell 1994; Hammack and Hesler 1996; Bartlett and Staten 1996; Floate et al. 2001; Ratcliffe et al. 2002; Guglielmone et al. 2004; Kaufman et al. 2005; Geden et al. 2006;

Taylor and Berkebile 2006; Mihok and Carlson 2007; Mihok et al. 2007). No single control method tested thus far is effective in decreasing stable fly populations below the economic injury threshold (Meyer et al.1990; Seymour and Campbell 1993; Cilek and Greene 1994; Thomas et al. 1996; Macedo 2004; Taylor and Berkebile 2006). The current procedure recommended as the most efficient means of stable fly control is an Integrated Pest Management approach, which stresses the importance of sanitation, and utilizes a combination of the methods listed above (Watson et al. 1994; Powell and Barringer 1995; Campbell and Thomas 1999; Campbell 2006).

Sanitation is an important control factor in feedlots and dairies. The removal of organic waste such as spilled feed and manure, regular cleaning, and good drainage decreases larval development sites. Manure can be spread out to dry, or piled in mounds with sufficient drainage. During wet weather the edges of the mounds should be scraped away in order to dry (Campbell 1995, 1997, 2006; Watson et al. 1994; Campbell and Thomas 1999).

Chemical controls can be effective for short periods, but require that the treatment be repeated regularly. Animal sprays give some relief, but are washed off when the cattle walk through damp grass or stand in water for avoidance (Campbell and Hermanussen 1971; Watson et al. 1994; Campbell 1997). Residual sprays applied to fly resting surfaces such as fences, buildings and vegetation can be effective for up to 14 days, provided that extensive alternate resting places are not accessible nearby. They may also be washed off in the rain or decomposed by direct sunlight (Watson et al. 1994; Campbell 1997; Campbell

and Thomas 1999). Area sprays are effective where flies congregate, but are not a

long term solution as they only kill the flies they contact (Watson et al. 1994;

Campbell 1997; Campbell and Thomas 1999).

Feed-through fly controls, which contain larvicides or insect growth

regulators, pass through the digestive system of the host animal and remain in the

feces. These controls are not effective for the control of stable flies, as they do

not oviposit in fresh cow manure (Campbell 1997).

The application of larvicides on fly breeding areas is not an effective

control method. The acidity of the substrate decomposes the larvicides rapidly,

and frequent application promotes insect resistance to the chemicals (Campbell

1997; Campbell and Thomas 1999).

Releasing parasitic wasps is not an effective means of stable fly control.

The numbers of flies are not significantly reduced, and the cost of the parasitoids

is more than their economic benefit (Andress and Campbell 1994; Campbell

1997, 2006).

Baits are not an effective method against stable flies, since they feed on

blood and are not attracted to the baits (Campbell 2006). Traps, however, have

been effective at capturing stable flies. They are attracted to certain olfactory

stimuli such as CO_2, ammonia, and phenylpropanoid compounds (Gatehouse

1967; Hammack and Hesler 1996), and visual stimuli such as Alsynite fiberglass

which reflects UV light (Gersabeck et al. 1982; Black and Krafsur 1985; Allan et

al. 1987) and the Nzi trap which attracts stable flies with a blue colored paint

(Mihok 2007; Mihok and Carlson 2007). Stable flies respond to wavelengths of

light in the UV range (360 nm) and the blue range (450-550 nm) (Allan *et al*. 1987).

A sterile insect release program combined with IPM practices was conducted in St. Croix, US Virgin Islands with some success (LaBrecque et al. 1981; Patterson et al. 1981; Williams et al. 1981; Willis et al. 1981, 1983; Bartlett and Staten 1996). However, the success of the sterile insect technique would be unlikely with stable flies on a large scale. Although 4 out of 5 feasibility factors for the method (Knipling 1955) apply to stable flies, the populations may be too large, and immigration of wild flies into an area would be a problem due to their long distance dispersal capability (Bailey et al. 1973; Hogsette and Ruff 1985).

STABLE FLIES AND DISEASE

In addition to being a pest of livestock and other animals, stable flies are known to be mechanical vectors of many diseases. In India, they are abundant pests of animals such as sambar, deer, mithan, blackbuck, and various carnivores, and are mechanical vectors of surra disease and equine infectious anemia (EIA) virus (Veer et al. 2002). In a study in the Czech Republic and Slovakia, two species of *Mycobacterium* were isolated from adult stable flies at a farm raising both cattle and pigs (Fischer et al. 2001). A laboratory study by the US Army Medical Research Institute of Infectious Diseases showed that stable flies can mechanically transmit *Bacillus anthracis*, the agent of anthrax, and Rift Valley fever virus (Turell and Knudson 1987). *Bartonella henselae* type M was isolated from stable fly DNA during a study in California (Chung et al. 2004).

Enteric bacteria are transmitted by stable flies, as could be expected from their association with animal feces. *Campylobacter* spp. were detected in stable flies collected from turkey production facilities in Arkansas (Szalanski et al. 2004). In a laboratory experiment in which stable flies were orally inoculated with *Enterobacter sakazakii*, over 50% of the flies still carried the pathogen 20 days after inoculation. *E. sakazakii* also had significant positive effects on stable fly development (Mramba et al. 2007). *Escherichia coli* have a positive effect on stable fly larval development when in a mixed bacterial community. The larvae ingest the *E. coli* but do not digest it readily, so cattle feeding on silage containing the infected larvae may ingest the bacteria (Rochon et al. 2004). Puparia of infected larvae have also been found to contain large amounts of *E. coli* (Rochon et al. 2005).

Some bacteria are pathogenic to stable flies, such as *Aeromonas* sp., *Pseudomonas aeruginosa* and *Serratia marcescens* (Lysyk et al. 2002). Further research on the efficacy of such pathogens in causing mortality in stable flies could be another potential control method.

STABLE FLY GENETICS

The majority of the genetic research on stable flies to date has been focused on genetic variation among or between populations, in an attempt to determine their origin and dispersal patterns, and genetic control strategies such as sterile male release programs.

Genetic control methods

Following the success of the screwworm (*Cochliomyia hominivorax*) sterile male release program on the island of Curacao, Knipling (1955) suggested several applications for this method, including the control of small numbers of naturally occurring pests, newly established populations of pests, or in conjunction with other integrated pest management practices (Knipling 1955). In 1974, a sterile male release program was initiated on St. Croix, US Virgin Islands, as a component of an integrated pest management program to control stable fly populations. At that time, populations averaged 9.5×10^5 during the wet season and 2.5×10^5 during the dry season (La Brecque et al. 1981). The program included mass rearing of 250,000-300,000 flies per day, with 70,000 required for colony maintenance. Males from 24-48 h old were sterilized by exposure to 2 kR of cobalt-60 gamma radiation (Williams et al. 1981). For 18 months during 1976-1977, sterile males were released at a rate of 1×10^5 per day, 5 days per week. By the end of the project, the stable fly population was reduced to ~350 flies, although not entirely eliminated. However, after cessation of the project, during just 3 generations, populations rose to 210,000 (Patterson et al. 1981).

During the 1980's, genetic mutations were investigated as potential control mechanisms. The stable fly has 5 pairs of chromosomes, with 4 recessive mutations being reported at the time. (Hunter et al. 1992) Chromosome 1 contains the sex locus, chromosomes 2, 3, and 4 contain the carmine eyes (ca) mutant, the black pupa (bp) mutant, and the rolled down wing (rd) mutant, respectively (Willis et al. 1981; Willis et al. 1983; Hunter et al. 1992). The fourth

recessive mutant, subcostal incomplete (sci) was reported in 1992 (Hunter et al. 1992). Possibilities for stable fly control using mutations consisted of DNA recombination techniques such as reciprocal translocations (Willis et al. 1981), and genetic sexing techniques to eliminate females (Willis et al. 1983; Seawright et al. 1986; Bartlett and Staten 1996). The latter was accomplished using chemical susceptibility genes or the black pupa mutant gene (Willis et al. 1983; Seawright et al. 1986; Bartlett and Staten 1996). These methods could be effective when combined with an integrated pest management program (Bartlett and Staten 1996).

Population genetics

Due to the ubiquity and pestiferous nature of stable flies, it would be an advantage to determine the origin of seasonal populations and their dispersal patterns. It has been shown that immature stable flies are able to overwinter in livestock areas, in build-ups of substrate that retain some heat, such as piles of wasted hay, silage, grass clippings and compost piles (Berkebile et al. 1994; Broce et al. 2005). However, the source of the populations remains unknown (Broce et al. 2005). Stable flies are able to fly long distances and to disperse with the wind (Bailey et al. 1973; Gersabeck and Merritt 1985; Hogsette and Ruff 1985; Beresford and Sutcliffe 2009). In a flight-mill test, a stable fly was reported to fly 29.11 km in 24 hours (Bailey et al. 1973). A study in Northwest Florida, in which stable flies were marked with fluorescent dust, released, then recaptured in Williams traps, reported a flight range of 225 km. In this area, stable fly populations become so dense on the beaches that tourism comes to a standstill. It

is believed that the flies migrate to the beach areas, since there are few breeding sites available (Hogsette and Ruff 1985).

Several studies of stable fly population genetics have attempted to determine dispersal patterns and sources of the populations. Jones et al. (1991) tested allele frequencies of 10 different enzymes using protein electrophoretic analysis. They collected 100 stable flies from each of 37 sample sites from 1982-1985, including 8 beach sites in Florida, 7 dairies in Florida, dairies in Indiana, Maryland, New York and Texas, and a feedlot in Nebraska. Their data showed very little variation among populations, suggesting a high level of gene flow across the United States. They suggested that the movement of stable flies is due to drifting on weather fronts rather than migratory behavior, and that flies on the Florida beaches could have originated as far away as Nebraska (Jones et al. 1987; Jones et al. 1991).

Szalanski et al. (1996) performed a study using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. They screened portions of the cytochrome oxidase (CO) I, II, and III mitochondrial DNA genes, NADH 4 and 5 genes, and nuclear ribosomal DNA genes. Their samples were primarily from Nebraska, with samples from Texas and Manitoba included. The results were similar to Jones et al. (1991), and they also reported very low levels of genetic differentiation among populations (Szalanski et al. 1996).

Gilles et al. 2007 also reported low levels of genetic differentiation in the stable flies on dairy farms on La Reunion Island, a small (2507 km^2) island in the

Indian Ocean east of Madagascar. They noted more differentiation at two sites which used dissimilar farming methods. The methods used in this study were the analysis of 7 microsatellite loci which had been sequenced previously (Gilles et al. 2004). The 7 loci were amplified by PCR and then sequenced (Gilles et al. 2007).

Contradictory data were reported by Marquez et al. (2007). They examined r16S and COI mitochondrial DNA loci by amplifying the DNA and sequencing the PCR products. They collected 20 samples (totaling 277 individual flies) from 11 countries: 4 in the Palearctic region, 3 in the Oriental region, 2 in the Ethiopian region, Brazil in the Neotropical region and the United States in the Nearctic region. This group reported considerable variation in the mtDNA of stable flies, with 22% variation in the 809 nucleotides observed (Marquez et al. 2007). The Nearctic samples were most closely related to the Palearctic and Neotropical samples. Their results support the hypothesis that stable flies were introduced into the New World during colonial times.

Dsouli-Aymes et al. (2011) reported similar results to those of Marquez et al. (2007). They acquired stable flies from 20 countries in 6 zoogeographical regions: 2 in the Oriental region, 10 in the Afrotropical region, 5 in the Palearctic region, Canada in the Nearctic region, Colombia in the Neotropical region and New Caledonia in the Oceanic region. They analyzed mitochondrial (COI, Cyt-b, ND1-16S) and nuclear (ITS2) DNA by amplification and sequencing. Their results showed the Oriental region diverging from the other regions as a separate, isolated lineage which did not contribute to dispersal of stable fly populations.

Instead, they suggest that Palearctic, Nearctic, Neotropical and Oceanic groups originated in the Afrotropical region.

Other genetic research

The hematophagous nature of stable flies generates interest in the innate immune responses developed by this insect to resist the pathogenic microbes it is exposed to during feeding. Of particular interest are the antimicrobial peptides (AMPs), most of which are produced by the fat body. Three AMPs specific to the stable fly have been sequenced, which are unique in that they are specific to the anterior midgut. Two are defensins: stomoxys midgut defensins (smd) 1 and 2 described by Lehane et al. (1997), which exhibit anti-Gram negative activity. The third, stomoxyn, was identified by Boulanger et al. (2002). It is a cecropin-like peptide which exhibits a wide spectrum of anti-microbial activity against bacteria, fungi, and trypanosomes (Boulanger et al. 2002).

GENETIC ANALYSIS TECHNIQUES

A variety of techniques are utilized in genetic studies, depending on the nature of the research, including the analysis of proteins, nuclear DNA and mitochondrial DNA (mtDNA). DNA technology advances rapidly, and methods are continually modified for optimum performance (Jones et al. 1987; Jones et al. 1991; Gilles et al. 2004). Interest in the mitochondrial genome dominates current research (Szalanski and Owens 2003; De Oliveira et al. 2005; Marquez et al. 2007), and in 2008, Oliveira et al. described the sequence of the entire mitochondrial genome of the horn fly, *Haematobia irritans* and the "almost complete" mitochondrial genome of the stable fly.

Protein Electrophoresis

Water-soluble proteins are extracted from the sample and absorbed onto a paper wick. The wick is placed in the well of a starch or acrylamide gel, and the gel is placed into a buffer. Electricity is applied to the gel buffer, and electrophoresis continues for several hours. The gel is then sliced horizontally and the thin slice is incubated with a stain that contains a substrate that is specific to the target enzyme. The gel is visualized in a light box and the bands are compared to known samples (Jones et al. 1987; Avise 2004). This method is simple and not too time consuming. Many different codominant alleles can be identified at numerous loci, and the data can be easily replicated, although there is only moderate resolution of genetic differences. This method is useful for studying population genetics and relationships between species (Avise 2004).

RFLP and PCR-RFLP

DNA is extracted from the sample and restriction enzymes are added to the DNA. Restriction enzymes cleave the DNA at specific sites which are usually 4, 5, or 6 nucleotides in length. The recognition for *Eco*RI, for example, is the base sequence 5'-GAATTC-3' (Avise 2004). The cleaved DNA is then electrophoresed on agarose or acrylamide gel to separate the different sized fragments. The gel is transferred to a nylon membrane, where radioactive probes are added. The probes bind to the DNA fragments, the gel is dried and an X-ray film is placed over it. When developed, the results are an autoradiograph on which the DNA fragments can be visualized. This method is called Southern hybridization, or Southern blot (Hoy 2003; Avise 2004).

The development of the polymerase chain reaction (PCR) in the 1990's facilitated the RFLP technique, allowing for extraction of DNA from much smaller samples. PCR is an automated method of exponentially amplifying DNA, which involves 20-30 cycles of 3 different temperatures: 94ºC for 20 seconds for denaturation of the template DNA, 55ºC for 20 seconds for annealing primers to the template, 72ºC for 30 seconds for extension of the DNA. The PCR cycles must be optimized for each organism. Reactions require a PCR mix containing dNTPs, *Taq* DNA polymerase, PCR buffer, $MgCl_2$, specific forward and reverse primers and template DNA. During the denaturation step, the double strands of the DNA separate. Specific primers (added to the PCR mix) anneal to the ends of the target sequence on the DNA, and the DNA between the primers is replicated. Each cycle doubles the amount of DNA, allowing for millions of copies to be replicated in a very short time (Hoy 2003; Avise 2004; Varsha 2006). The Southern blot has been replaced by the addition of dyes such as ethidium bromide to the gel, which can then be visualized under UV light (Clark 2000).

PCR-RFLP is used for both nuclear and mitochondrial DNA analysis (Szalanski et al. 1996). It requires more time than other techniques, but the data can be replicated without difficulty. The bands indicate codominant alleles using nDNA and maternal alleles using mtDNA. Usually few loci are assayed, but many alleles per loci can be identified (Avise 2004).

RAPDs

Randomly amplified polymorphic DNA (RAPD) is a technique in which universal primers are used to amplify unknown DNA sequences. Short primers

are used which have the ability to generate multiple fragments. Polymorphisms are detected when the PCR product is separated by gel electrophoresis. Using the RAPD technique, one is able to detect small differences in populations, species, and individuals because it generates numerous DNA fragments and many loci can be analyzed in one reaction. It requires only a small amount of DNA and is relatively inexpensive (Hoy 2003; Christen 2008). This method is a popular method in population biology, but is not very reproducible and only reveals dominant markers (Jones et al. 1997).

AFLP

Amplified fragment-length polymorphism is a technique that combines the RFLP and RAPD methods (Bensch and Akesson 2005). Genomic DNA is digested by two restriction enzymes, usually *Eco*RI and *Mse*I. Adapters (short DNA fragments complimentary to the loci cut by the enzymes) are ligated to the ends of each fragment. Then a preamplification is run with primers that are complimentary to the adapter and the enzyme, plus an additional nucleotide. Following preamplification, a small amount of the product is added to a PCR mix containing specific primers, amplified and separated on polyacrylamide gel (Avise 2004; Bensch and Akesson 2005).

AFLP is a relatively inexpensive method of screening a large number of loci, and has become a useful tool in the field of population genetics (Bensch and Akesson 2005). Although it reveals only dominant markers, and is based on the presence or absence of a band (Bonin et al. 2007), it has been shown to be as

efficient as techniques that separate co-dominant markers, such as microsatellites (Bensch and Akesson 2005).

DNA sequencing

Historically, two methods were developed for DNA sequencing, one by Maxam and Gilbert, the other by Sanger. Each involved isolating and denaturing the DNA, labeling the ends with radioactive primers, and separating the fragments by gel electrophoresis. Each method required four reactions, one for each deoxynucleotide (Avise 2004). Currently, sequencing is predominantly automated, using PCR thermal cyclers connected to sequencing machines (Avise 2004).

Other techniques

Other methods of DNA analysis could be useful in analyzing stable fly population genetics. Short interspersed elements (SINEs), single-strand conformational polymorphism (SSCPs), single nucleotide polymorphism (SNPs), techniques described in Avise (2004), are methods that could be employed in population genetics. Essentially, experimental designs should consist of available techniques that provide the maximum quantity and quality of data with the least expenditure of time and money.

CONCLUSIONS

Effective control strategies for stable fly populations are of primary importance in the livestock industry, as well as other affected areas such as tourism on Florida beaches and the convergence of urban and agricultural habitats. Because stable flies are distributed world-wide, and have the ability to

travel long distances, single control methods have unsatisfactory results. The application of Integrated Pest Management strategies appears to be the most successful approach so far. Improved sanitation, such as removal of manure and wasted feed, washing of stalls and milking areas, and good drainage systems are important practices to eliminate breeding areas and larval development sites. Insecticides, traps and biological controls aid in reducing fly numbers. However, these practices do not reduce stable fly numbers to an acceptable level.

An enormous amount of possibilities exist in the study of stable flies and their control, and the logical direction is to implement control strategies at their source. On a local scale, more efficient methods could be developed to eliminate larval development and overwintering sites. New biological control methods could be investigated, such as recombinant DNA technologies that block the production of essential hormones or antimicrobial peptides. Further study on dispersal patterns using release and recapture techniques could aid in locating local sources of stable fly outbreaks.

Further research is needed in stable fly population dynamics, to investigate dispersal patterns and possible sources of stable fly populations. To date, studies have indicated low differentiation and high gene flow among populations, even on a small island scale (Szalanski et al. 1996; Gilles et al. 2007), although Marquez et al. (2007) and Dsouli-Aymes et al. (2011) reported a high level of variation in stable fly mitochondrial genomes on a global scale. Each group used different DNA analysis techniques and different sample areas. Szalanski et al. (1996) examined populations in Nebraska by PCR-RFLP; Gilles et al. (2007)

concentrated on La Reunion Island using microsatellites which were amplified and sequenced. Recently, two studies have been done on the global scale. Marquez et al. (2007) acquired samples from 11 different countries in 5 biogeographical regions, using direct sequencing of mtDNA as their analysis technique; Dsouli-Aymes et al. (2011) acquired samples from 20 countries distributed among 6 biogeographical regions and used amplification and sequencing of both mitochondrial and nuclear genes. The global studies differ from the others in that they found greater differentiation between subpopulations than those at the local scale. Different techniques could generate more informative data on stable fly population dynamics. Larger sample sizes could prove beneficial, and many more loci could be evaluated using AFLP, including DNA sequences that are as yet undescribed. Examining populations on a global scale, such as the research by Marquez et al. (2007) and Dsouli et al., would be more likely to show differentiation between populations, so it would be logical to begin at a large scale and work toward a smaller scale.

Technology in the fields of genetics and molecular biology advances rapidly, and concomitantly, the capability for further understanding of stable fly biology and habits increases. Since the purpose of stable fly research is population control, advancement is a step toward success. The more knowledge we acquire about stable fly population genetics, the further we are toward understanding the methods to control their numbers. While investigating control methods, we may also discover beneficial ecological niches for the stable fly that compel us to more readily accept their presence.

SIGNIFICANCE

If the results support the null hypothesis, that no genetic variation exists between stable fly populations, it will support the aforementioned studies that assume a high level of gene flow between populations. However, if my hypotheses are supported, and there is a level of genetic variation between populations, possibilities for further research into genetic variations will be revealed. Patterns in the phylogenetic relationships between populations may occur, which may lead to locating the sources of stable fly populations and subsequently the development of more efficient methods of control.

RESEARCH OBJECTIVES

More research is needed in the population genetics of stable flies to increase our knowledge of their dispersal patterns and the sources of outbreaks. Advancements in DNA technology over the past decade offer more efficient methods of screening a larger number of genetic loci with an equivalent input of time and expenses. Describing variations in population distribution will expand our understanding of the population dynamics of the stable fly. The goal of this research is to investigate genetic variation in stable fly populations across the United States and on a global scale.

Objective 1:

Validate the shipping protocol for samples collected for this research.

Objective 2:

Analyze genetic variations of stable fly populations across the United States, in north-south, east-west, northwest-southeast, and southwest-northeast transects.

Objective 3:

Analyze genetic variations in stable fly populations from several geographic areas, including numerous locations in the United States, and other locations where samples may be obtained, such as Central and South America, Africa, Europe, and Australia.

BIOLOGICAL QUESTIONS

1. Is the shipping protocol used for sample acquisition adequate to retain the quality of the DNA during several days in transit?

2. Are stable fly populations homogeneous across the United States, or is there variation due to geographical barriers?

3. Do stable fly populations vary genetically between zoogeographical regions, or is there one global population due to uninhibited gene flow?

DISSERTATION HYPOTHESES

Chapter 2:

Null hypothesis: The shipping protocol is adequate to retain the quality of the DNA if the samples are soaked in 95% ethanol for any length of time before shipping.

Alternative hypothesis: Quality and quantity of the DNA is enhanced if samples are soaked for at least 24 hours before shipping.

Chapter 3:

Null hypothesis #1: There will be no genetic differentiation between populations of stable flies in the United States.

Alternative hypothesis: Genetic differentiation will be found in populations across the United States when divided by geographical barriers such as large mountain ranges.

Null hypothesis #2: Minimal genetic variation will be found, which will support the results of Jones *et al.* (1991), Szalanski et al. (1996), and Gilles et al. (2007), and suggest that stable flies are a global population.

Alternative hypothesis: AFLP will reveal greater genetic variation than previous experiments and genetic differentiation will be evident across geographical barriers. This hypothesis would support the results of Marquez et al. (2007) and Dsouli-Aymes et al. (2011), and suggest that there are isolated sub-populations of stable flies.

CHAPTER 2:

An Ethanol Preservation Technique Suitable for Shipping Stable Flies Long

Distance While Maintaining the Quantity of DNA

INTRODUCTION

The stable fly, *Stomoxys calcitrans,* is a haematophagous pest of livestock, pets and humans, which is distributed worldwide. Its parasitism of livestock causes a decrease in weight gain and milk production due to blood loss, stress and avoidance behaviors (Campbell 1992; Campbell et al. 1997; Catangui et al. 1993), with economic losses estimated at > $1 billion annually in the United States (Taylor and Berkebile 2006). Therefore, more efficient control methods of this insect are continuously being sought. Genetic studies are at the forefront, with population genetics being of primary interest due to the dynamics of stable fly behavior. Stable flies are known to fly long distances and disperse with the wind (Fye et al. 1980; Hogsette et al.1989), which suggests the possibility of gene flow between distant areas. Population genetic studies have been conducted at the local and regional level to determine the origin of stable fly outbreaks, immigration vs. overwintering, and genetic variation within regions (Szalanski et al. 1996; Taylor et al. 2007; Oliviera et al. 2008). Recently, global population studies have been conducted to examine the evolutionary origins of stable flies and the global population differentiation (Marquez et al. 2007; Dsouli-Aymes et al. 2011).

Successful genetic research requires the acquisition of samples containing quality DNA. Various sample collection methods are used for stable flies, including sweep nets, traps and sticky cards (Taylor and Berkebile 2006). Good quality DNA can be obtained using these methods if the samples are preserved soon after collection. Preservation methods include freezing at -80ºC,

lyophilization (freeze drying), and soaking in 95% ethanol. If collections are made at a local scale, any of these methods are efficacious and the DNA will remain undamaged. However, global population studies require acquiring samples from distant countries, and shipping regulations put restraints on the methods that can be used (Clark et al. 2009)..

Storing samples at -80ºC is a very effective preservation method, but not for samples to be shipped long distances. If put on dry ice, the samples would remain cold for some time, but sublimation may occur before arrival at the destination. Additionally, it is not permissible to ship dry ice through the postal system, which would eliminate this method for global studies (USPS 2008).

Lyophilization is effective, and dried samples can be shipped through the mail. Lyophilization is a method commonly used for plant tissue and bacterial samples, and samples preserved with this method yield large amounts of DNA. However, it is an expensive process and many locations from which samples are desired would not have access to the equipment needed for lyophilization (Clark et al. 2009).

Soaking in 95% ethanol is an inexpensive, efficient method of preservation. Small vials of ethanol can be carried into the field and samples preserved immediately after collection, eliminating the possibility of decomposition beginning between collection and deposition in a -80ºC freezer. It is prohibited to ship ethanol by air transportation through the postal service (USPS 2008). This study examines a method of preserving specimens in ethanol

before shipping which is sufficient to retain the DNA quality and quantity until reaching the destination laboratory.

MATERIALS AND METHODS

Experimental design

A protocol was developed for shipping stable fly samples long distances while retaining the quality of the DNA. Samples are placed in a vial of 95% ethanol immediately after collection, or as soon as possible thereafter. The samples are imbibed in the ethanol for 24 h. The ethanol is poured off and a cotton ball is inserted into the vial before sealing. The vials are shipped, and ethanol is added to the vials upon arrival at the destination laboratory.

The efficacy of this shipping method was tested by performing a time-point experiment which attempted to duplicate preservation and shipping conditions. Stable flies were obtained from a laboratory colony and all individuals were from the same cage, eliminating differences in DNA due to population variation and condition of the samples, and the adult stable flies were placed in 15 ml tubes containing 95% ethanol immediately after collection. The variables were the time that the adults were allowed to soak in 95% ethanol (6, 12, 24 and 48 h), and time between removal and reconstitution of ethanol to simulate shipping times (2, 4, 6 and 8 days). This experiment was replicated 4 times. Ten stable flies were put into each tube, and 5 of the 10 were randomly chosen for DNA extraction.

Table 1. Experimental design: A Randomized Complete Block Design with subsamples was used to test a shipping method for insect samples acquired globally. Effects of hour and day were analyzed as well as interactions between hour and day in a 4x4 factorial Analysis of Variance.

_____

	Hours soaked in ethanol before pour-off			
Days after pour-off	6hr	12hr	24hr	48hr
2d	6h/2d	12h/2d	24h/2d	48h/2d
4d	6h/4d	12h/4d	24h/4d	48h4d
6d	6h/6d	12h/6d	24h/6d	48h/6d
8d	6h/8d	12h/8	24h/8d	48h/8d

*Each time/day combination was replicated 4 times.

DNA extraction

DNA was extracted using the CTAB (Hexadecyltrimethylammonium bromide) method modified from Doyle and Doyle (1987). Samples were washed in autoclaved double distilled water for 10 minutes. The head, abdomen, wings and legs were detached from the thorax, and the gut was removed. The thorax was homogenized in 250µl CTAB buffer (100 mM Tris-HCl, 1.4M NaCl, 0.02 M EDTA, 2% CTAB, 0.2% β-mercapto ethanol) in a 1.5ml microcentrifuge tube, then an additional 250µl CTAB was added for a total of 500µl CTAB. Fifteen microliters of RNaseA was added to each tube, and tubes were incubated on heat blocks for 2h at 65ºC. Samples were mixed every 20 min by inversion of the tubes. After 2h, 10µl Proteinase K was added to each tube and they were incubated at 37ºC for 1h.

After incubation, samples were centrifuged for 5 min. at 14,000rpm and 20ºC to separate tissue from supernatant. The supernatant was transferred to a new autoclaved 1.5ml tube and the tissue was discarded. Five hundred microliters

of chloroform: isoamyl alcohol (24:1) were added to each sample and centrifuged at 14,000rpm and 20ºC for 20 min. This forms 2 layers, the aqueous layer on top and chloroform layer on the bottom. The aqueous layer was removed and transferred to a new 1.5ml tube and the bottom layer was discarded. The chloroform step was then repeated, after which the aqueous phase was transferred to a new tube and 400ml chilled (-20ºC) isopropanol was added to precipitate the DNA. Samples were stored at 4ºC over night.

Samples were centrifuged for 30 min. at 14,000rpm and 4ºC, at which time a white pellet of DNA formed at the bottom of the tube. The isopropanol was poured off, the samples were washed with chilled absolute ethanol and centrifuged for 5 minutes at 14,000rpm and 4ºC. The ethanol wash was repeated using 70% ethanol. After the second wash the ethanol was poured off, the remainder was removed with a pipet and the samples were allowed to air dry. After drying, the DNA was re-suspended in 50µl 1X TE buffer and stored at -80ºC.

Data collection

The amount of DNA in ng/µl was determined from readings with the Nanodrop® spectrophotometer (ThermoScientific), and used as data. Each of the 5 sub-samples (individual flies) from all tubes were analyzed for quantity of DNA extracted, and 3 readings were taken from each sub-sample. The pedestals of the Nanodrop® were cleaned after each reading with a dry tissue. After each group of 20 flies the pedestal of the spectrophotometer was cleaned with nanopure dH_2O and a new blank was applied. For optimum accuracy, a P2 pipet was used to

transfer 1.5µl of DNA to the pedestal. If the sample did not form a bead on the pedestal, the pedestal was cleaned and the sample was re-loaded.

Statistical analysis

Data were analyzed using the PROC MIXED procedure (SAS Institute 2009). One data point was missing, so 959 of 960 data points were used in a Randomized Complete Block Design with subsamples and a 4 x 4 factorial analysis, where hours soaked are the blocks, the days after pour-off are fixed factors, and the subsamples are the 10 individual flies in each hour/day combination.

RESULTS

There were no interaction effects between hours and days (Table 2, Fig. 1) so the Differences of Least Square Means was used to analyze differences between the hours soaked in 95% ethanol and the time after pour-off. The samples soaked for 6 h contained a higher amount of DNA than 12, 24 or 48 h

Table 2. The Type 3 Test of Fixed Effects generated by SAS indicates no significant interaction between hours and days (P=0.3086), so the Difference of Least Square Means could be used to analyze the differences between hours soaked in 95% ethanol and time after pour-off.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Hour	3	45	4.35	0.0089
Day	3	45	5.20	0.0036
Hour*Day	9	45	1.22	0.3086

No hour*day interaction: P = 0.3086.
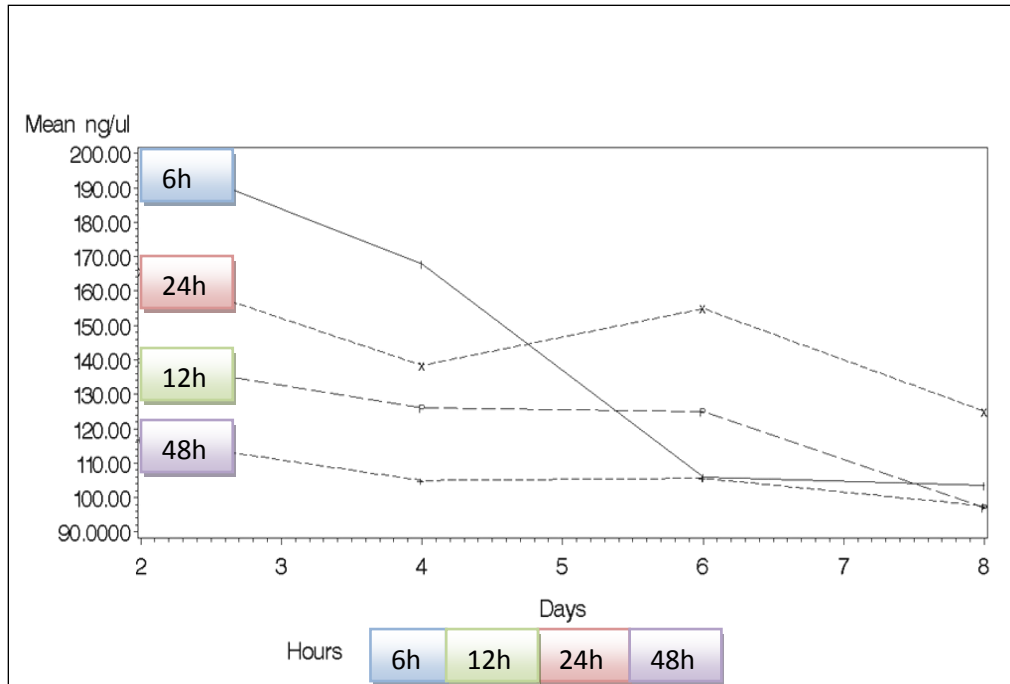
Fig. 1. Plot of hours soaked in 95% ethanol by days after pour-off generated by PROC PLOT. The graph indicates no significant interaction between hours soaked and days after pour-off. The amount of DNA (ng/µl) drops dramatically in the 6h samples after 4 days.

at the 2d and 4d points, but dropped off considerably at 6d. The 24h samples produced higher amounts of DNA than 12h and 48h at all days, and more than 6h at 6d and 8d, while the 48h samples produced the least amount of DNA at all days. The amount of DNA produced by the 6h and 24h samples was significantly (P = 0.0051 and P = 0.0035 respectively) higher than those soaked 48h. The samples left for 2d after pour-off produced significantly more DNA than those left for 6d and 8d (P = 0.0152 and P = 0.0004 respectively), and the samples left for 4d after pour-off produced significantly more DNA (P = 0.0325) than those left for 8d (Table 3).

Table 3. Results from the Differences of LSMeans. Amount of DNA extracted from samples soaked for 6h and 24h was significantly higher than the amount of DNA from samples soaked for 48h (P=0.0051 and P=0.0035 respectively). Significant differences between days after pour-off occurred 2dx6d (P=0.0152), 2dx8d (P=0.0004), and 4dx8d (P=0.0325).

Differences of Least Squares Means									
Effect	**Hour**	**Day**	**_Hour**	**_Day**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
Hour	6		12		22.4088	12.9185	45	1.73	0.0897
Hour	6		24		-1.7637	12.9185	45	-0.14	0.8920
Hour	6		48		38.0044	12.9188	45	2.94	0.0051
Hour	12		24		-24.1725	12.9185	45	-1.87	0.0678
Hour	12		48		15.5956	12.9188	45	1.21	0.2337
Hour	24		48		39.7681	12.9188	45	3.08	0.0035
Day		2		4	21.1140	12.9188	45	1.63	0.1092
Day		2		6	32.5990	12.9188	45	2.52	0.0152
Day		2		8	49.6240	12.9188	45	3.84	0.0004
Day		4		6	11.4850	12.9185	45	0.89	0.3787
Day		4		8	28.5100	12.9185	45	2.21	0.0325
Day		6		8	17.0250	12.9185	45	1.32	0.1942

*Significant at P < 0.05.

DISCUSSION

These results support the alternative hypothesis and suggest that the method used for sample acquisition from distant countries, soaking in 95% ethanol for 24h before pour-off, was overall the best method. The 24h samples consistently produced more DNA than soaking for 12h or 48h, and 24h soaking produced more DNA than the 6h samples at 6d and 8d. However, within the United States or over short distances, where shipping can be expedited, soaking for 6h would be sufficient, because the 6h samples produced the highest amount of DNA at 2d and 4d.

It was unexpected that the 48h samples yielded the least amount of DNA; it would seem that soaking for a longer period of time would preserve the samples more thoroughly. This experiment could be replicated to determine if other factors were influencing the 6h and 48h results, and to test longer periods of pour-off, such as 14 or 21 days. The condition of the samples could also be considered in a similar experiment. In this test, all flies were collected from the same rearing cage and killed immediately in 95% ethanol. However, samples collected in the field may not be handled in the same manner. If they are collected from traps or sticky cards, they could be dead before collection, and may not be imbibed in ethanol until return to the laboratory, or longer. It would be more representative of field collections to design a similar experiment using collection method and time after collection before soak in addition to the factors used herein.

CHAPTER 3:

Genetic variability of the stable fly, *Stomoxys calcitrans* (L.) (Diptera:

Muscidae) assessed on a global scale using Amplified Fragment Length

Polymorphism

INTRODUCTION

Stomoxys calcitrans is a haematophagous pest with a global distribution. In North America it is the primary pest of livestock, causing > \$1 billion in economic losses annually (Taylor and Berkebile 2006). It is a synanthropic pest, and as the human population expands into agricultural areas, it is becoming a cause of dissention between farmers and new residents at the urban/rural interface (Thomas and Skoda 1993). Therefore the majority of research on this pest has concentrated on its control in livestock facilities. Other research has focused on finding the origin of stable fly outbreaks on beaches in Florida, New Jersey, and the Great Lakes, where the flies prove detrimental to the tourist trade (King and Lenert, 1936; Simmons and Dove 1941, 1942; Dove and Simmons, 1942; Simmons 1944; Hansens, 1951; Williams and Rogers 1976; Hogsette and Ruff 1985; Jones et al. 1991; Koehler and Kaufman 2006)..

There is a paucity of information as to the effect of stable flies on wildlife. In 1962 a stable fly outbreak devastated the lion population in the Ngorongoro Crater in Africa (Fosbrooke 1963). Recently, stable flies were implicated in the transmission of West Nile Virus to pelicans in Montana. It was found that they are capable of mechanical, but not biological, transmission of the virus (Doyle et al. 2011). These reports indicate that stable fly parasitism may have significant effects on global ecology as well as economy.

Stable fly research has been ongoing for a century, but as yet no efficient method of control has been developed. Integrated pest management techniques which implement a high level of sanitation have produced the best results, but

stable fly numbers have not been significantly reduced (Campbell 1995). More information on the source of stable fly outbreaks would be useful when developing new control methods. Resistance to certain pesticides has been reported in stable flies (Mount 1965; Marçon et al. 1997), and discovering the genetic basis for this resistance would be helpful.

Genetic research is at the forefront of pest management studies, and new methods are being developed on a regular basis to more efficiently analyze insect genomes. The most frequently used methods have been microsatellite analysis of genomes and direct DNA sequencing of mitochondrial genomes (Gilles et al. 2004; Marquez et al. 2007; Dsouli-Aymes et al. 2011). Conversely, plant scientists have been using AFLP, and recently insect researchers have become aware of the potential of the technique, especially for population genetics.

As new methods are developed for DNA analysis, many studies are conducted to compare the efficiency of one against another. Gerber et al. (2000) compared AFLPs which use dominant markers with microsatellites which are co-dominant, to reconstruct parentage in a stand of oak trees. In this case the co-dominant markers were found to be more efficient. Garcia et al. (2004), however, compared AFLP, RFLP, RAPD and SSR markers to evaluate the genetic diversity of inbred maize lines. They found that AFLP was the most efficient method for this type of research.

Hardy (2003) developed a new estimator of pairwise relatedness between individuals using dominant markers, and suggested that AFLP may be as valuable as microsatellites for studying isolation-by-distance processes.

Entomologists are becoming aware of the benefits of AFLP for the study of insect population genetics. Martinelli et al. (2007) studied the genetic structure of the Fall Army Worm, *Spodoptera frugiperda* and the similarity between those feeding on maize and cotton in Brazil. Krumm et al. (2008) studied the genetic variability of the European Corn Borer, *Ostrinia nubilalis* in the Midwestern United States. Alamalakala et al. (2009) used AFLP to find markers that would differentiate the New World Screwworm, *Cochliomyia hominivorax,* from the Secondary Screwworm, *C. macellaria.*, since the larvae of these species are impossible to differentiate by morphological methods.

Recently, Lindroth (2011) studied the genetic variation of Western Bean Cutworm, *Striacosta albicosta,* across the United States; Tiroesele (2011) examined the genetic variation of the Bean Leaf Beetle throughout the Midwest; and Kneeland et al. (2011) evaluated the genetic variation between laboratory populations and field populations of the Spined Soldier Bug, *Podisus maculiventris,* in Missouri.

AFLP has become recognized as an efficient method for population genetics analysis for animals as well as plants. It is less expensive than other methods and generates a large number of markers which can be scored using computer software. AFLP is more reproducible than RAPDs and requires only a minute amount of DNA (Bensch and Åkesson 2005). The benefits of AFLP make it an attractive method for analyzing a large number of samples such as in global population studies.

Previous research on the global population structure of stable flies utilized direct sequencing of mitochondrial genes (Marquez et al. 2007) or both mitochondrial and nuclear genes (Dsouli-Aymes et al. 2011). Direct sequencing of previously reported loci undoubtedly produces more accurate information than a random method such as AFLP. However, using AFLP more information can be gained due to the large number of loci that can be analyzed. Results from this project may not be complimentary to those discussed above due to the differences in technique. AFLP is used to analyze restriction fragments of total genomic DNA rather than specific mitochondrial genes. However, the additional information gained from using a new technique for a global study will increase the bank of knowledge that has been gained so far on the population dynamics of stable flies.

MATERIALS AND METHODS

Samples

Samples were generously supplied by colleagues around the world. Due to the diversity of locations and donors, different collection methods were used, including sweep netting and sticky traps. Some specimens had been preserved from previous research. However, the preservation and shipping method in chapter 2 were followed by all collectors.

Immediately upon arrival, all vials or tubes containing samples were refreshed with 95% ethanol and stored at 4℃ until DNA extraction. In most cases an abundance of samples were received. Some locations were unable to collect the requested 50 flies, but only one location (Idaho, 11 samples) had less than 20

flies that were analyzed. When possible, 40 flies from each location were

analyzed. Figure 2a shows the global collection sites for this project, and Figure

2b shows the collection sites of Marquez et al. (2007) and Dsouli-Aymes et al.

(2011). Figure 2c is a map of the North American collection sites used in this

project. Table 4 is a complete list of the samples collected globally, and Table 5

shows the geographical coordinates and elevation of sampling locations.
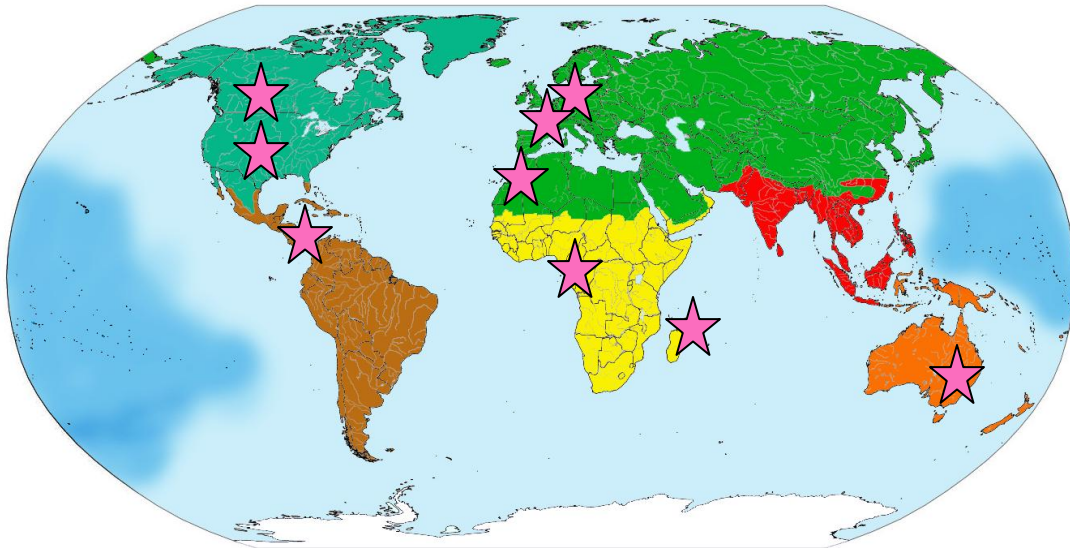
Fig. 2a. Stable fly sample sites by country and biogeographical region. Stars represent countries
only. Multiple samples were acquired from several locations but are not indicated on the map.
An expanded map of North America (Fig. 2c) shows the states and provinces from which samples
were acquired.
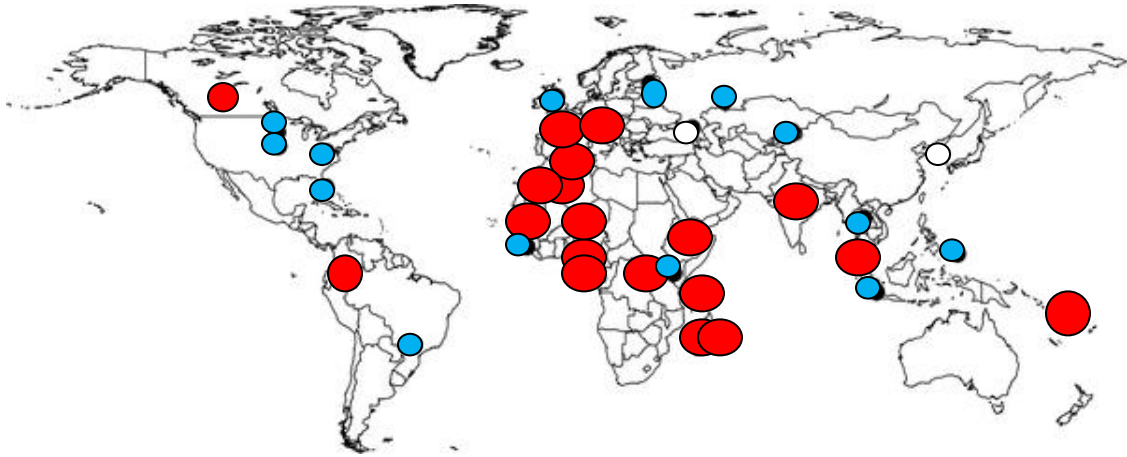
Fig. 2b. Sample locations of stable flies by Marquez et al. (2007) are represented by blue dots, and locations sampled by Dsouli-Aymes et al. (2011) are represented by red dots. Map is from Marquez et al. (2007).
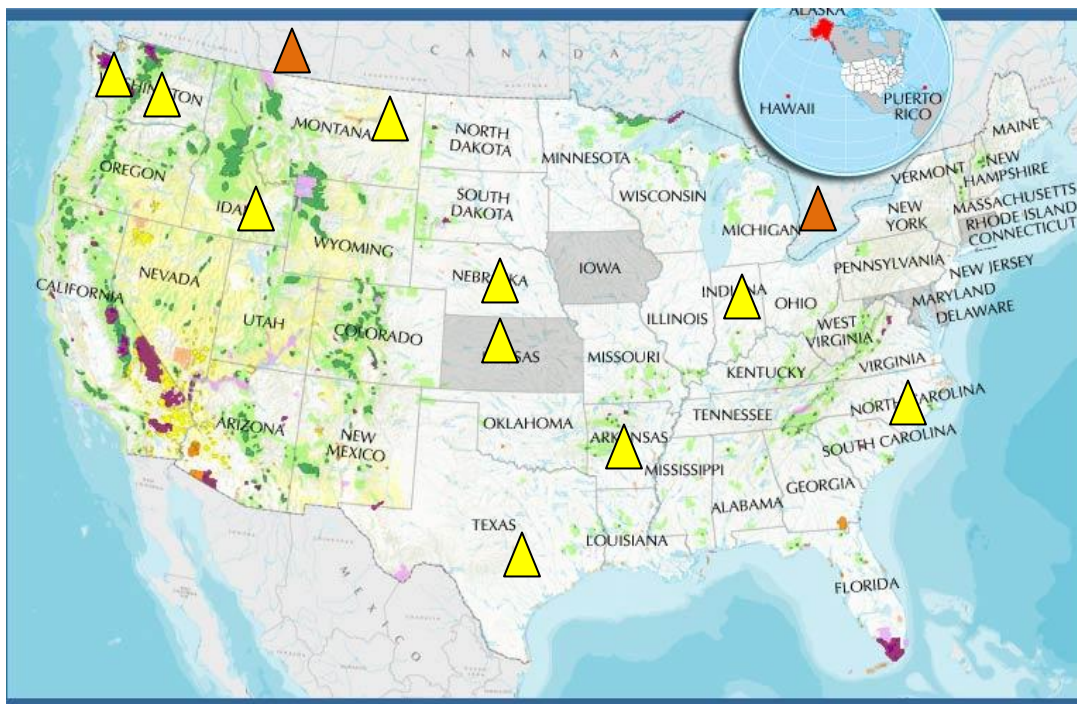
Fig. 2c. Stable fly sample locations within North America. Yellow triangles represent states and brown triangles represent Canadian provinces.

Table 4. Collection sites of stable fly samples grouped by biogeographical region, country, and city or locality.

Region	Country	Location, City, State	No. of Samples
Afrotropical	Gabon	Makokou	40
	La Reunion	Plaine des Cafres	40
Australasian	Australia	Gatton,Qld	12
		Pinjarra Hills, Qld	9
Nearctic	Canada	Lethbridge, Alberta	40
		Russell, Ontario	40
	USA	Jonesboro, Arkansas – ASU farm	40
		Idaho Falls, Idaho	12
		Jasper Co., Indiana	40
		Manhattan, Kansas – KansasStateUniversity	40
		Montana – Medicine Lake NWR	40
		Lincoln, Nebraska – North Platte lab colony	40
		Raleigh, North Carolina – NCSU Dairy	40
		Kerrville, Texas –Knipling-Bushland Livestock Insects Research Lab	24
		Washington 1 (eastern) Frazier (Moxee) Ferguson (Moxee) Russell (Prosser) Stark	32
		Washington 2 (western) Carstens Silvana	9
Neotropical	Panama	Potrerillos Arriba, Dolega, Chiriqui	80
Palearctic	Denmark	Fuglebjerg	40
		Næstved	40
		Roskilde	40
		Hyllinge	40
		Hyllinge	40
		Lynge	40
		Slangerup	40
		Olstykke	40
	France	Le Faut, near Seyne-les-Alpes	40
	Morocco	Gharb area north of Kenitra	40

Table 5. Geographical coordinates and elevation of stable fly collection sites. Locations south of the equator are represented by a "-" to conserve the N-W designation.

Location, City, State	Coordinates	Elevation
Makokou	0.532158N, 12.829655W*	480m (1574.8ft)
Plaine des Cafres	-21.2192N, 55.5590W*	1293m (4242.1ft)
Gatton,Qld	-27.333158N, -152.163266W	107m (351ft)
Pinjarra Hills, Qld	-27.322043N, -152.535131W	36.6m (120ft)
Lethbridge, Alberta	49.413656N, 112.503062W	893.0m (2930ft)
Russell, Ontario	45.152633N, 75.213009W	68.9m (226ft)
Jonesboro, Arkansas – ASU farm	35.502237N, 90. 421540W	97.2m (319ft)
Idaho Falls, Idaho	43.275969N, 112.020289W	1433.2m (4702ft)
Jasper Co., Indiana	39.031979N, 88.052427W	165.8m (544ft)
Manhattan, Kansas –	39.110099N, 96.341801W	361.2m (1185ft)
Montana – Medicine Lake NWR	47.585650N, 107.563472W	865.0m (2838ft)
Lincoln, Nebraska –	40.495959N, 96.393773W	353.6m (1160ft)
Raleigh, North Carolina – NCSU Dairy	35.494691N, 78.430072W	109.7m (360ft)
Kerrville, Texas –	30.0428N, 99.3854W	536.8m (1761ft
Washington 1 (eastern)		
Frazier (Moxee)	46.528205N, 120.374317W*	347.8m (1141ft)
Ferguson (Moxee)	46.528205N, 120.374317W*	347.8m (1141ft)
Russell (Prosser)	46.133357N, 119.442946W*	205.7m (675ft)
Stark	46.506087N, 120.193906W*	449.3m (1474ft)
Washington 2 (western)		
Carstens	48.4877N, 121.703W*	211.2m (693ft
Silvana	48.241N, 122.369W*	30.8m (101ft)
Potrerillos Arriba, Dolega, Chiriqui	8.410396N, 82.290556W	869.3m (2852ft)
Fuglebjerg	55.316263N, 11.494282W*	21.3m (70ft)
Næstved	55.189888N, 11.799569W*	14.9m (49ft)
Roskilde	55.550185N, 11.94664W*	41.1m (135ft)
Hyllinge	55.69786N, 11.848336W*	17.4m (57ft)
Hyllinge	55.705953N, 11.925521W*	17.4m (57ft)
Lynge	55.846009N, 12.251469W*	47.9m (157ft)
Slangerup	55.834287N, 12.155846W*	27.7m (91ft)
Olstykke	55.806833N, 12.147198W*	18.0m (59ft)
Le Faut, near Seyne-les-Alpes	44.341025N, 6.406077W*	1340m (4396.3ft)
Gharb area north of Kenitra	34.687207N, -6.005123W*	30m (98.4ft)

DNA Extraction

DNA was extracted using the CTAB (Hexadecyltrimethylammonium bromide) method modified from Doyle and Doyle (1987). Samples were washed in autoclaved double distilled water for >10 minutes. The head, abdomen, wings and legs were detached from the thorax, and the gut was removed. The thorax was homogenized in 250μl of CTAB buffer (100 mM Tris-HCl, 1.4M NaCl, 0.02 M EDTA, 2% CTAB, 0.2% β-mercapto ethanol) in a 1.5ml microcentrifuge tube, then an additional 250μl CTAB was added for a total of 500μl CTAB. A few grains of sea sand (Fisher Scientific) were added to facilitate homogenization. Fifteen microliters of RNaseA was added to each tube, and tubes were incubated on heat blocks for 2h at 65ºC. Samples were mixed every 20 min by inversion of the tubes. After 2h, 10μl Proteinase K was added to each tube and they were incubated at 37ºC for 1h.

After incubation, samples were centrifuged for 5 min. at 14,000 rpm and 20ºC to separate tissue from supernatant. The supernatant was transferred to a new autoclaved 1.5ml tube and the tissue was discarded. Five hundred microliters of chloroform: isoamyl alcohol (24:1) were added to each sample and centrifuged at 14,000rpm and 20ºC for 20 min. Two layers are formed, the aqueous layer containing the DNA is the top layer, and the chloroform is on the bottom. The aqueous layer was removed and transferred to a new 1.5ml tube and the bottom layer was discarded. The chloroform step was then repeated, after which the aqueous phase was transferred to a new tube and 400ml chilled (-20ºC)

isopropanol was added to precipitate the DNA. Samples were stored at 4ºC over night.

Samples were centrifuged for 30 min. at 14,000rpm and 4ºC, at which time a white pellet of DNA formed at the bottom of the tube. The isopropanol was poured off, the samples were washed with chilled absolute ethanol and centrifuged for 5 minutes at 14,000rpm and 4ºC. At this point, very large DNA pellets could be observed in nearly every sample. The ethanol wash was repeated using 70% ethanol. After the second wash the ethanol was poured off, the remainder was removed with a pipet and the samples were allowed to air dry. After drying, the DNA was re-suspended in 50μl 1X TE buffer and stored at -80ºC.

After the DNA had been extracted, all samples were analyzed for DNA quality and quantity on the Nanodrop® spectrophotomer (ThermoScientific). The pedestal was cleaned with a kimwipe after each sample, and the pedestal was washed with autoclaved nanopure water after each group of 20 samples. After each location (40 samples), the pedestal was washed and the spectrophotometer was re-blanked with 1X TE.

AFLP-PCR

AFLP-PCR was performed using a protocol modified from Vos et al. (1995). Three different thermal cyclers were used: PTC-200 (MJ Research), GeneAmp 2700 and GeneAmp 9700 (Applied Biosystems). The 4200 DNA analyzer (Licor Biosciences, Lincoln, NE) was used for polyacrylamide gel electrophoresis.

AFPL Step 1: Restriction digestion

Restriction digestion mix was made for 1000 reactions, the entire sample

set plus extra to allow for loss during pipetting.

NEB buffer 4	1250 µl
MseI restriction enzyme	125 µl
EcoRI restriction enzyme	62.5 µl
Bovine Serum Albumin (BSA)	125 µl
dH$_2$O	3940 µl

The restriction digestion was transferred in 5.5 µl aliquots into .2ml PCR

tubes (Midwest Scientific, St. Louis, MO), and 7 µl DNA was added to each tube

for a total volume of 12.5 µl. Samples from 2 locations (80 samples) were

prepared at one time, and run on the "RESTDIG" program on the thermal cycler

which consists of one cycle of 37ºC for 2.5hr, 70ºC for 15 min. and a 4ºC hold.

AFLP Step 2: Adapter ligation

The adapter ligation mix was also prepared for 1000 samples.

EcoRI prepared adapter	500 µl
MseI prepared adapter	500 µl
T4 DNA ligase	150 µl
T4 DNA ligase buffer	500 µl
dH$_2$O	3350 µl

After restriction digestion, 5 µl of the adapter ligation mix was added to

the restriction digest. Samples were incubated at 25ºC for 8 hr, and left over night

at 4ºC.

AFLP Step 3: Diluting the ligation mix.

A 1:10 dilution of the ligated product was made by adding 135 µl 1X TE buffer to each tube from step 2. Tubes were vortexed to mix well, then stored at 4ºC until the next step.

AFLP Step 4: Preamplification

The preamplification mix was not prepared all at once due to the cost of the mix and the taq polymerase, and the large amount of both needed per sample. Enough for 100 samples was prepared each time until all samples were run.

Preamplification primer mix II	1000 µl
10X PCR buffer	125 µl
25mM MgCl$_2$	75 µl
Bullseye Taq polymerase	25 µl

The preamplification mix was transferred to new .2 ml PCR tubes in 12.25 aliquots, and 1.25 µl of the template DNA from step 3 was added to each tube. Samples were run on the "preamp" thermal cycler program consisting of 20 cycles of 94ºC for 30s, 56ºC for 1 min and 72ºC for 1 min. with a 4ºC hold.

AFLP Step 5: Checking preamp DNA

This step was omitted due to lack of equipment to visualize an agarose gel.

AFLP Step 6: Dilution of preamplification product.

A 1:20 dilution was prepared by adding 195 µl dH$_2$O to the preamplification product. Tubes were vortexed to mix well and stored at 4ºC.

AFLP Step 7: Selective amplification.

Before proceeding with step 7, a primer test was performed to determine which primer pairs would work best for stable fly DNA (Fig. 3). A matrix of the available primers was created and primer pairs were tested using DNA from a previous project known to be of good quality.

EcoR1	MseI							
	CAA	CAC	CAG	CAT	CTA	CTC	CTG	CTT
AAC	■ blue	■ green	■ red		■ green	■ green	■ blue	
AAG								
ACA	■ blue	■ blue	■ red		■ blue	■ green	■ blue	
ACG	■ blue	■ red	■ red		■ red	■ blue	■ red	
ACT	■ blue	■ red	■ red		■ blue	■ blue	■ red	
AGC								
AGG								

Fig. 3. Chart used for primer test. Colored boxes represent primer pairs that were tested. Green = primer pairs chosen, red = did not work, blue = worked but not as well as primer sets chosen.

Based on the test above, the primer sets chosen for this project were:

Primer set 1   M-CAC/E-AAC

Primer set 2   M-CTA/E-AAC

Primer set 3   M-CTC/E-AAC

Primer set 4   M-CTC/E-ACA

The sequences of the oligonucleotides used in this project are shown in Table 6.

Table 6. Nucleotide sequences of adapters, preamplification primers and selective primers used. Sequences were described by Vos et al. (1995). EcoRI selective primers (E-AAC and E-ACA) were tagged with fluorescent dye.

Primer ID	Primer Type	Sequence
EcoRI-1 (forward)	Adapter	5-CTCGTAGACTGCGTACC-3
EcoRI-2 (reverse)	Adapter	5-AATTGGTACGCAGTCTAC-3
MseI-1 (forward)	Adapter	5-GACGATGAGTCCTGAG-3
MseI-2 (reverse)	Adapter	5-TACTCAGGACTCAT-3
E (N+0)	Preamp Primer	5-GACTGCGTACCAATTC-3
M (N+1)	Preamp Primer	5-GATGAGTCCTGAGTAAC-3
M-CAC	Selective Primer	5-GATGAGTCCTGAGTAACAC-3
M-CTA	Selective Primer	5-GATGAGTCCTGAGTAACTA-3
M-CTC	Selective Primer	5-GATGAGTCCTGAGTAACTC-3
E-AAC	Selective Primer	5-GACTGCGTACCAATTCAAC-3
E-ACA	Selective Primer	5-GACTGCGTACCAATTCACA-3

Before preparing selective amplification mixes, optimization tests were performed to determine the correct amount of reagents to use to obtain the best results with stable flies. Components tested were Taq polymerase, dNTPs and primers. The following selective mix is the result of these tests.

Selective amplification mixes were prepared all at once, enough for 1000 reactions.

10X PCR buffer	1200 μl
$MgCl_2$	720 μl
Amplitaq 360 taq polymerase	90 μl
10mM dNTPs	400 μl

M-primer	750 μl
E-primer	300 μl
dH$_2$O	6790 μl

Four selective mixes were prepared, each with a different primer combination. Ten microliters of the selective mix was aliquoted into .2 ml PCR tubes and 2 μl of diluted template DNA was added to each tube. Two sample locations were prepared at one time. Samples were run on the PCR program "selective" or "touchdown" which have the same parameters: 1 cycle of 94ºC for 30s, 65ºC for 30s, and 72ºC for 1 min; 12 cycles of 94ºC for 30s, 65ºC for 30s (with the 65ºC annealing temperature decreasing by 0.7ºC each cycle to a final temperature of 56ºC) and 72ºC for 1 min., 23 cycles of 94ºC for 30s, 56ºC for 30s and 72ºC for 1 min; 4ºC hold.

AFLP Step 8: Denaturation

Following the selective amplification, samples were denatured by adding 2.5 μl blue stop solution to each tube and running on the "denature" program, 95ºC for 3 min with a 4ºC hold. After the PCR steps were completed, samples were stored at 4ºC until loaded on gel. The step 3 and step 6 products were stored at -20ºC.

Gel electrophoresis

Gels were prepared as per the directions in Appendix V. Gels were washed with 70% isopropanol, polished with furniture polish, and then washed with 100% isopropanol. Bind silane was prepared by mixing 100 μl bind silane (diluted in ethanol) with 100 μl 10% acetic acid. The binding solution was

applied to the glass plates in the area where the comb would be inserted. Spacers were placed on the edges of the plates, and they were clamped together with gel rails.

Gel was prepared by mixing 20 ml gel matrix, 150 µl Ammonium persulfite solution and 15 µl Temed. Half of the gel was poured into the plates with a 10 ml pipette, the comb was positioned, and the remainder of the gel was poured. It was allowed to set for at least 1.5hr.

When the gel had set, 1L of 1X TBE buffer was prepared from the 10X stock solution and poured into the lower buffer tank of the scanner. The gel was placed into the machine and rails were tested for stability. The upper buffer tank was inserted at the top of the gel, clamped tightly and filled with buffer. The lids were placed on the buffer tanks and the power cord was plugged in. The lid was closed and the pre-run was started.

A 48-well comb was used, so gels were loaded with 40 samples (one location) per gel plus a negative control sample in the last lane. If two locations had only 20-24 samples, they were run together on the gel, and there was a negative control in the final lane of each location. Two runs were made on each gel, at which time a new gel was prepared. Running a gel more than twice can result in too many artifacts from the previous gels left behind in the third gel.

Data analysis

When the gels had finished running, the E-SEQ (Licor Biosciences, Lincoln, NE) program was opened and the gels were saved into a file that could be accessed from SAGA Generation 2 software. See Appendix VI for saving and scoring gels. Once SAGA accepted the gels, they were scored and the scoring

was confirmed. The reports were not generated until all locations in one primer set were scored, in case bins were added during scoring. If this happens, SAGA adds the bins to every gel in that primer set, so it is necessary to go back and re-score previously confirmed gels.

After scoring, the reports were generated in the Phylip format and saved as text files. The data matrices were set up as per each program's specifications and the data were processed in each software program. A graph showing the number of markers used and the % coefficient of variation (Fig.4) was generated using the dboot program (Coelho 2001). This analysis calculates the percent of variation accounted for by the number of markers in the analysis. Results for the Analysis of Molecular Variance (AMOVA), the number of usable and polymorphic loci, Tajima's D and Fu's FS tests of neutrality, mismatch distribution and the Mantel test were generated using the Arlequin software (Excoffier et al. 2005). The PASSaGE program (Rosenberg and Anderson 2011) was used for the Mantel test. Popgene (Yeh 1997) was used to analyze Nei's genetic diversity (G-statistics) and to generate dendrograms based on Nei's genetic distance using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The dendrograms were processed in *MEGA* version 4 (Tamura et al. 2007) to create high quality figures. Appendix VIII gives a detailed description of analyzing data using Arlequin and Popgene.

RESULTS

Four primer combinations were chosen for this project but, due to difficulties as yet unresolved, only 2 primer sets and 20 sub-populations were

used in the final analysis, with a total of 662 individuals and 191 markers per

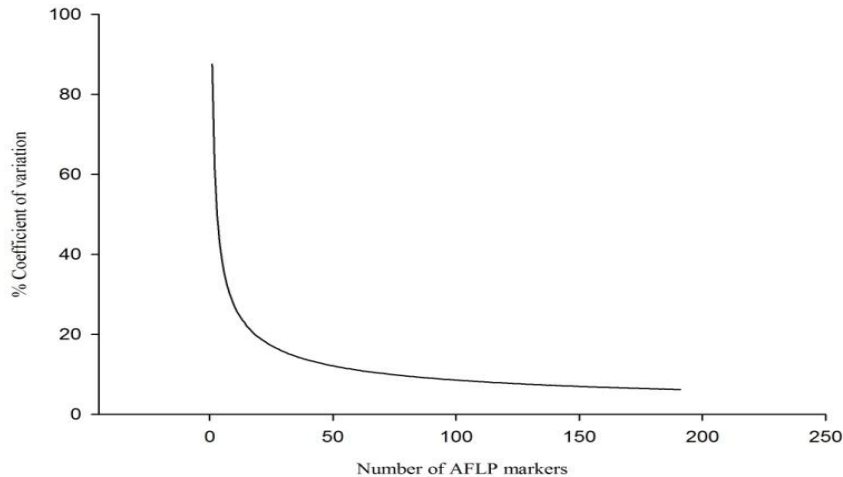individual analyzed. Table 7 shows the groupings used for the AMOVA analysis.

Fig. 4. Graph of number of AFLP markers and the % coefficient of variation calculated using the DBOOT (Coelho 2001) program. The graph shows that 94.6% of the variation in the stable fly population is accounted for by the number of markers generated using 2 primer sets for AFLP. The graph was generated using SigmaPlot (Systat Software Inc 2011).

Arlequin analyses

Table 7. Groups used for the Analysis of Molecular Variance. Australia and Panama are grouped together to avoid having 2 groups with single locations.

Nearctic	Palearctic	Neotropical/ Australian	Afrotropical
Alberta	Denmark1	Panama1	Gabon
Arkansas	Denmark2	Panama2	LaReunion
Idaho	France	Australia	
Indiana	Morocco		
Kansas			
NCarolina			
Nebraska			
Ontario			
WashE			
WashW			

AMOVA

Table 8. The Analysis of Molecular Variance (AMOVA) showing the majority of the genetic variation to be within populations (66.96%), and only a small amount among groups (5.21%) The fixation index (F_{ST}) is low (0.33035), suggesting a high level of gene flow between locations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	4	2479.640	2.09226 Va	5.21
Among populations within groups	14	5245.253	11.17206 Vb	27.82
Within populations	590	15863.734	26.88769 Vc	66.96
Total	608	23588.627	40.15200	

Fixation Indices
FSC : 0.29354
FST : 0.33035
FCT : 0.05211

According to the analysis of molecular variance, the majority of the variation in the stable fly samples analyzed occurs within populations (66.96%), with 27.82% of the variation occurring among populations within groups. A very low percentage (5.21%) of variation occurs among groups. The F-statistics represent the inbreeding coefficient (Wright 1950) where T = total population, S = a subpopulation and C = a colony within the subpopulation. Values of this statistic range from 0-1, where 0 is a population with no inbreeding and 1 is an isolated inbreeding population. The low F_{ST} value suggests a large amount of gene flow between locations (Table 8).

Table 9. Number of usable and polymorphic loci and expected heterozygosity in the stable fly population by location. The majority of the loci are polymorphic.

Location	No. of loci	Usable loci	Polymorphic loci	Exp. Heterozygosity
Alberta	191	189	165	0.30852
Arkansas	191	183	126	0.24477
Australia	191	172	147	0.31161
Denmark1	191	188	170	0.25279
Denmark2	191	190	126	0.17275
France	191	188	149	0.25696
Gabon	191	191	155	0.25018
Idaho	191	178	127	0.27647
Indiana	191	191	178	0.30883
Kansas	191	190	177	0.33426
LaReunion	191	186	157	0.28025
Morocco	191	191	152	0.25877
NCarolina	191	191	143	0.25441
Nebraska	191	191	172	0.33816
Ontario	191	191	167	0.30999
Panama1	191	191	181	0.36485
Texas	191	187	149	0.25094
WashE	191	191	169	0.29219
WashW	191	190	162	0.34171
Mean				0.28469
s.d.				0.04563
Total				0.41122

Out of 191 loci scored, 188 were usable for analysis (Table 9). Loci are removed

by Arlequin if they have too much missing data. Eight of the 19 locations had no

loci removed, whereas the other 11 had only a small number that were not usable.

Of the188 loci analyzed, the majority were polymorphic.

Tests of neutrality

Tajima's D and Fu's F were used to test for neutrality. Strong negative

results in Fu's FS suggest population expansions, and large numbers suggest

population bottlenecks. According to Tajima (1989), if D is negative it suggests a

recent bottleneck or large insertion-deletion polymorphisms. If D is positive it

suggests small insertion-deletion or restriction site polymorphisms. It would also

suggest population expansion.

Table 10. Tajima's D and Fu's FS tests of neutrality. Both the high D value and significantly
negative FS values suggest a population expansion. High D values can be due to polymorphic
restriction sites.

Location	Tajima's D(P-value)	Fu's FS(P-value)
Alberta	1.46809 (P=0.92)	-5.22550 (P=0.02)
Arkansas	1.26179 (P=0.92)	-5.33427 (P=.02)
Australia	1.35882 (P=0.91)	-4.17903 (P=0.04)
Denmark1	0.77800 (P=0.77)	-13.13378 (P=0.002)
Denmark2	0.37170 (P=0.70)	-16.01568 (P=0.00)
France	1.46987(P=0.93)	-12.82544 (0.002)
Gabon	1.14843 (P=0.87)	-13.09049 (P=0.00)
Idaho	0.78860(P=0.83)	-0.57453 (P=0.22)
Indiana	1.50742(P=0.91)	-11.00265 (P=0.004)
Kansas	1.96853(P=0.95)	-10.28905 (P=0.004)
LaReunion	1.49190(P=0.92)	-10.45486 (P=0.003)
Morocco	1.41473(P=0.93)	-12.72990 (P=0.006)
NCarolina	1.12177(P=0.90)	-5.33460 (P=0.015)
Nebraska	2.21061(P=0.97)	-10.17311 (P=0.004)
Ontario	1.87249(P=0.95)	-10.17311 (P=0.002)
Panama1	2.36058(P=0.97)	-9.53384 (P=0.008)
Texas	0.74552(P=0.80)	-5.04453 (P=0.03)
WashE	1.13882(P=0.86)	-6.29277 (P=0.017)
WashW	0.82699(P=0.80)	-.21480 (P=0.33)
Mean	1.32794(P=0.88)	-8.54801(P=0.038)
s.d.	0.52489(P=0.07)	4.45043 (P=0.087)

The results of the neutrality test show positive deviations from 0 for D, but they

were not significant. Fu's FS are significantly negative, suggesting population

expansion (Table 10).

Mismatch distribution

Similar to neutrality tests, the mismatch distribution compares the

observed to the expected mismatch distribution in each sample in expanding or

stationary populations. The demographic expansion estimates deviation from the

sudden expansion model. The spatial expansion estimates deviation from the

continent-island model. Significant SSD values infer deviation from the

expansion model. A small raggedness index (<0.05) suggests population

expansion whereas larger raggedness values (>0.05) suggest stationary

populations or bottlenecks (Rogers and Harpending 1992; Cordaux et al. 2003).

Table 11a. Mismatch Analysis: Demograpic Expansion Test. This analysis is based on the distribution of differencesin pairs of haplotypes. A small raggedness index suggests population expansion whereas asmall raggedness index indicates a stationary population or a bottleneck. The demographic expansion test is based on the sudden expansion model.
_____
                      Demographic Expansion

_____

Location	SSD (P-value)	Raggedness Index (P-value)
Alberta	0.002 96(0.70)	0.00679 (0.37)
Arkansas	0.0135 (0.03)	0.00719 (0.65)
Australia	0.00384 (0.75)	0.00722 (0.58)
Denmark1	0.00218 (0.88)	0.00375 (0.47)
Denmark2	0.00213 (0.35)	0.00375 (0.47)
France	0.00154 (0.48)	0.00341 (0.29)
Gabon	0.01463 (0.00)	0.00344 (0.81)
Idaho	0.01465 (0.67)	0.03967 (0.41)
Indiana	0.00111 (0.84)	0.00239 (0.77)
Kansas	0.00123 (0.97)	0.00245 (0.79)
LaReunion	0.00144 (0.87)	0.00214 (0.98)
Morocco	0.00283 (0.39)	0.00422 (0.32)
NCarolina	0.00349 (0.95)	0.00373 (0.99)
Nebraska	0.00166 (0.69)	0.00310 (0.31)
Ontario	0.00126 (0.86)	0.00248 (0.72)
Panama1	0.00234 (0.46)	0.00345 (0.31)
Texas	0.00432 (0.86)	0.00572 (0.77)
WashE	0.00219 (0.86)	0.00368 (0.90)
WashW	0.01618 (0.54)	0.02512 (0.89)
Mean	0.00492 (0.63947)	0.00697 (0.63579)
s.d.	0.00530 (0.29022)	0.00942 (0.24412)

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Table 11b. Mismatch Analysis: Spatial Expansion Test. This analysis is based on the distribution of differences in pairs of haplotypes. A small raggedness index suggests population expansion whereas asmall raggedness index indicates a stationary population or a bottleneck. The spatial expansion test is based on the continent-island model.

	Spatial Expansion	
Location	SSD (P-value)	Raggedness Index (P-value)
Alberta	0.002301(0.57)	0.00679 (0.32)
Arkansas	0.0309 (0.65)	0.00719 (0.54)
Australia	0.00384 (0.74)	0.00722 (0.67)
Denmark1	0.00218 (0.90)	0.00256 (0.64)
Denmark2	0.00213 (0.31)	0.00375 (0.44)
France	0.00154 (0.44)	0.00341 (0.29)
Gabon	0.00117 (0.83)	0.00344 (0.24)
Idaho	0.01465 (0.60)	0.03967 (0.39)
Indiana	0.00113 (0.83)	0.00239 (0.83)
Kansas	0.00139 (0.71)	0.00245 (0.66)
LaReunion	0.00095 (0.96)	0.00214 (0.94)
Morocco	0.00185 (0.32)	0.00422 (0.06)
NCarolina	0.00286 (0.73)	0.00373 (0.96)
Nebraska	0.00172 (0.56)	0.00310 (0.31)
Ontario	0.00133 (0.72)	0.00248 (0.72)
Panama1	0.00247 (0.24)	0.00345 (0.26)
Texas	0.00432 (0.86)	0.00572 (0.79)
WashE	0.00225 (0.78)	0.00368 (0.83)
WashW	0.01620 (0.51)	0.02512 (0.90)
Mean	0.00358 (0.64526)	0.00697 (0.56789)
s.d.	0.00428 (0.2082)	0.00942 (0.27393)

Only 2 locations show a significant SSD, Gabon and Arkansas. All samples have a very low raggedness index, which indicates population expansion (Table 11).

Mantel test

The Mantel test compares a genetic distance matrix with a geographical distance matrix to test for correlation between genetics and geographical location.

Table 12. Mantel test, based on the correlation between 2 matrices, in this case a genetic distance matrix and a geographical distance matrix. The Mantel test performs permutations on one matrix while holding the other constant. The correlation coefficient r is very low (0.063186), indicating that there is no correlation between genetic and geographic distance in the stable fly samples analyzed.

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Mantel test

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Mean value Y	0.317885
Sums of squares Y	1.363524
Mean value X1	7205.272398
Sums of squares X1	45299154755.654533
ZY1	407370.183892
Sum of products (SP(Y,X1))	15703.604864
Regression coefficient (bY1)	0.000000
Correlation coefficient (rY1)	0.063186
Determination of Y by X1(%)	0.003993

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Table 12 shows the Mantel test results generated in Arlequin. The genetic

distance matrix was used as the Y matrix and geographical distance was used for

X1. Therefore, the mean value of Y is the mean genetic differences found

between my samples, and mean value X1 is the mean distance in km between

collection locations. The Z statistic represents the Hadamard product of the 2

matrices ($Z_{XY} = \mathbf{X*Y} = \sum_{i=1}^{N} . \sum_{j=1}^{i} . x_{ij}\, y_{ij}$) and Y was held constant. Determination

of Y by X1 indicates the % influence X had on Y, or the geographical location

had on the genetic difference, and the value is very low at 0.003993 (Excoffier et

al. 2005). This and the low correlation coefficient (0.063186) indicate that there is

no correlation between geographical location and genetic distance in these

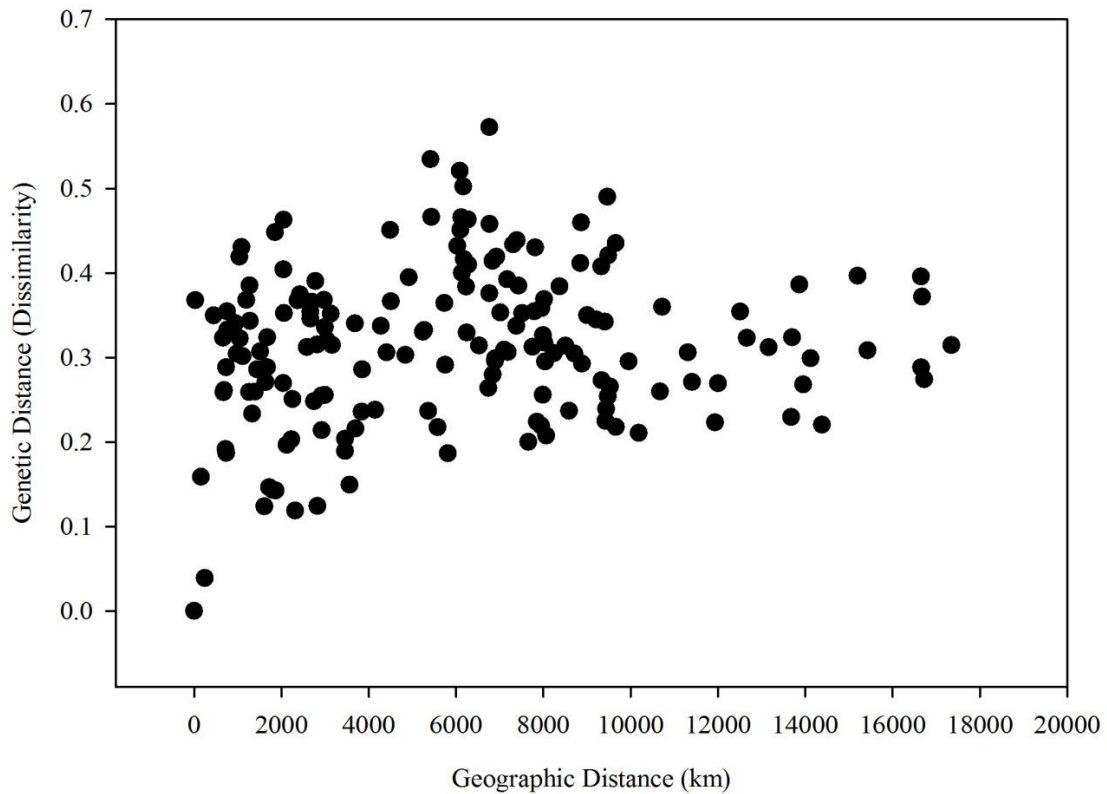samples, and these results are supported by the scatter plot in Fig. 5.

Fig. 5. Scatter plot of Mantel test created in SigmaPlot. The shotgun pattern of scatter indicates No correlation between stable fly genetic distances and geographic locations.

POPGENE

Nei's genetic diversity

Nei's genetic diversity (G_{ST}) is comparable to Wright's F_{ST}. Nei (1973) refers to G_{ST} as the "coefficient of gene differentiation". Whereas F_{ST} measures differentiation in sub-populations, it is only applicable when there are only 2 alleles at a locus. $G_{ST,}$ however, measures the degree of differentiation in multiple populations.

Table 13. Analysis of Nei's genetic diversity in subdivided populations. The low G_{ST} values suggest diversity among populations, and very high Nm values (20.5945 for North America) indicate a high level of gene flow between stable fly populations.

	H_t	H_s	G_{st}	G_{cs}	$N_m(G_{st})$	$N_m(G_{cs})$	F_{st}
North America	0.3472	0.3389	0.0237	0.1835	20.5945	2.2254	0.27325
Old World	0.4065	0.3842	0.0546	0.2433	8.6501	1.5552	0.36499
All samples	0.3940	0.3765	0.0444	0.2469	10.7504	1.5254	0.36650

**H_t = Total diversity
   H_s = Diversity within populations
   G_{st} = Diversity among populations
   G_{cs} = Diversity due to colonies within subpopulations
   N_m = Estimate of gene flow based on G_{st}
   F_{st} = Fixation index

The very low G_{ST} values and high Nm values (estimate of gene flow), suggest that there is a high amount of gene flow between locations and very little genetic differentiation (Table 13).

Dendrograms

Some of the dendrograms created in Popgene were skewed until certain locations were removed. An example is shown here and the complete set of dendrograms can be viewed in Appendix IX. The UPGMA method was used to construct the dendrograms but this method assumes that evolutionary (mutational) rates are equal for each group (Michener and Sokal 1957). Other methods could have been used for more accurate dendrograms. The neighbor-joining method does not assume the same rate of evolution for each lineage, and usually produces accurate trees (Saitou and Nei 1987). The maximum parsimony method considers all differences and similarities and may be a more accurate method for creating

phylogenetic trees (Felsenstein 1978; Kolaczkowski and Thornton 2004). Outliers were used as a control to demonstrate the efficacy of the UPGMA method of building the dendrograms, and were not included in data analysis.
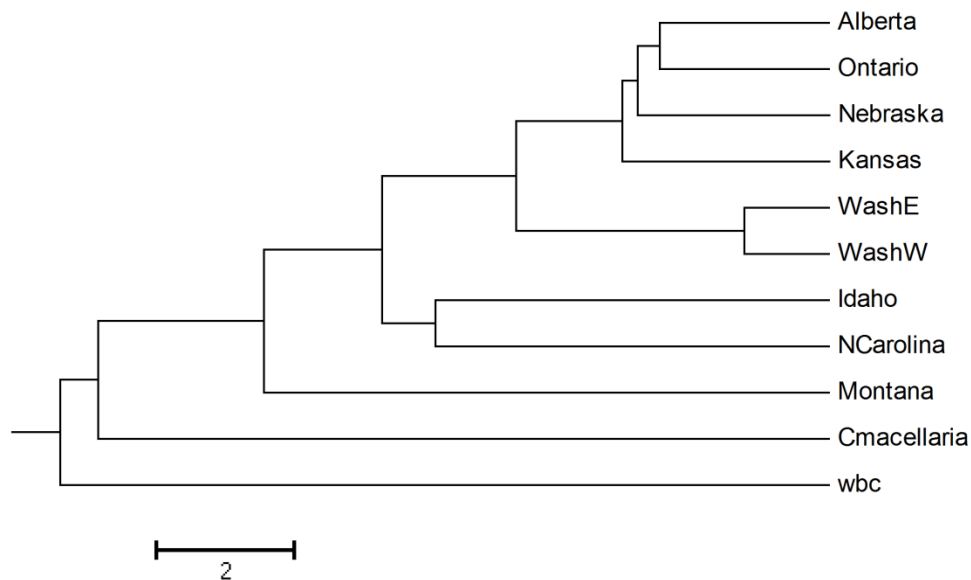

Fig. 6. Dendrogram generated using the North American *Stomoxys calcitrans* samples with *Cochliomyia macellaria* (Diptera: Calliphoridae) and the Western bean cutworm, *Striacosta albicosta* (Lepidoptera: Noctuidae) as outliers. Some locations were removed due to skewedness.

DISCUSSION

The results of this project support the results of Marquez et al. (2007) and Dsouli-Aymes et al. (2011) in many respects. Both authors suggested a separation between stable flies in the Oriental biogeographical region and the rest of the world. This project did not analyze any samples from the Oriental region. We did have samples from Australia, which the other two lacked. No differentiation was found between Australia and the other regions, perhaps because it was colonized by Europeans similarly to North America. Marquez et al.

(2007) analyzed a total of 277 individual flies, an average of 25 flies per country. Dsouli-Aymes et al. (2011) reported sequencing "one to ten specimens for each population". This project analyzed 662 individuals from 20 locations, an average of 33 flies per location. Using 2 primer pairs and the AFLP technique, 191 loci were generated per individual, with a total of 126,442 loci analyzed.

The Analysis of Molecular Variance (AMOVA) reported the majority of the genetic variation to be within populations (66.96%), with 27.82% among populations within groups, and a very small amount of variation (5.21%) among groups. F_{ST} was low at 0.33035, and is calculated by permuting haplotypes among populations among groups. The F_{CT} (0.05211) is calculated by permuting populations among groups, and the F_{SC} (0.29354) is calculated by permuting haplotypes within populations among groups (Excoffier et al. 2005). These results suggest a large amount of gene flow between locations, even on a global scale. Similar results have been reported at a local scale (Szalanski et al. 1996; Gilles et al. 2007). Dsouli-Aymes et al. (2011) showed nearly opposite results, with the majority of the variation in their samples occurring among groups (85.14% for COI, 71.73% for CytB, and 77.06% for NDI-16S). However, they grouped their samples into one group containing the Oriental samples and one group containing all others. If the Oriental population is a separate lineage, as they suspect, grouping in this manner would affect the AMOVA results.

The tests of neutrality, Tajima's D and Fu's FS, suggest that these populations may have experienced (or are experiencing) population expansion. Strong positive FS results indicate population subdivisions or bottlenecks,

whereas strong negative results suggest population expansion. According to

Tajima (1989), negative values of D suggest recent bottlenecks or large insertion-

deletion polymorphisms, while positive D values suggest restriction site or small

insertion-deletion polymorphisms. The raggedness index in the mismatch

distribution is also an indication of population expansion or bottlenecks, where a

small value (<0.05) indicates expansion and large values (>0.05) indicate a

stationary population or a bottleneck. My Tajima's D results are all high, with P-

values approaching 1, which suggests population expansion. Considering that the

AFLP technique produces restriction site fragments for analysis, the large D

values could be due to restriction site polymorphisms. The results of Fu's FS are

significantly negative, indicating population expansion, except for two locations,

Idaho and Western Washington. In the mismatch distribution, the raggedness

index values are very low both in the demographic expansion, based on the

sudden expansion model, and in the spatial expansion, based on the continent-

island model. The small raggedness values are indicative of population

expansion. These results support the hypothesis that stable flies originated in one

region and populations expanded over time.

Nei's genetic diversity tests support a high level of gene flow between

these locations. The G_{ST} and G_{CS} values are low (0.0444 and 0.2469 respectively),

and the Nm is very high, especially for North America, with a value of 20.5945.

The F_{ST} is also low, with a total F_{ST} of 0.36650, which agrees with the AMOVA

F_{ST} of 0.33035. These F_{ST} values are not as low as the G_{ST} values, and suggest a

small amount of genetic differentiation. F_{ST} applies to populations with only two

alleles at a locus, whereas G_{ST} can be applied to any population, with multiple alleles at each loci, therefore G_{ST} may be a more accurate representation of gene differentiation. If there are only two alleles, F_{ST} and G_{ST} are equivalent.

A Mantel test was used to investigate a correlation between genetic distance and geographical distance. The correlation coefficient, *r,* is very low at 0.063186. This number and the graph generated by SigmaPlot indicate that there is no correlation between genetic distance and geographical distance in these samples.

The tests of neutrality and mismatch distributions suggest that stable flies have experienced or are experiencing population expansion. The F-statistics and Nei's genetic diversity suggest that there is a large amount of gene flow occurring globally, which could indicate a panmictic population except for the Oriental region which seems to be isolated (Marquez et al 2007; Dsouli-Aymes et al. 2011). However, results generated by genetic analysis software are based on 1000 or more computer simulations and may not represent all of the underlying factors affecting the genetic structure of this insect.

The results from our dendrograms seemed to be skewed until some of the populations were removed. The dendrogram of North American samples shown above had the most logical distribution, but several locations were removed to get these results. However, several pairs of locations grouped together no matter what groupings were used. These include the two Denmark samples, eastern and western Washington, Nebraska and Kansas, Alberta and Ontario, Idaho and Montana (although North Carolina for some reason grouped with this pair), and

surprisingly, Australia and Morocco. In some groupings Texas and Arkansas grouped together. The majority of the North American samples which grouped together were geographically near to each other.

The most confusing aspect of the dendrogram results is that when Panama 1 and Panama2 were grouped with North America to form a "New World" group, the two always split up, with several American locations between them. But when grouped with the "Old World", the Panama samples grouped together. Also, the "Old World" always grouped in a logical order until all samples were grouped together, at which time the dendrogram became completely skewed, with Gabon and La Reunion grouping between North American samples. The outliers sometimes were skewed as well, grouping at the bottom of a sub-tree and not the entire tree. These results could be due to some of the gels being very good and others not, or it could be inconsistencies in the scoring, although the gels were scored a second time after the skewed dendrograms were produced. Scoring the gels again very carefully did not change the skewed results. It could be a combination of gel results and underlying genetic factors in stable flies.

The results could be indicative of the origins of New World stable flies. Since the Old World samples grouped in a logical order, there could have been several introductions into the New World from different areas. They would likely have been introduced from the Palearctic region due to colonization of North America by the Europeans. There could also have been introductions from Africa during slave trading activities. The skewedness of the dendrograms when the New World is included suggests multiple origins and a large amount of gene flow

between New World areas. Australia and Morocco grouping together may also be a result of colonization. Australia was colonized by the British at a time when they controlled many regions in Africa, so it seems likely that there would have been some travel between the colonies.

Considering that the results from the Arlequin analyses and Nei's genetic diversity were consistent throughout, there could be explanations for the skewed dendrograms.   The Panama samples, for example, could be genetically similar to the North American samples and the splitting apart could be due to having the same polymorphisms as some of the North American samples. It would not be surprising that gene flow is occurring between North and Central American locations. This could also apply to Old World samples. If stable flies are a panmictic population, with no isolated sub-populations to separate out, samples from any geographical location could be similar.

Dsouli-Aymes et al. (2011) suggested an African origin for stable flies in the other biogeographical regions. La Reunion and Gabon mixing into the New World group in my dendrograms could support this hypothesis. Panama being split into two groups could support the hypothesis of Palearctic origins for New World flies, suggesting that Panama samples were similar to both New and Old World groups.

My results suggest that human migration and colonization may have affected the expansion of the stable fly population. *Stomoxys calcitrans* is a synanthropic pest and a generalist pest of livestock. For thousands of years humans have traversed the globe, accompanied by their livestock. Nomadic tribes

wandered continuously, searching for sustenance. During the Greek and Roman empires, armies consisting of thousands of men on horseback traveled hundreds of miles to conquer new lands. The Romans traveled as far as the British Isles, the Vikings invaded Britain and parts of Europe, and Genghis Khan united nomadic tribes and conquered most of Eurasia. With the multitude of horses and other livestock accompanying man in these endeavors, it is likely that stable flies followed as well.

In more recent times, there has been an influx of humans and their livestock into the New World from the Palearctic region. As the human population spread across the Americas, the stable fly population expanded as well, feeding on livestock and increasing in numbers. Now, with humans and stable flies occupying the entire continent, stable fly populations continue expanding due to the movement of livestock for economic and recreational activities. Considering the dynamic movement of humans and livestock, it is not unexpected that stable fly populations are dynamic, with a very high degree of gene flow across the New World and globally.

It seems that stable flies are not differentiated across geographical barriers. However, one of the two locations that did not have a significant Fu's FS value was western Washington. This is not enough evidence to indicate that the Cascade Mountains are a barrier to gene flow, but it would be interesting to analyze a large number of populations from both eastern and western Washington to investigate the possibility. In this project, the western Washington sample was

small. The extracted DNA was of good quality and the gels were some of the best that were run, but a larger sample size would be more revealing.

One aspect of this project that should be reported, and usually is omitted from dissertations, is the unexplainable problems encountered during the research process. Human error is always a factor, especially when working with a subject such as DNA. Sterile techniques must be strictly followed to prevent contamination. Protocols must be optimized for each organism. Care must be taken to preserve the integrity of reagents such as enzymes and oligonucleotides. But following laboratory procedures diligently does not always guarantee good results. This project began optimistically, with all of the DNA extractions producing very large, very clean pellets of DNA. According to the Nanodrop® results, the DNA was of good quality and more than sufficient quantity. PCR was begun with enthusiasm and the first few gels that were run were excellent, with very clear, defined bands. Then results began to deteriorate. A gel would come out with every 8th individual amplified and no bands on the others. Then, by the end of the project, most of the gels were coming out white, with the sizing standard and the primer front visible but no bands (Appendix XI). It was suspected that the thermal cyclers may be malfunctioning, but it was not logical that they would all malfunction at the same time. During the last week of running gels, the thermal cycler that was used most often (because it was the most trusted) died completely. Although this could explain many of the gels that did not work, some of the "white" gels were run on different machines. Logically they would not all break down at once, although they have been used continuously for several

years and it could be a possibility. It was a random occurrence that could not be solved with the process of elimination, since the thermal cyclers seemed to work at times and not at others. The only choice was to persevere until enough good gels were produced to run the analyses.

In spite of the problems encountered, the results are consistent throughout the different analyses. They concur with the results of Marquez et al. (2007) and Dsouli-Aymes et al. (2011) despite the different analysis techniques. We suggest that, except perhaps for the Oriental region, the stable fly population is a panmictic population with gene flow occurring across geographical locations and barriers. However, samples from geographical locations near to each other tend to be more similar than more distant samples, with some exceptions, and this would be consistent with a high level of gene flow.

This project revealed significant information on the origin of stable flies in certain areas such as Australia and the New World. It showed that geographical features such as mountain ranges may not be a barrier to gene flow, and there are no isolated populations within the areas analyzed. This information could be a benefit when managing the pest populations, since pest management strategies should have a similar effect on stable flies in all locations. This project did not address the origin of stable flies in local areas, but some patterns arose in the dendrograms that may be useful for further research in that area. Alberta, Ontario, Nebraska and Kansas, always group together. These results could be explained by trade in the cattle industry between the Midwestern United States and Canada.

Further research could be conducted with larger sample sizes and different geographical locations. Locations such as eastern and western Washington could be analyzed with large sample sizes at a local scale. It would be a daunting task to acquire enough representative samples from every biogeographical region to answer questions such as would areas that we did not analyze have more differentiation; are there isolated populations across larger geographic barriers like the Himalayan Mountains, as suggested by Dsouli-Aymes et al. (2011). Larger-scale global projects could provide insight into stable fly population dynamics both on the local scale and globally.

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APPENDIX I

Extraction Protocol DNA

<u>**Specimens:**</u>

Samples were preserved in 95% ethanol following the procedure in chapter 2, and stored at 4ºC until processing. Prior to extraction, samples were transferred to 1.5ml microcentrifuge tube and washed in dH_2O for >10 min. The head, abdomen and appendages were removed from the thorax, the thorax was opened with a scalpel and the gut was removed to avoid contamination with extraneous DNA.

DNA EXTRACTION

CTAB METHOD: ORIGINAL PROTOCOL

<u>**Procedure:**</u>

➤ Place specimen in an autoclaved 1.5 ml microcentrifuge tube, add 250 µl CTAB buffer and homogenize with pestle. Then add another 250 µl CTAB and mix well by low speed vortexing.

➤ Add 10 µl proteinase K (stock conc. 20 mg/ml), vortex at low speed. Incubate 1-2 h at 65 ^{o}C. Gently mix the homogenate by inverting the tube after every 20 min.

➤ Add 15 µl RNase A, (stock conc. 50 mg/ml) incubate for at least 2h at 37 ^{o}C by gently mixing the homogenate after every 20 min by inverting the tube several times. Do not vortex.

➤ Centrifuge for 5 min at 14,000 rpm. Remove supernatant, transfer to new autoclaved tube.

- ➤ Add 500 µl chloroform: isoamyl alcohol mixture (24:1). Mix the organic and aqueous phases by inverting the tube several times. Do not vortex as DNA may shear.

- ➤ Centrifuge for 20 min at 12,000 rpm (room temperature).

- ➤ Collect the upper aqueous phase, transfer to a new autoclaved eppendorf tube and repeat the chloroform: isoamyl alcohol extraction step again.

- ➤ Transfer the aqueous phase into a new eppendorf tube without disturbing the interface. Add 400 µl of chilled isopropanol (-20 ^{o}C). Mix gently and keep the samples at 4 ^{o}C for at least 2 h or leave overnight.

- ➤ Centrifuge for 30 min in a cooled microcentrifuge (4 ^{o}C) at max speed/12,000 rpm. At this stage DNA becomes visible as a white/transparent pellet at the bottom of the tube.

- ➤ Retain the pellet and carefully discard the supernatant.

- ➤ Wash the pellet in 500 µl chilled absolute ethanol. Tap the tube until the pellet comes free from the bottom of the tube. Centrifuge for 5 min at 4 ^{o}C, max speed. Decant the supernatant and wash pellet in cold 70 % ethanol.

- ➤ Centrifuge for 5 min max speed in a cooling centrifuge. Remove as much 70% ethanol as possible with a pipetteman. Air-dry pellet (30 min). Make sure that ethanol has completely evaporated, then, add 50-100 µl autoclaved 1X TE buffer. Re-suspend overnight at 4 ^{o}C.

- ➤ DNA can be stored at 4 ^{o}C for short term and at –20 ^{o}C for long term.

Changes to original protocol

1. A few grains of autoclaved washed sea sand (Fisher Scientific) were added to the tubes to facilitate homogenization.

2. RNaseA was added to samples and incubated at 65ºC for 2 hours, as per manufacturer's package insert. Stock solution was diluted 1:100 as per personal communication with Applied Biosystems technician.

3. Proteinase K was added after RNaseA, and incubated at 37ºC as per manufacturer's package insert. Stock solution was diluted 1:10 as per personal communication with Applied Biosystems technician.

4. Centrifugation was always performed at 14,000rpm.

5. Ethanol washes were 400µl.

6. Pellets were allowed to air dry for several hours under the fume hood. To make sure all ethanol has evaporated, the tube can be flicked. If ethanol is still present it will splatter on the sides of the tube.

APPENDIX II

Reagent and buffer preparation

SOLUTIONS REQUIRED FOR CTAB METHOD

I. CTAB buffer

Component	Mol. Wt.	Qty. needed	
		100 ml	200 ml
100 mM Tris-HCl	157.59	1.576 g	3.152 g
1.4 M NaCl	58.44	8.182 g	16.363 g
0.02 M EDTA	372.2	0.744 g	1.489 g
2% CTAB (Hexa decyl trimethyl ammonium bromide)		2.000 g	4.000 g
0.2% β-mercapto ethanol		0.2 ml	0.4 ml

For a 200 ml solution, weigh out all the components except β-mercapto ethanol and dissolve in 150 ml nanopure water. Adjust the pH to 8.0 and then make up the volume to 200 ml. Autoclave the solution and add 0.4 ml (400 µl) β-mercapto ethanol after cooling. Store at 4 ^{o}C*.

NOTE: CTAB buffer stored at 4 ^{o}C forms a precipitate, therefore before using the buffer for DNA extraction, heat the solution at a low temperature to dissolve CTAB and then use the solution.

*Change in protocol: CTAB was transferred to small glass bottles at 20 ml per bottle before autoclaving. B-mercaptoethanol was not added until just prior to use, at 40μl/bottle. It is efficacious to prepare 800 ml at one time, which makes 40 bottles of 20 ml, enough for 40 extractions of 40 samples each (or 80 extractions of 20 samples, etc.).

II. Proteinase K (Stock 20 mg/ml): Stored at −20 ^{o}C.

Weigh 0.02g Proteinase K powder. Add 600 μl of autoclaved nanopure water. Mix thoroughly till proteinase dissolves. Then add 400 μl autoclaved glycerol. Store at − 20 ^{o}C. This is the stock solution. It must be diluted 1:10 for a working solution.

Alternatively, we can buy 20 mg/ml Proteinase K solution.

III. RNase A   (Stock 50 mg/ml): Stored at −20 ^{o}C

Weigh 0.05g RNase powder. Add 600 μl of autoclaved nanopure water. Mix thoroughly till the enzyme dissolves in water. Then add 400 μl autoclaved glycerol. Store at −20 ^{o}C. This is the stock solution. It must be diluted 1:100 for a working solution.

Alternatively, we can use the RNase solution.

IV. 1X TE buffer

10 mM Tris-Cl
0.1 mM EDTA

Weigh 0.03582g EDTA. Dilute in small beaker with 10ml dH$_2$0.

Weigh 0.1576g Tris-HCl in small beaker with 10ml dH$_2$0.

Combine 2-10ml portions with 900ml dH$_2$0 in 1000ml beaker.

Use pH meter to adjust pH to 8.0

Pour into screw cap bottles, autoclave, store at 4ºC.

ORGANIC REAGENTS

The **organic reagents** required for DNA extraction are found in the cupboard below the fume hood. Always use these reagents in a fume hood.

- Chloroform: Isoamyl alcohol (24:1)
- Measure 240 ml chloroform into a beaker and add 10 ml isoamyl alcohol. Mix and store in a reagent bottle at room temperature in the fume hood.

- **Isopropanol (Isopropyl alcohol):** Store in a bottle at – 20 ^{o}C

- **Absolute (99.9%) Ethanol**: Store in a bottle at – 20 ^{o}C

- **70% Ethanol:** Mix 70 ml 99% ethanol with 30 ml double distilled water and store in a bottle at - 20 ^{o}C

APPENDIX III

AFLP Protocol: Lab version

AFLP-PCR

STEP 1: RESTRICTION DIGESTION

Component	Conc. Needed	1 Rxn	Supplier
One Phor all buffer	1x	1.25	Amersham
Mse I enzyme	1.25U	0.32	NEB
Eco RI enzyme	1.25U	0.08	Amersham
BSA	1.25μg	0.125	NEB
Autoclaved nanopure water	-	3.725	-

✓ Dispensed 5.5µl into each tube

✓ Added 7.0 µl template DNA

✓ Re-digest for 2.5 hours at 37^oC in Perkin Elmer Gene Amp PCR System 9600. Also enzyme denature at 70^0C for 15 minutes in the same system. Used the following program

      3 temperatures PCR

      37^oC 60 min, 37^oC 90 min, 70^oC 15 min

Step 2: ADAPTER LIGATION

ADAPTER PREPARATION (DURING OR BEFORE DIGESTION)

Component	1 Rxn	Supplier
EcoRI Adapter	0.5	OP
Mse I Adapter	0.5	OP
T_4DNA ligase buffer	0.5	NEB
T_4 DNA Ligase	0.15	NEB
Autoclaved nanopure water	3.35	-

Eco R1 Adapter	For 100 ligations	For 200 ligations
Eco R1.1 (1µg/ µl)	1.40 µl	2.8 µl
Eco R1.2 (1µg/ µl)	1.25 µl	2.5 µl
OPA	2.50 µl	

STEP 3: DILUTING THE LIGATION MIXTURE

A 1:10 dilution of the ligation mixture was performed by transferring 135 µl of TE buffer to the 0.5 ml microcentrifuge tube containing approximately 15 µl of the digest/Ligation mixture, and mixing well. Leave it overnight in 4°C.

STEP 4: PREAMPLIFICATION (from Licor protocol)

The following components were added to the PCR tubes (new tubes)

Component	Stock conc.	Conc. Needed	1 Rxn	Supplier
Pre amp primer mix II	10X	-	10.0	BRL
10X PCRbuffer+ Mgcl$_2$ 15 mm	10X	1X	1.25	PE
AmpliTag DNA polymerase	10U/µl	1.25U	0.25	PE

✓ Dispensed 11.50 µl into each tube and added 1.25 µl diluted template (step 3)

✓ Mixed gently and performed 20 cycles of amplifications.

Amplificaiton Conditions:

94°C for 30 s

56°C for 1 min

72°C for 1 min

Soak at 4°C

Note: Calculations shown here represent ½ volume of the volume used in the Licor protocol.

STEP 5: AGAROSE GEL ELECTROPHORESIS to check pre-amplifications
Condition:

▪ 1% agarose gel

▪ Buffer: 1x TAE

▪ Electrode : 60 Volt

▪ **Period: 10-15 min**

▪ **1** µl pre-amplified product mixed by 1 µl dye loaded

STEP 6: DILUTION OF PREAMPLIFIED PRODUCT

A 1:20 dilution of pre-amplified product was performed by adding 190 µl

autoclaved ddH$_2$Oo to approximately 10µl of pre-amplified mixture

STEP 7: SELECTIVE AMPLIFICATIONS

(from LICOR handout)

✓ Prepared master mix separately for each primer combo

✓ Master mixture was prepared for all reactions to compensate for pipetting error.

Component	Conc. Needed	1 Rxn	Supplier
Mse I primer (CTA)	-	2.0	BRL
*IRD-EcoRI Primer(AAG)		0.50	Licor
10X PCRbuffer	1X	1.20	PE
AmpliTag DNA polymerase	1.25U	0.06	PE
Autoclaved nanopure water	-	4.80	-

8.5 µl of the mixture was dispensed into each tube containing 2.0 template (1:20

diluted pre-amp mix). DNA was added, mixed and PCR amplified.

Selective PCR Amplificaiton

Conditions **(TOUCHDOWN PROGRAM)**

1 cycle   94°C for 30 s

                               65°C for 30 s

                               72°C for 1 min

12 cycles   94°C for 30 s

                               65°C- 56°C   for 30 s

                               72°C for 1 min

23 cycles	94°C for 30 s
	56°C for 30 s
	72°C for 1 min
Soak	4°C

*Note: the IRD label is light sensitive, so keep the samples protected from light by covering with an aluminium foil form this step on

Reagent preparation

Tris-Borate (TBE)*

Working solution	Concentrated stock solution pH 8.0 (per liter)	
1X	5X	**10X**
	54 g Tris-base	108 g Tris-base
	27.5 g Boric acid	55 g Boric acid
	20 ml 0.5M EDTA solution (pH8.0)	40 ml 0.5M EDTA solution (pH8.0) 7.44 g salt

* A precipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid problems, store the 5X or 10X solution in glass bottles at room temperature and discard any batches that develop a precipitate.

Bind Silane

Add 50 µl bind silane to 10 ml absolute ethanol. Store at 4ºC in amber jar or wrap with foil.

Ammonium persulfite solution

0.1g Ammonium persulfite powder

1 ml nanopure H$_2$0

Prepare in 1.5ml microcentrifuge tube. This will make 6 gels. To prepare the amount for one gel, weigh 0.015g ammonium persulfite powder directly into the microcentrifuge tube. Add 150 μl nanopure H$_2$0 Mix by flicking and inverting the tube.

Polyacrylamide gel solution (lab protocol)

19 ml KBPlus gel matrix (Licor)

128.4 μl ammonium persulfite solution

12.85 μl Temed

Mix by swirling and stir with pipette.

Polyacrylamide gel solution (LICOR product protocol)

20 ml KBPlus gel matrix (Licor)

150 μl ammonium persulfite solution

15 μl Temed

Adapter preparation

EcoRI   **100 ligations**

EcoRI-1forward adapter (100μM)   5μl

EcoRI-2 reverse adapter (100μM)   5μl

NEB Buffer 4	2.2µl
dH$_2$0	87.8µl

MseI	**100 ligations**
MseI-1 forward adapter (100µM)	50µl
MseI-2 reverse adapter (100µl)	50µl
NEB Buffer 4	2.2µl

Denature the adapters for 3 min at 95ºC using the "denature" program on the thermal cycler.

Note: New England Biolabs (NEB) Buffer 4 has been substituted for "One Phor All Buffer", since that product was discontinued. Online searches and personal communication with Invitrogen and New England Biolabs revealed that these products contained the same ingredients. NEB Buffer 4 is included in the package when MseI restriction enzyme is purchased. This substitution also applies to step 1 and step 2 of the AFLP protocol.

Primers

Primers were supplied by Licor until 2009, when they discontinued the sale of AFLP-ready primers. Integrated DNA Technologies (IDT) acquired the license for IRDye-labeled primers previously supplied by Licor. To order from IDT, custom oligonucleotides must be specified, and the primer sequence supplied. The primers must be resuspended to a stock solution of 100µM by adding 10X the weight of 1X TE. For example, a tube containing 13ng of primer would be resuspended in 130µl 1X TE buffer. To make the working solution, the

100μM stock solution is diluted 1:100 with 1X TE buffer or nanopure H_20. The EcoRI primers are IRDye-labeled.

Resuspension and dilution of primers must be done very carefully under the clean hood using strict sterile techniques to avoid contamination. It must also be performed in the dark due to the light-sensitive nature of the labeled primers.

Primers may be ordered lab ready, meaning that they are resuspended to 100μM by IDT.

Primers ordered from IDT.

Name	Sequence	No. of Bases	Recognition Site
Adapters			
EcoRI-1 (forward)	5-CTCGTAGACTGCGTACC-3	17	
EcoRI-2 (reverse)	5-AATTGGTACGCAGTCTAC-3	18	
MseI-1 (forward)	5-GACGATGAGTCCTGAG-3	16	
MseI-2 (reverse)	5-TACTCAGGACTCAT-3	14	
Selective Primers			
E-NNN	5-GACTGCGTACCAATTCNNN-3	19	G*AATTC
M-NNN	5-GATGAGTCCTGAGTAANNN-3	19	T*TAA

The "NNN" notation for selective primers denotes the variable bases which are chosen for each primer. If the extension "ACT" is chosen for an EcoRI primer, it would be named "E-ACT". Any of the four bases can be substituted for "N" except at the first position, where the base must be complimentary to the preamplification primer. Using EcoRI and MseI, the first base of the E-extension

will be an "A" and the first base of the M-extension will be a "C", eg. E-**A**AC or M-**C**AG.   If different restriction enzymes are used, the first base in the extension would be complimentary to a different restriction site (Vos et al. 1995).

NOTE: IDT primers do not contain dNTPs. These must be added to the selective primer mix.

APPENDIX IV

Optimized AFLP Protocol

As noted previously, several changes have been implemented in the lab protocols due to discontinuation of products, different suppliers, and research of manufacturer suggestions for product use. Following is the protocol used during this research based on these changes. Only the optimized AFLP protocol is documented below. The CTAB extraction protocol had minor points which were discussed in Appendix I.

STEP 1: RESTRICTION DIGESTION

Component	Stock conc.	Conc. needed	1 Rxn	100 Rxn	Supplier
NEB buffer 4	10X	1X	1.25 µl	125 µl	NEB
MseI enzyme	4U/µl	1.25 U	0.32 µl	32 µl	NEB
EcoRI enzyme	15U µl	1.25 U	0.08 µl	8 µl	NEB
BSA	10mg/ml	1.25 µg	0.125 µl	12.5 µl	NEB

✓ Dispense 5.5µl into each tube

✓ Add 7.0 µl template DNA

✓ Re-digest for 2.5 hours at 37°C in Perkin Elmer Gene Amp PCR System 9600. Also enzyme denature at 70^{0}C for 15 minutes in the same system. Use the following program (usually designated "restdig" on thermal cycler)

> 3 temperatures PCR
>
> 37°C 60 min
>
> 37°C 90 min

70°C 15 min

Step 2: ADAPTER LIGATION

Component	1 Rxn	100 Rxn	Supplier
EcoRI Adapter	0.5	50 µl	IDT
Mse I Adapter	0.5	50 µl	IDT
T$_4$DNA ligase buffer	0.5	50 µl	NEB
T$_4$ DNA Ligase	0.15	15 µl	NEB
dH$_2$O	3.35		-

Dispense 5 µl of ligation mix into tubes containing digestion product from step 1.

Incubate on the thermal cycler at 25ºC for 8 hr. Program: ADAPLIG.

ADAPTER PREPARATION

Eco R1 Adapter   **100 ligations**

Eco R1-1 forward (100µM)   5 µl

Eco R1-2 reverse (100µM)   5 µl

NEB Buffer 4   2.2 µl

dH$_2$O   87.8 µl

MseI Adapter   **100 ligations**

MseI-1 forward (100µM)   50 µl

MseI-2 reverse (100µM)   50 µl

NEB Buffer 4   2.2 µl

Denature adapters for 3 min at 95ºC. Store at -20ºC.

STEP 3: DILUTING THE LIGATION MIXTURE

A 1:10 dilution of the ligation mixture is performed by transferring 135 µl of TE buffer to the 0.5 ml microcentrifuge tube containing approximately 15 µl of the digest/ligation mixture, and mixing well. The dilutions may be stored at 4°C.

STEP 4: PREAMPLIFICATION (from Licor protocol)

The following components are added to new PCR tubes:

Component	1 Rxn	100 Rxn	Supplier
Pre amp primer mix II	10.0	1000 µl	Licor
10X PCRbuffer	1.25	125 µl	Included w/taq
25mM MgCl$_2$	.75 µl	75 µl	Included w/taq
Taq DNA polymerase	0.25	25 µl	AB or Midsci

✓ Dispense 12.25 µl into each tube and add 1.25 µl diluted template DNA from step 3.

✓ Mix gently and perform 20 cycles of amplification using the "preamp" program.

Amplificaiton Conditions:

94°C for 30 s

56°C for 1 min

72°C for 1 min

Soak at 4°C

Note: Calculations shown here represent ½ volume of the volume used in the Licor protocol.

STEP 5: AGAROSE GEL ELECTROPHORESIS (to check preamplifications)

This step was omitted due to having no UV box in which to visualize the gel.

STEP 6: DILUTION OF PREAMPLIFIED PRODUCT

A 1:20 dilution of pre-amplified product was performed by adding 190 μl autoclaved dH$_2$O to approximately 10μl of pre-amplified mixture. If this results in too much DNA, the amount of water can be adjusted.

STEP 7: SELECTIVE AMPLIFICATION

✓ Prepare master mix separately for each primer combination

Component	1 Rxn	100 Rxn	Supplier
Mse I primer	.75 μl	75 μl	IDT
*IRD-EcoRI Primer	.4 μl	40 μl	IDT
10X PCR buffer	1.2 μl	120 μl	Included
25mM MgCl$_2$	.72 μl	72 μl	Included
10mM dNTPs	.24-.4 μl		AB, NEB, MidSci
Taq DNA polymerase	0.09μl	9 μl	AB, MidSci
DNA (from step 6)	2 μl		
dH$_2$O	6.79 μl	679 μl	

NOTE: The selective mix protocol must be optimized for each organism.

Dispense 10 μl of the mixture into new PCR tubes. Add 2 μl DNA from step 6.

Run selective amplification using the following parameters. Different

designations are used for the selective program. "Touchdown", "selective", or

just numbers corresponding to annealing temperature "52" are programmed on the thermal cyclers. View the programs before use to check the parameters.

Selective PCR Amplificaiton

1 cycle	94°C for 30 s
	65°C for 30 s
	72°C for 1 min
12 cycles	94°C for 30 s
	65°C - 56°C for 30 s
	72°C for 1 min
23 cycles	94°C for 30 s
	56°C for 30 s
	72°C for 1 min
Soak	4°C

STEP 8: DENATURE

✓ Add 2.5 µl blue stop solution to the selective products

✓ Denature for 3 min at 95ºC using the "denature" program.

✓ Samples are ready to load on gel.

APPENDIX V

Gel Preparation

- Measure 20 ml (6.5%) gel matrix (4 C).

- Allow it to warm to room temperature (approximately 15 min)

- Meanwhile prepare gel plates

- Arrange the plates on the Styrofoam blocks as indicated below, with the flat edge facing up. Clean plates by washing with 70% isopropanol on each side. Then polish with Pledge or other furniture polish on both sides, followed by a last wash with 100% isopropanol. At this point, plates should be literally squeaky clean.

- Prepare bind silane solution by pipetting out 100 ul of 10% acetic acid and 100 ul of bind silane into a clean, autoclaved microcentrifuge tube and mixing it well.

- Apply bind silane to the plates in the area where the comb is inserted and allow it to dry. Bind silane allows proper well formation.

- Arrange the plates by placing the spacer and fixing the plates with the gel rails. Over-tightening the screws on the clamps may crack the plates.

- Prepare 10% Ammonium persulfite solution (we usually prepare 1ml of 10% APS by dissolving 0.1 g APS in 1 ml autoclaved double-distilled water.

For preparing the acrylamide gel

1. Align the plates properly, with the diagonal face up. Diagram of the plates:
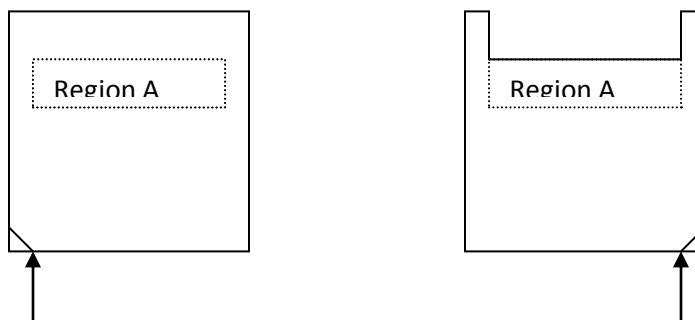
2. Prepare a solution of

   20 ml 6.5% KBPlus Gel matrix

   150 μl Ammonium persulfate

   15 μl TEMED.

3. Pour the gel with a 10 ml pipet. Pour 10 ml of the gel, insert the comb, then pour the remainder of the gel solution

4. Allow gel to set for 1 ½ - 2 hours

Loading and Running the Gel

Set up Licor scanner

➤ Prepare 1L of 1X TBE buffer from the 10X stock solution by mixing 100 ml stock with 900 ml nanopure water.

o Place the bottom buffer tank into the machine. Add 1X TBE to the fill line.

o Place gel into the machine, making sure that the rail arms are straight on the hooks.

• Place the upper tank into the gel rails at the top of the gel. Tighten the clamps.

• Add 1X TBE to the upper tank, making sure that the buffer covers the wells. It should be above the bottom fill line but doesn't have to reach the top fill line. Usually in the center between fill lines is plenty of buffer.

• Place the lids on both buffer tanks. Make sure they are properly plugged in to the power sources. Plug in the short power cord to the bottom of the upper buffer tank and the power source at the top right of the machine. Close the scanner lid.

- Open an internet browser such as Google Chrome. Type the number from the machine window into the browser address bar. Since the browser will recognize the address, usually typing "1" or "192" will bring up the address. Go to the website.

- A white window appears with a menu bar at the left. The first time a link is clicked, a password window will be displayed.

- After entering the password, click on "Collect Image". A window appears where the run is given a name. Click on "Create Run". The Prerun window will appear. Start the prerun, which will run for 15 min. If an error occurs during the prerun, the error log found in the menu will indicate the problem.

Load the samples

When the prerun has finished, it will show <<LOAD SAMPLES>>. At this time, disconnect the power cord, remove the lid from the top buffer tank and load 1 µl PCR product to each well. Do not begin loading in the first well. This will be used for the sizing standard, as will the well after the last sample.

- Load the samples and sizing standards, place the lid on the buffer tank and connect the power cord. Close the lid. On the computer, click "Collect Image". The gel is now running.

- Check the gel after ~ 1 hr to make sure it is working. Click on "Current Image". The gel will be displayed in the window. "Page up" to see the gel.

- Run the gel for 2 hr. In this time there will be markers as large as 500 bp. If ran longer, larger markers will be scanned, but usually there are not many scorable markers after 500 bp.

- To stop the gel, simply click on "Done Collecting".

APPENDIX VI

Scoring the Gels

Gels are scored using the SAGA Generation2 software from Licor. Detailed instructions on each step of the scoring process, including photographs of the computer window at each step, can be found in Lindroth (2011) and Krumm (2005). This paper will summarize the steps involved in scoring a gel.

Open the E-SEQ software on the desktop to save the gel. When first saving the gel, open it from the scanner. The gel will be saved in a previously opened file.

Open the SAGA Generation2 software. The server may have to be started before the software will run. There are 2 shortcuts on the lab computer, one for the server and one for the software.

A new project must be created in SAGA, which can be used for all gels in that project. When the software is opened, a project manager appears which contains a marker manager, primer manager, bin manager and gel manager.

Begin with the marker manager, the first button on the right. Each marker of the sizing standard must be entered into the manager one at a time. Then go to the primer manager. Here each primer will be entered and the primer pairs used in the project will be designated.

The bin manager will not be used at this time. Open the gel manager. Each sample name in the project must be entered into the manager individually.

Build the gel by adding the marker to the first line, then transferring the samples from the list on the left to the list on the right which represents the

current gel. The marker will also be put in the last line. For example, a gel with 20 samples will have the marker on line 1, samples on line 2-21, and the marker on line 22. Next, add the primer pair to lines 2-21. Do not add it to the markers. When the gel is finished it will show in the project manager as "ready to run".

Import the gel into SAGA by clicking on the gel template that is "ready to run", go to "file" and click on "import". The gel template will move to the "gels" tab and go through the process of reading the gel. The process includes "getting image", "lane analysis" and "genotyped". If for some reason the software doesn't accept the gel, it will either display "error" or become stuck on "lane analysis". The program will not allow the gel to be deleted when it is stuck on "lane analysis". If this occurs, the computer may have to be restarted, after which "lane analysis" will usually convert to "error".

Gels with the status of "genotyped" are ready to score. Four steps are involved in scoring. The first is to make sure the lanes are straight and in the correct place. One lane should lie in the center of each gel lane, including the sizing standards. When the gel is first opened, the button at the top left of the screen should be in "lane" mode. Click this button to enter "calibration" mode. Red lines each designated with a marker size will be displayed. Each line must be correctly placed on the gel.

Click the same button again to get to the "desmile" mode. Yellow lines allow the gel image to be straightened. It is helpful to also display the calibration lines so the same angle can be followed with the desmile lines. Clicking the same button one more time will display the "scoring" mode, where scores, bins, bin

lines and markers can be displayed. Many loci will automatically be marked with

"+" or "-". If the software is unsure of a locus it will display a box which can be

manually scored.

When scoring is finished, confirm the gel. The program manager will then

display the status of the gel as "confirmed".
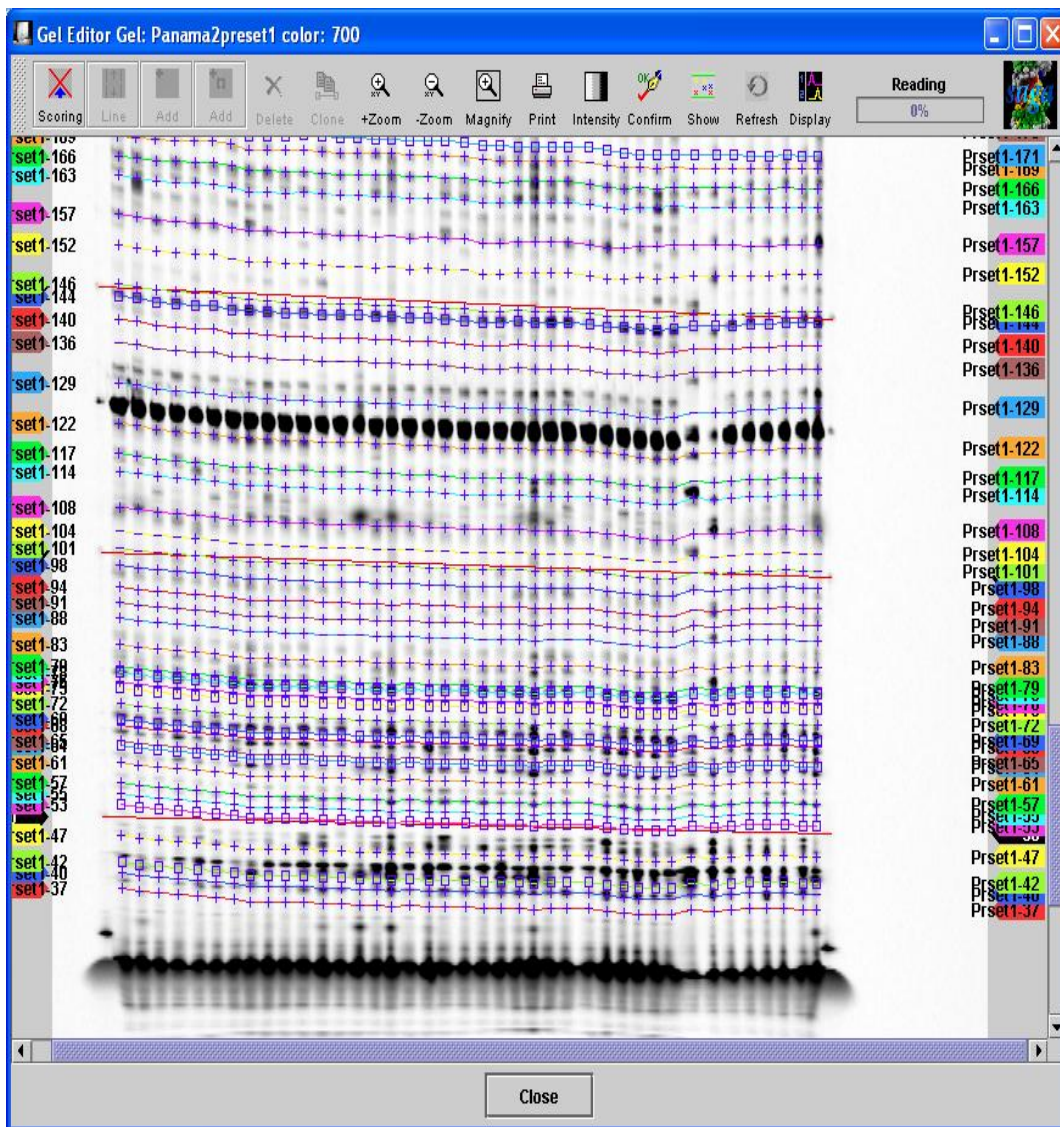
Fig. 7. Scoring an AFLP gel in SAGA Generation2 software.

APPENDIX VII

Examples of gels used in this project
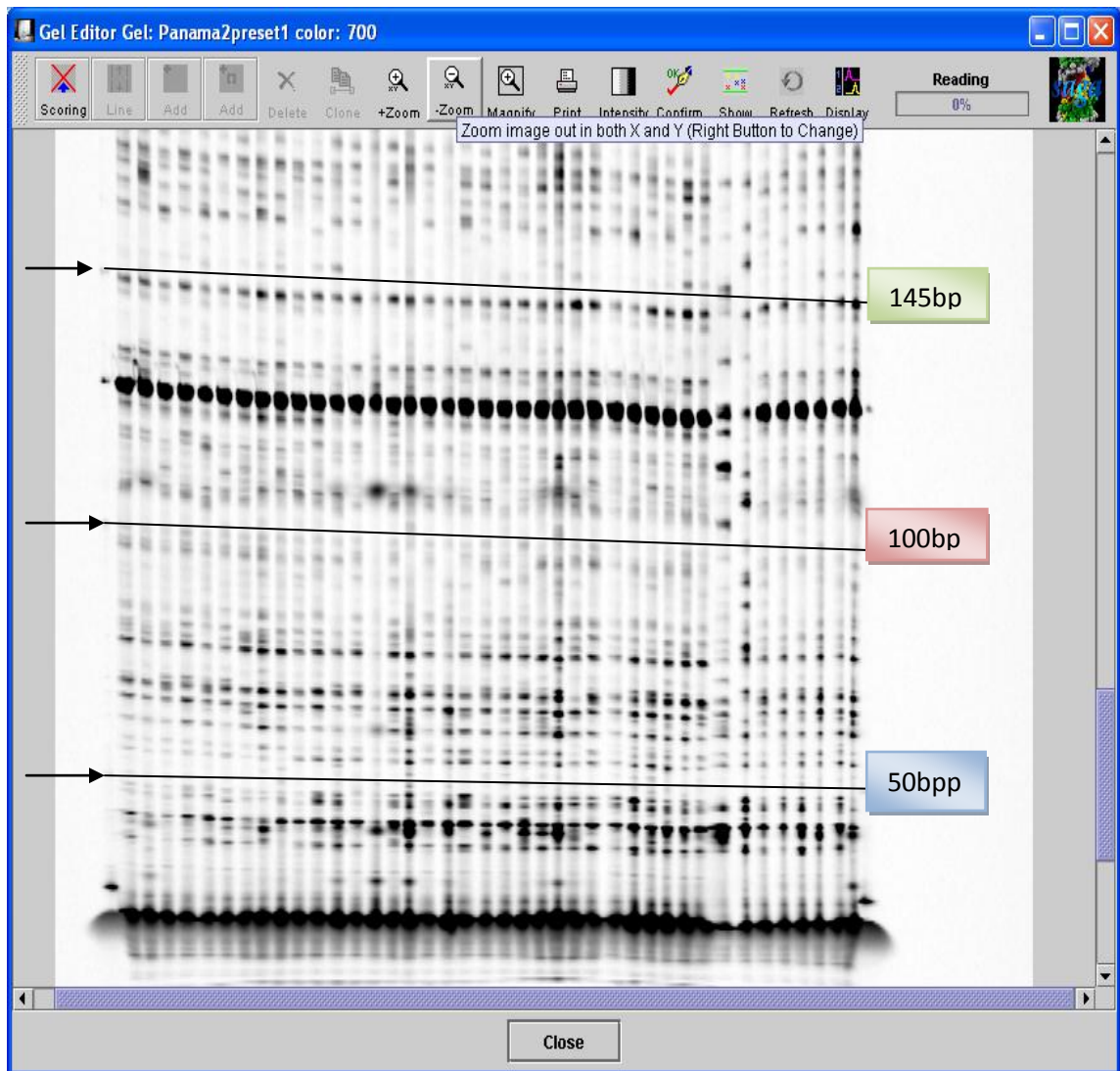
Fig 8. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected in Panama.
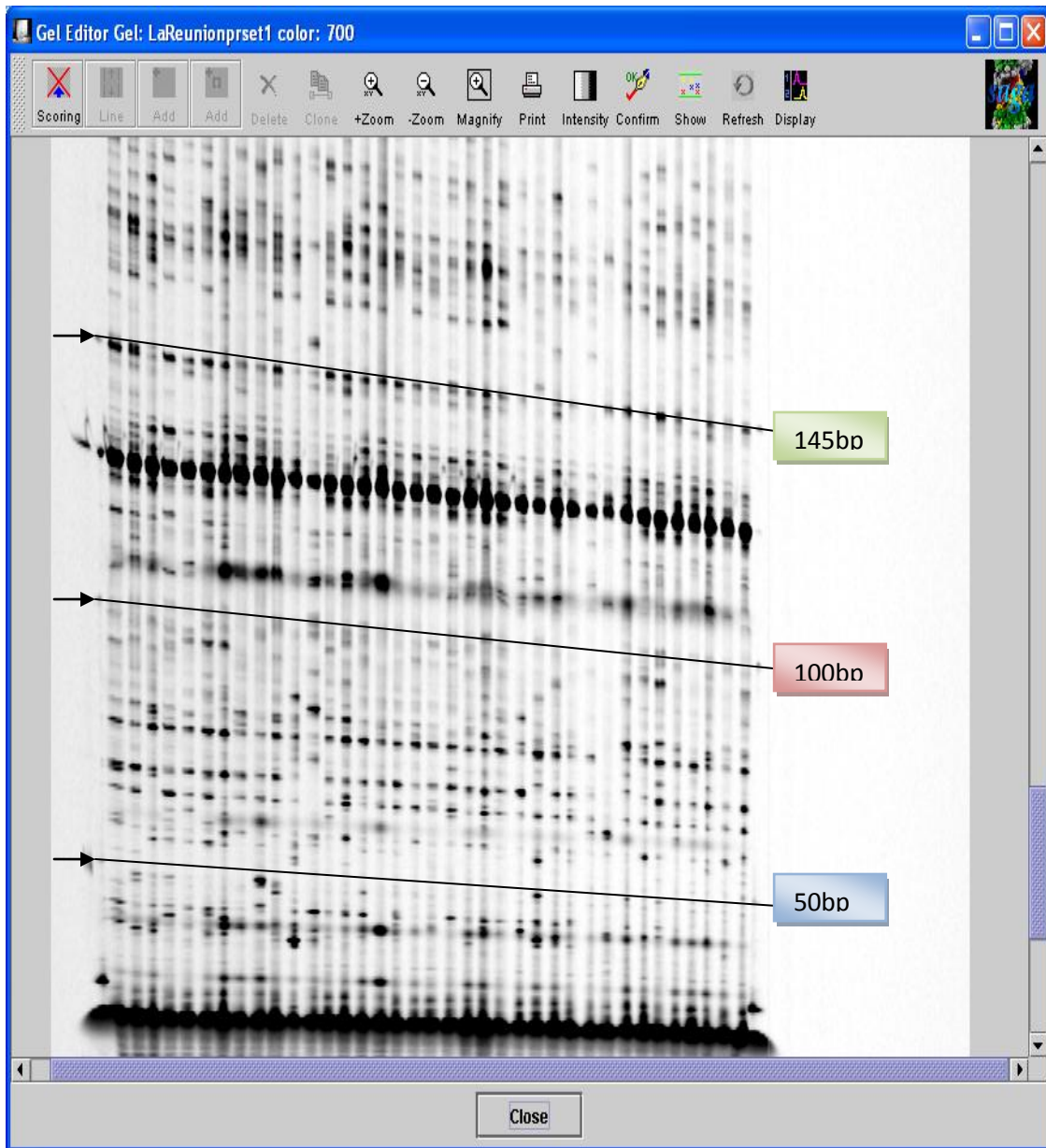
Fig. 9. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected on La Reunion Island.
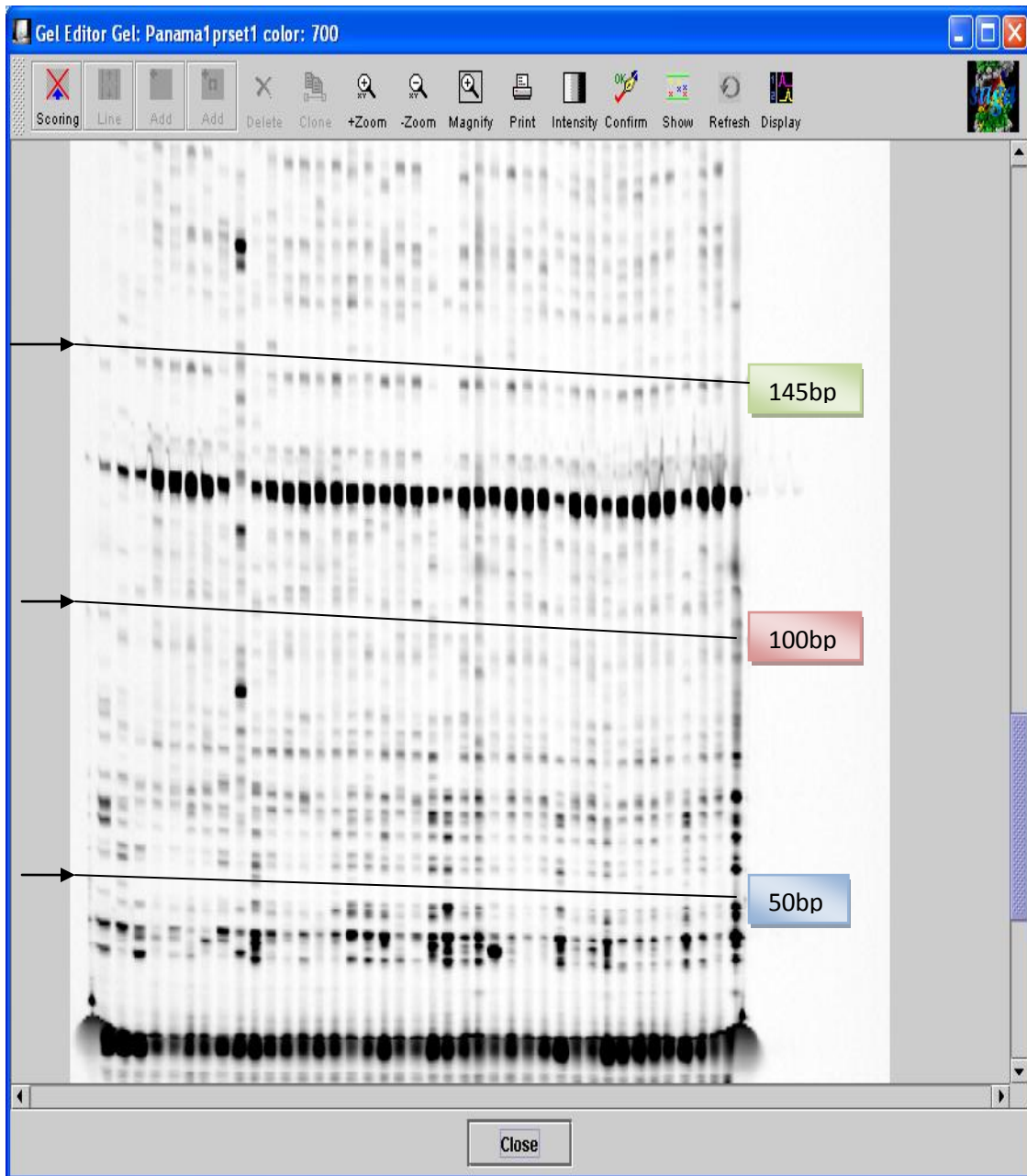
Fig. 10. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a second sample collected in Panama (Panama2).
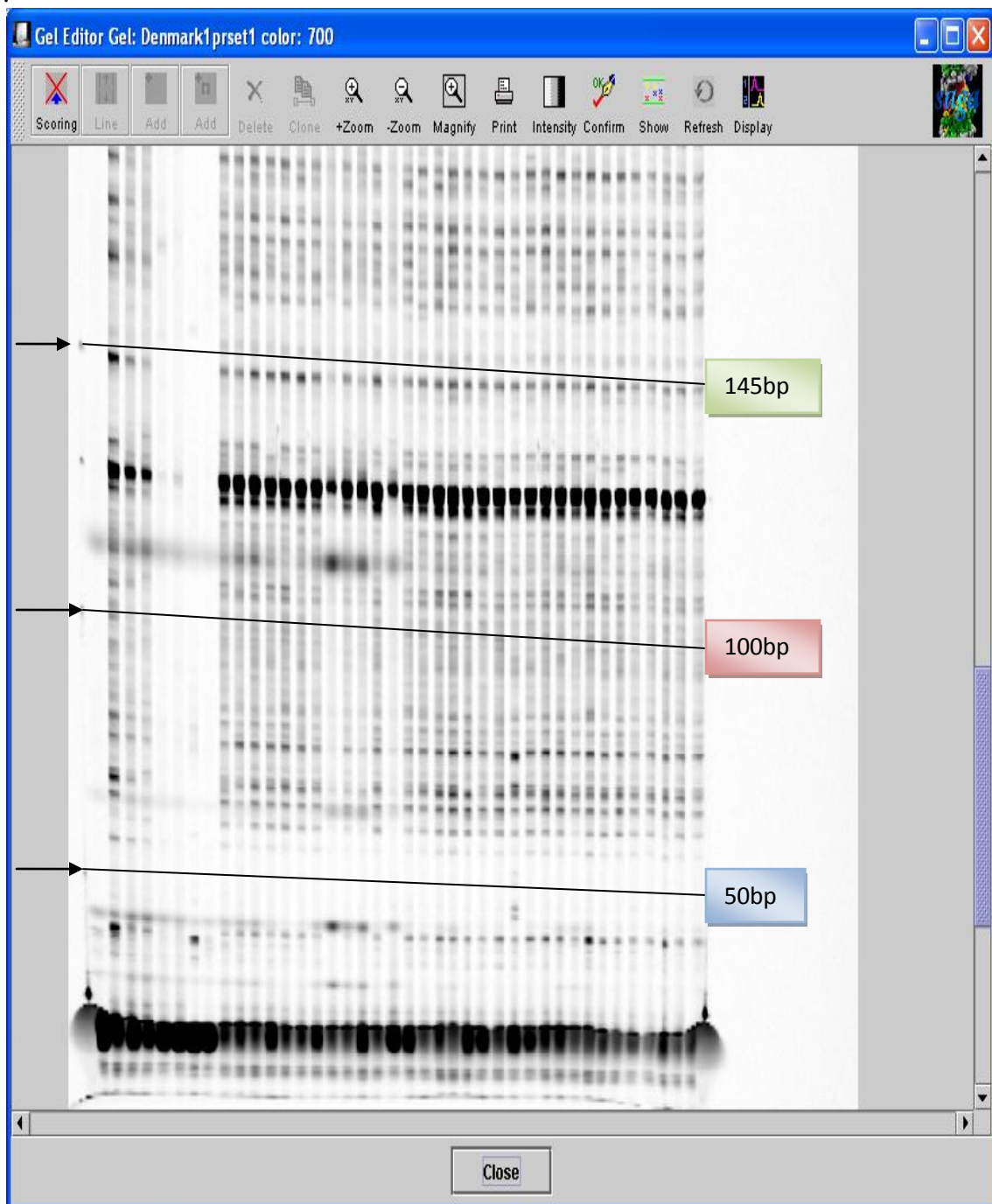
Fig. 11. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected in Denmark, designated Denmark 1.
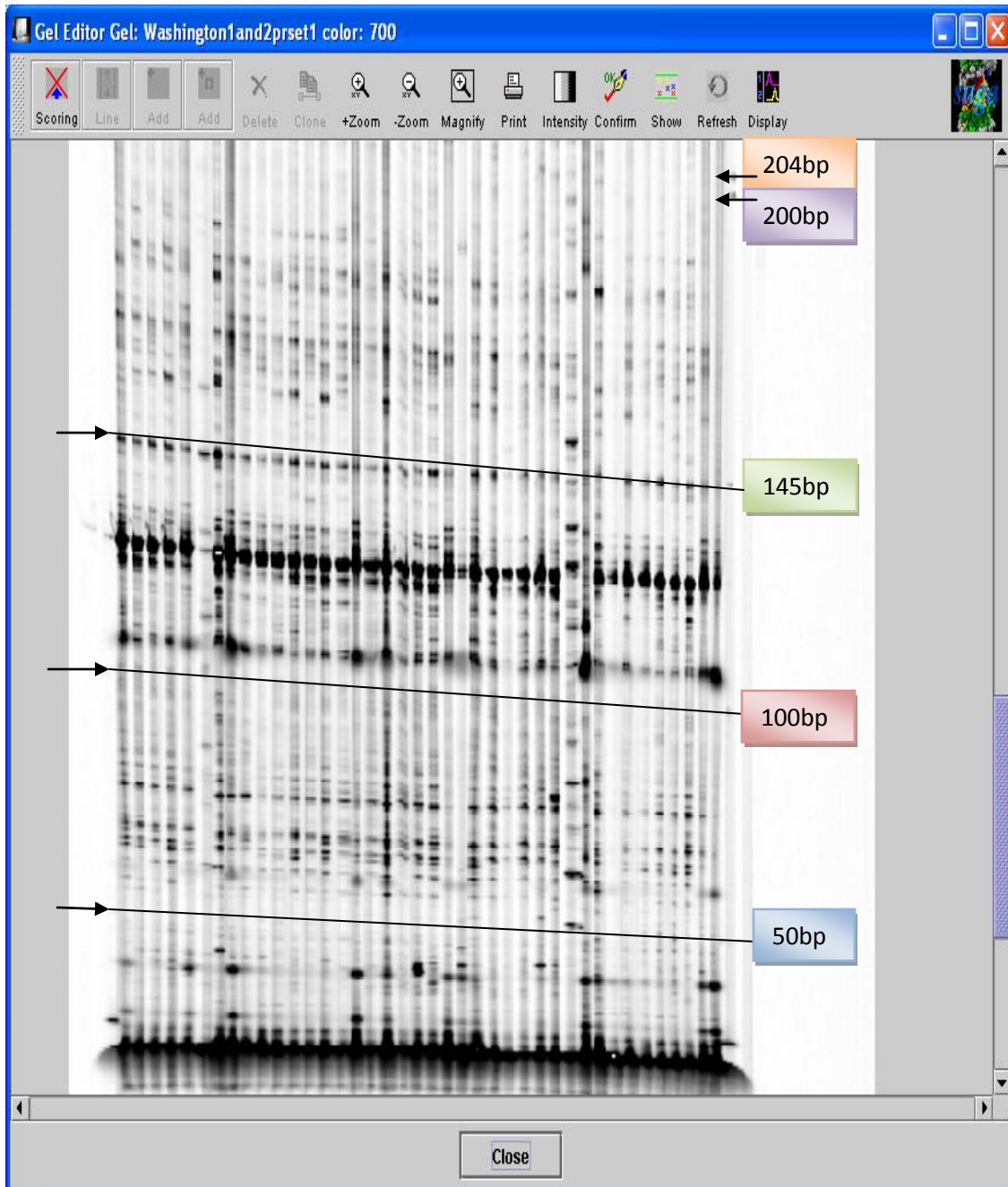
Fig. 12. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected in Washington State.
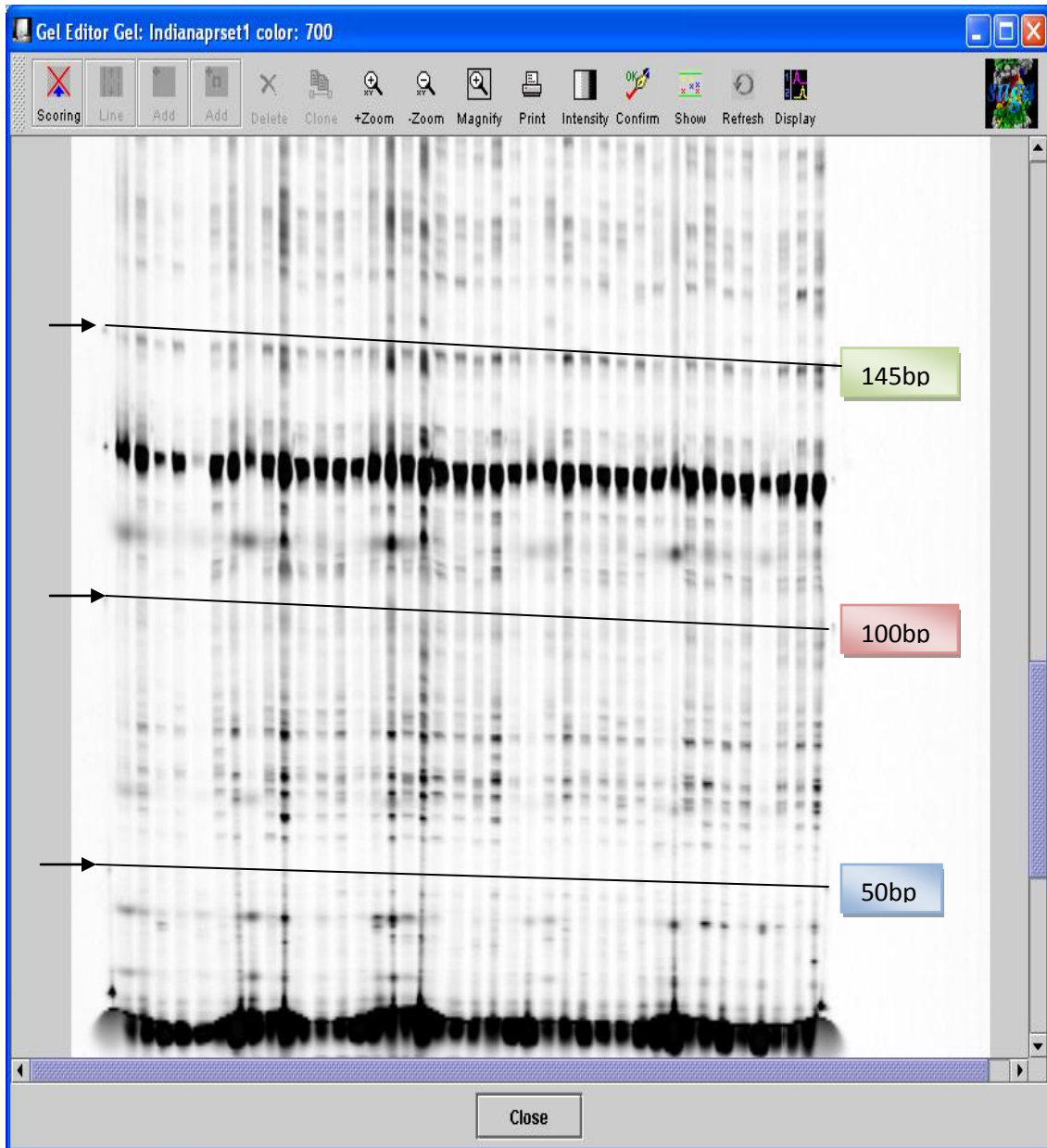
Fig. 13. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected in Indiana.
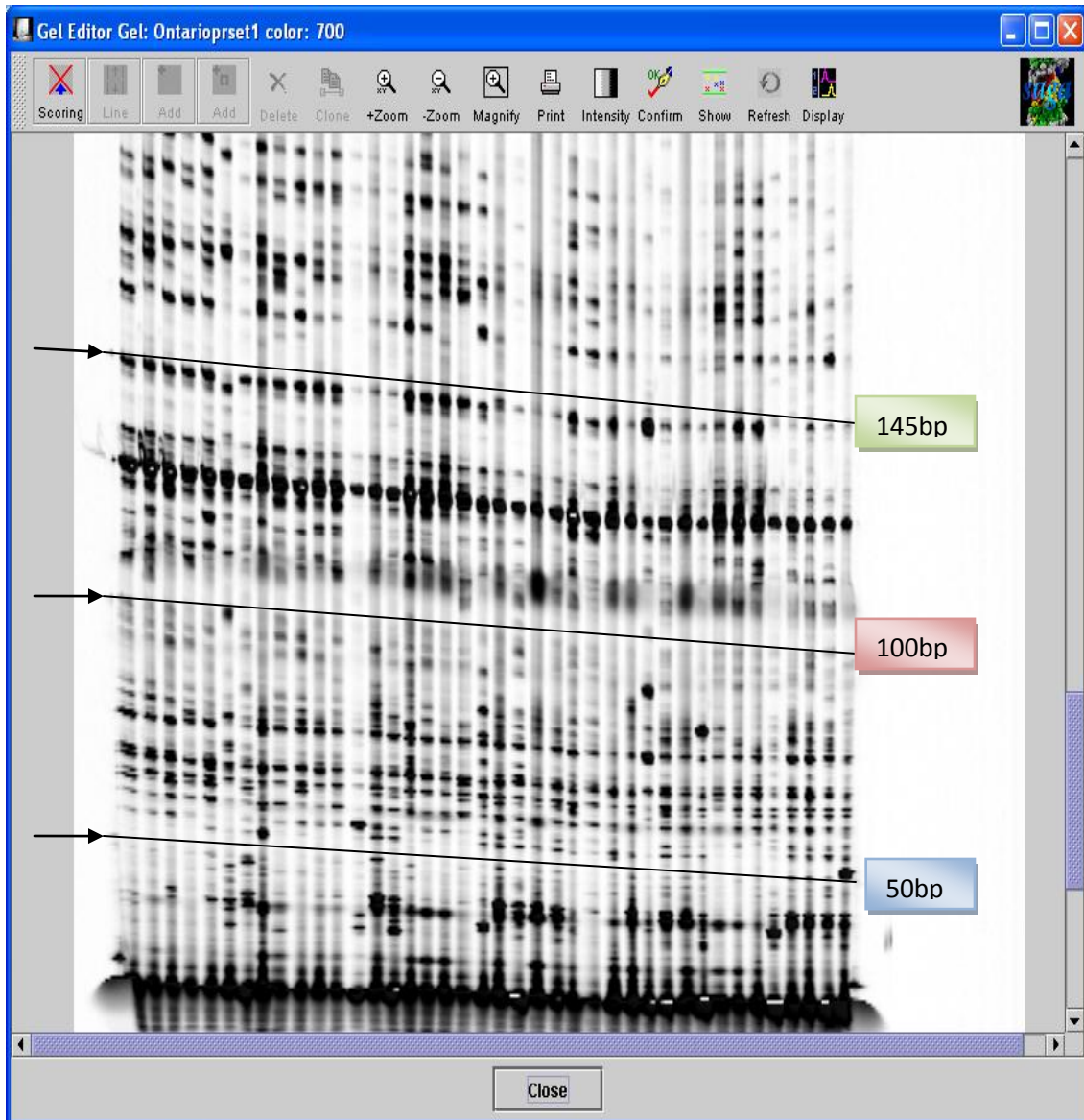
Fig. 14. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected in Ontario.
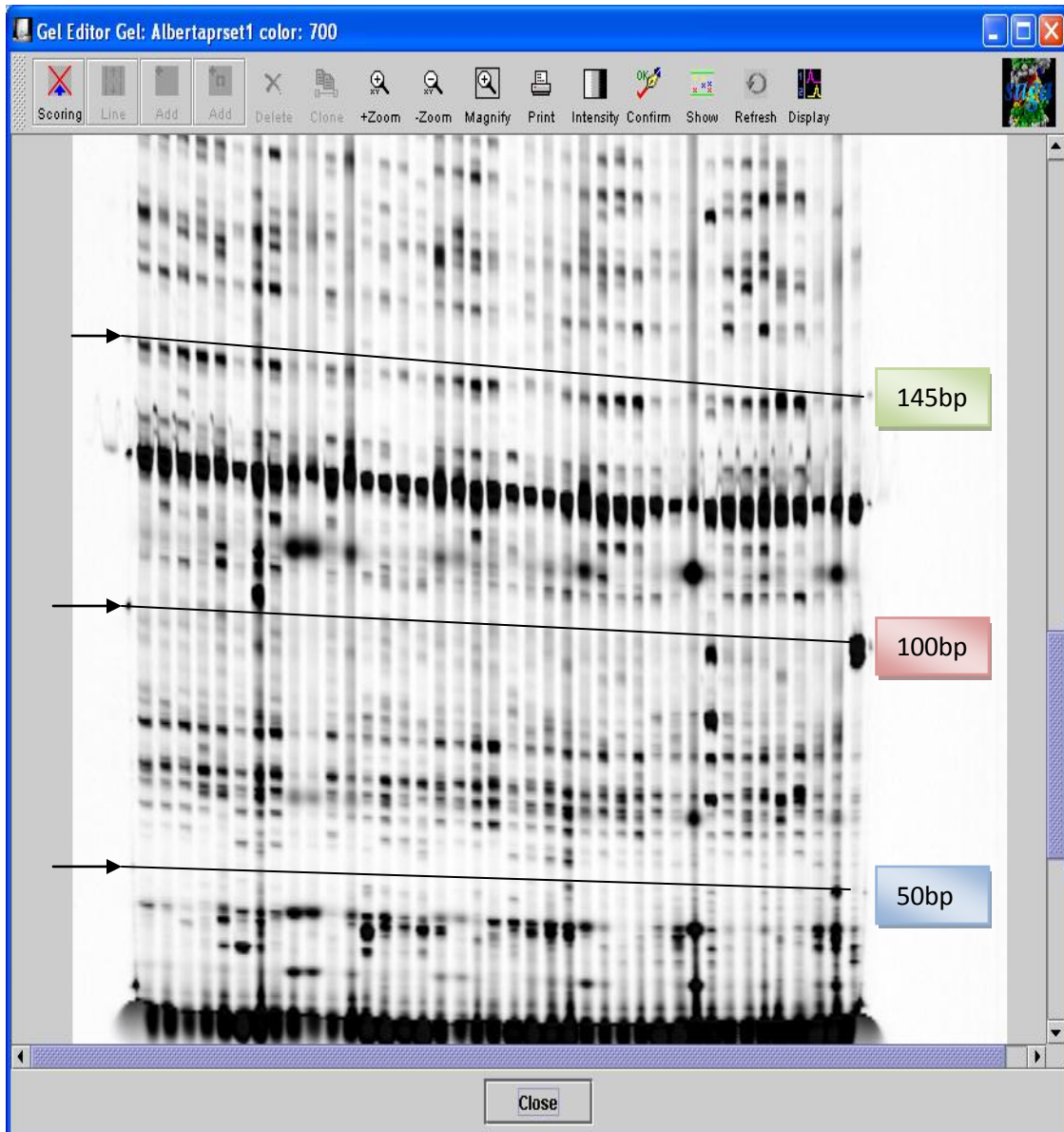
Fig.15. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected in Alberta.
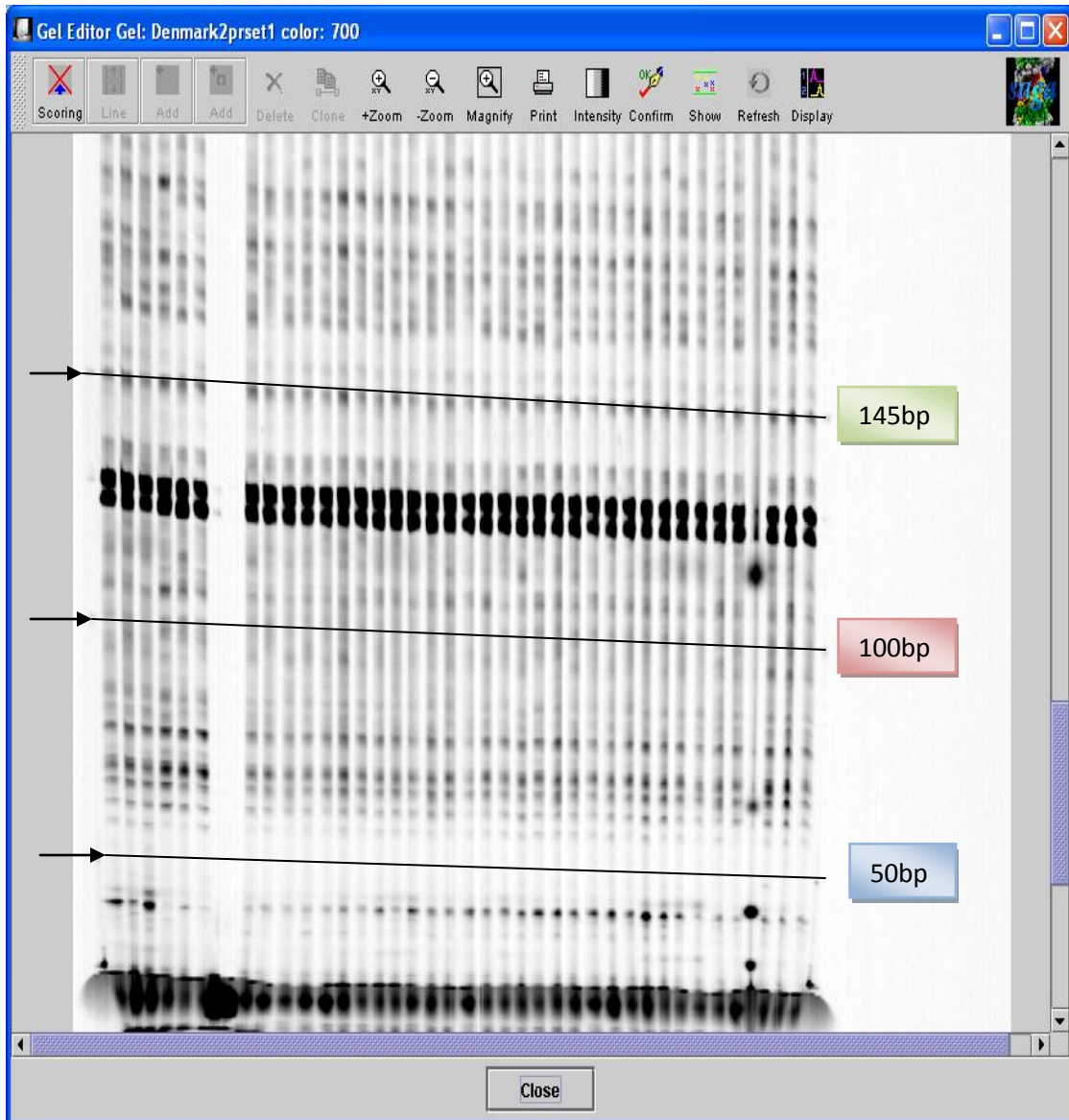
Fig.16. AFLP markers generated using the primer combination M-CAC/E-AAC
(primer set 1) from a sample collected in Denmark, designated Denmark 2.
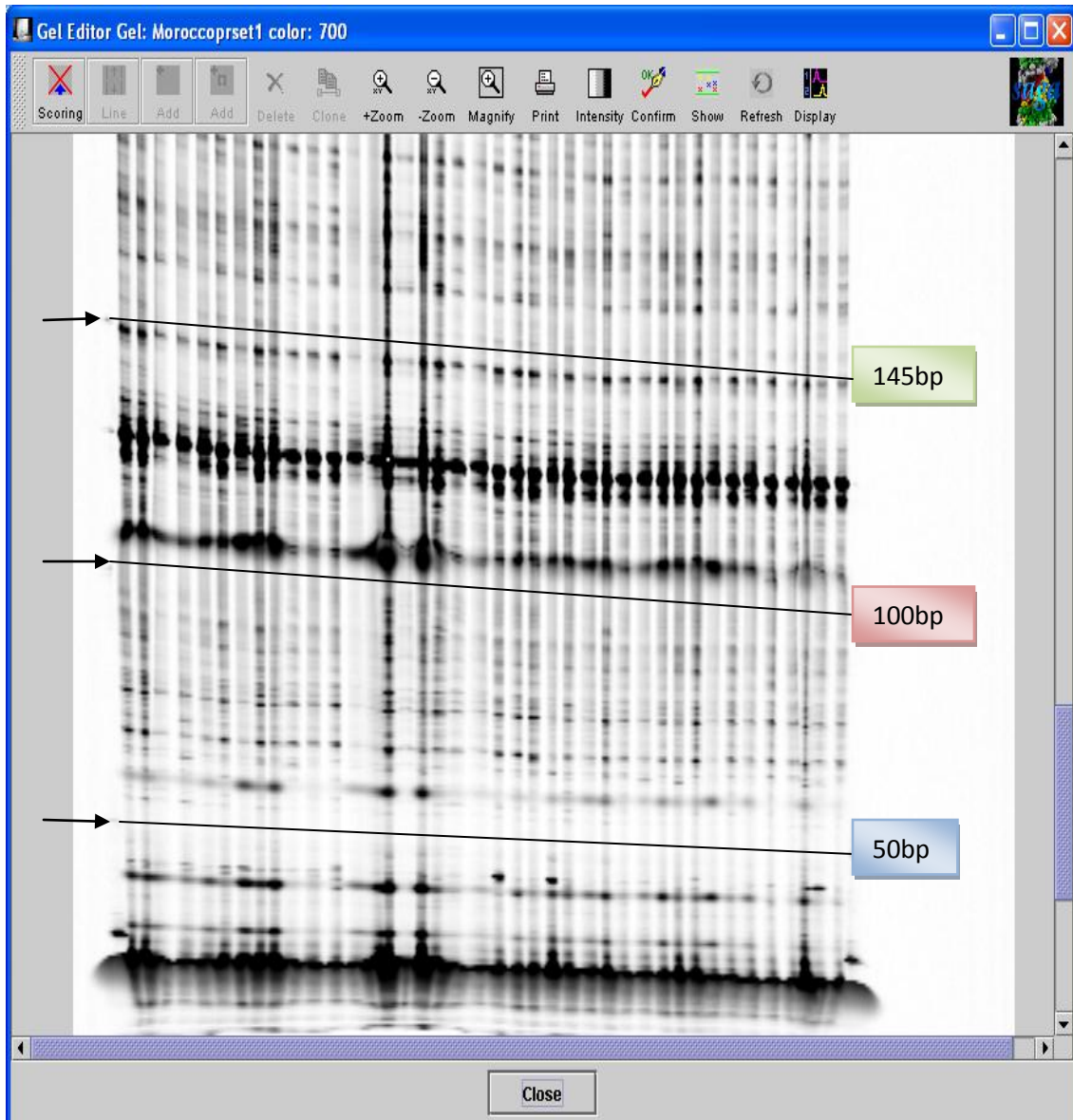
Fig.17. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected in Morocco.
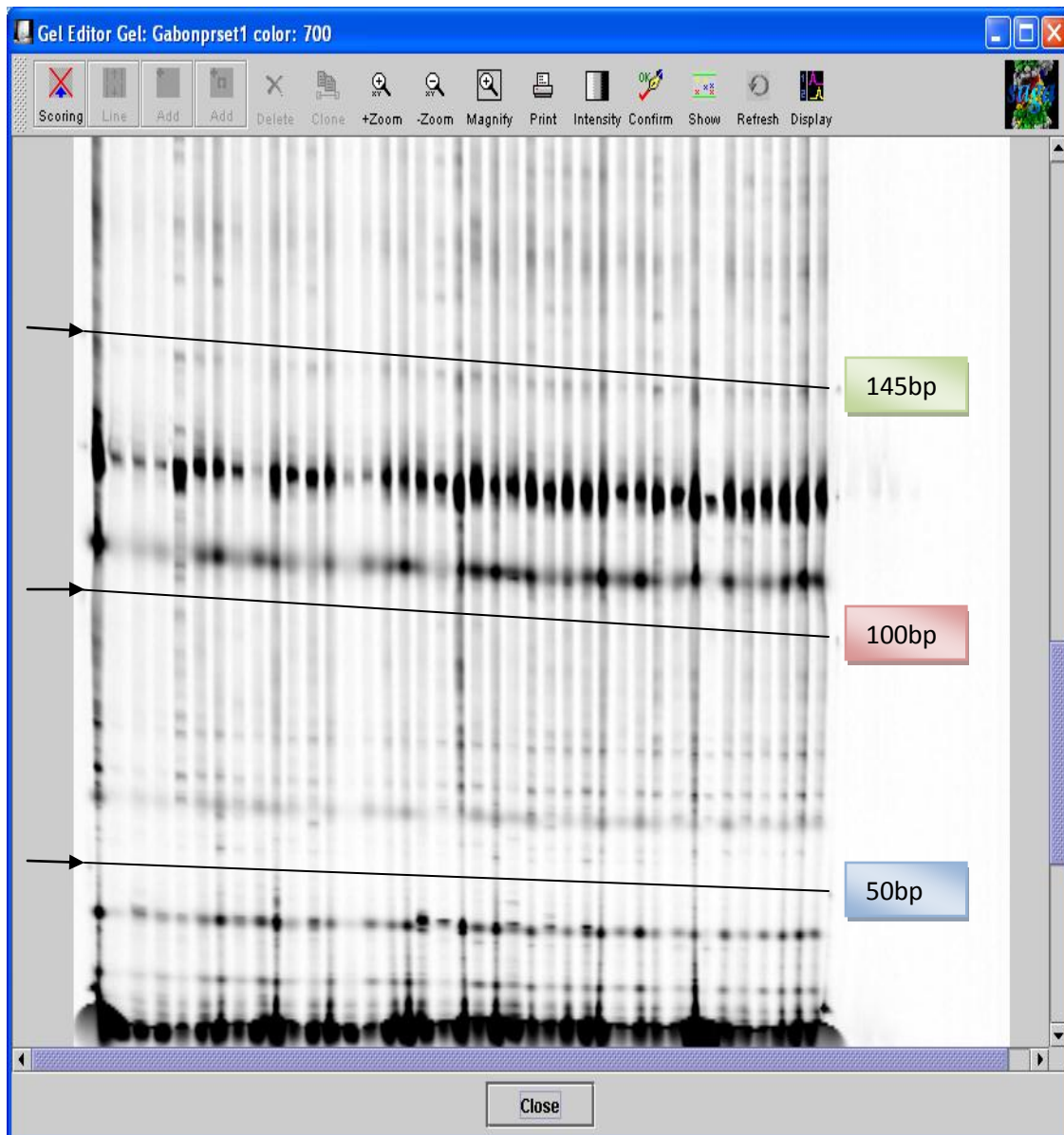
Fig.18. AFLP markers generated using the primer combination M-CAC/E-AAC (primer set 1) from a sample collected in Gabon.
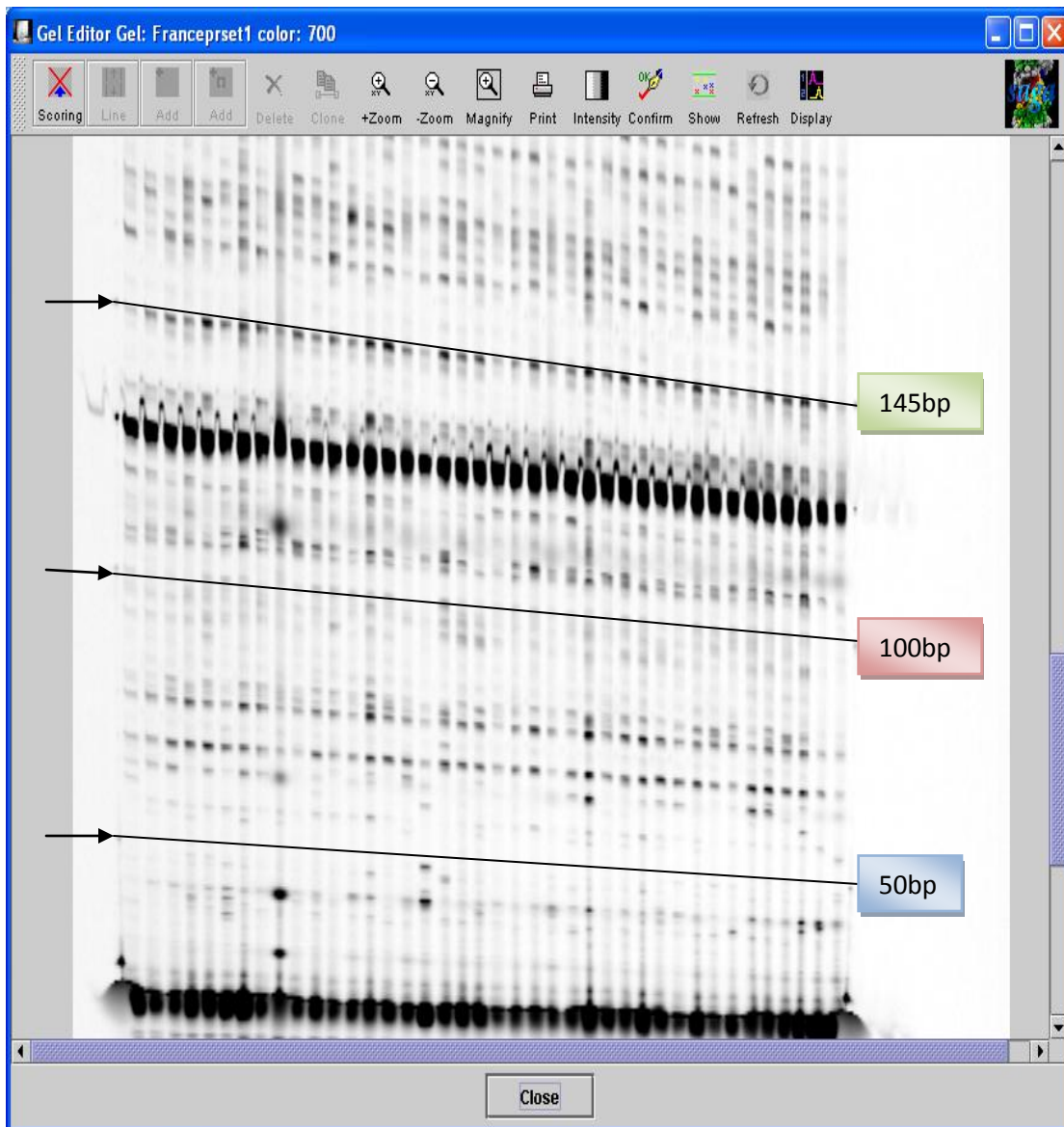
Fig. 19. AFLP markers generated using the primer combination M-CAC/E-AAC
(primer set 1) from a sample collected in France.
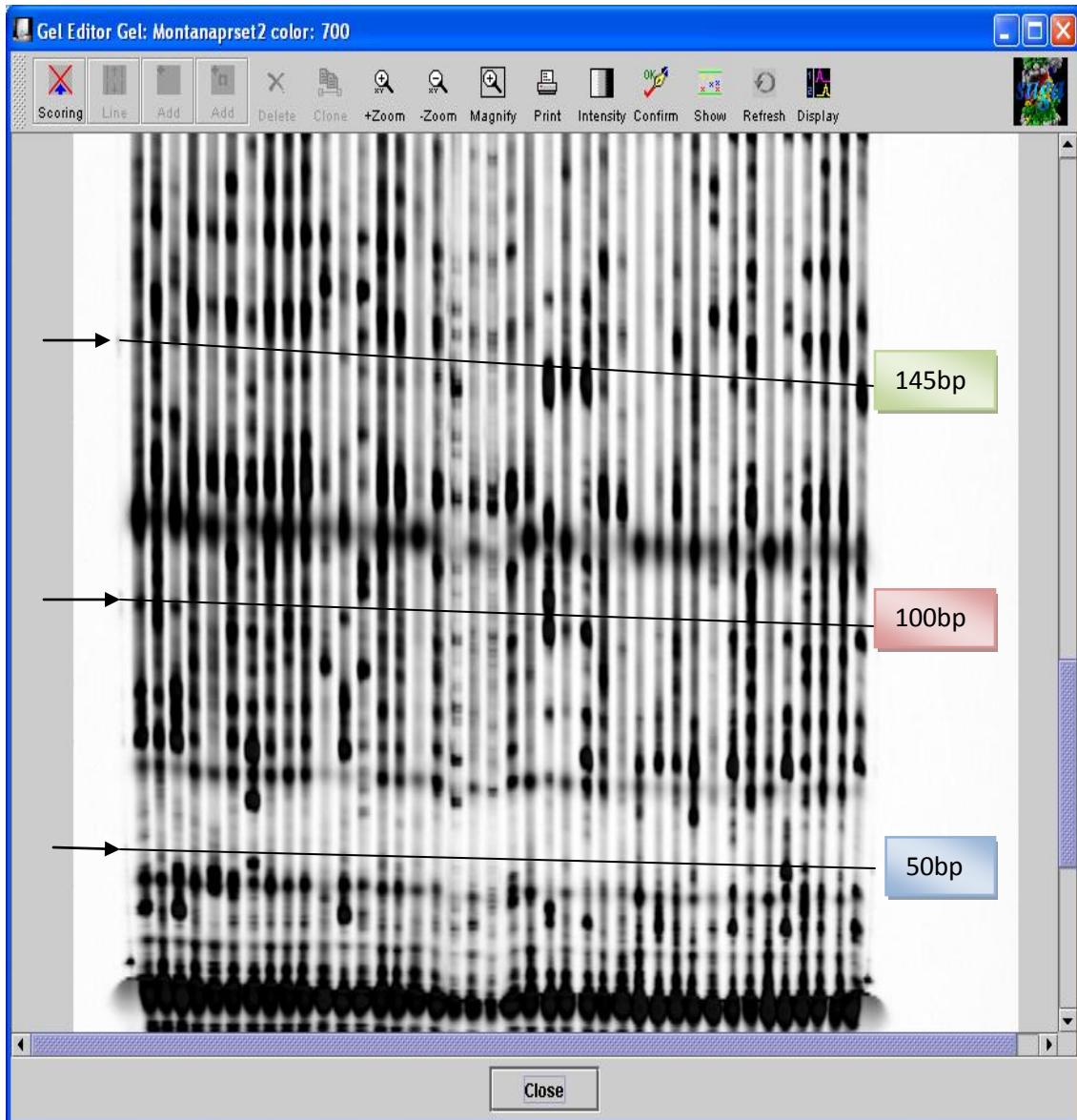
Fig. 20. AFLP markers generated using the primer combination M-CTA/E-AAC (primer set 2) from a sample collected in Montana.
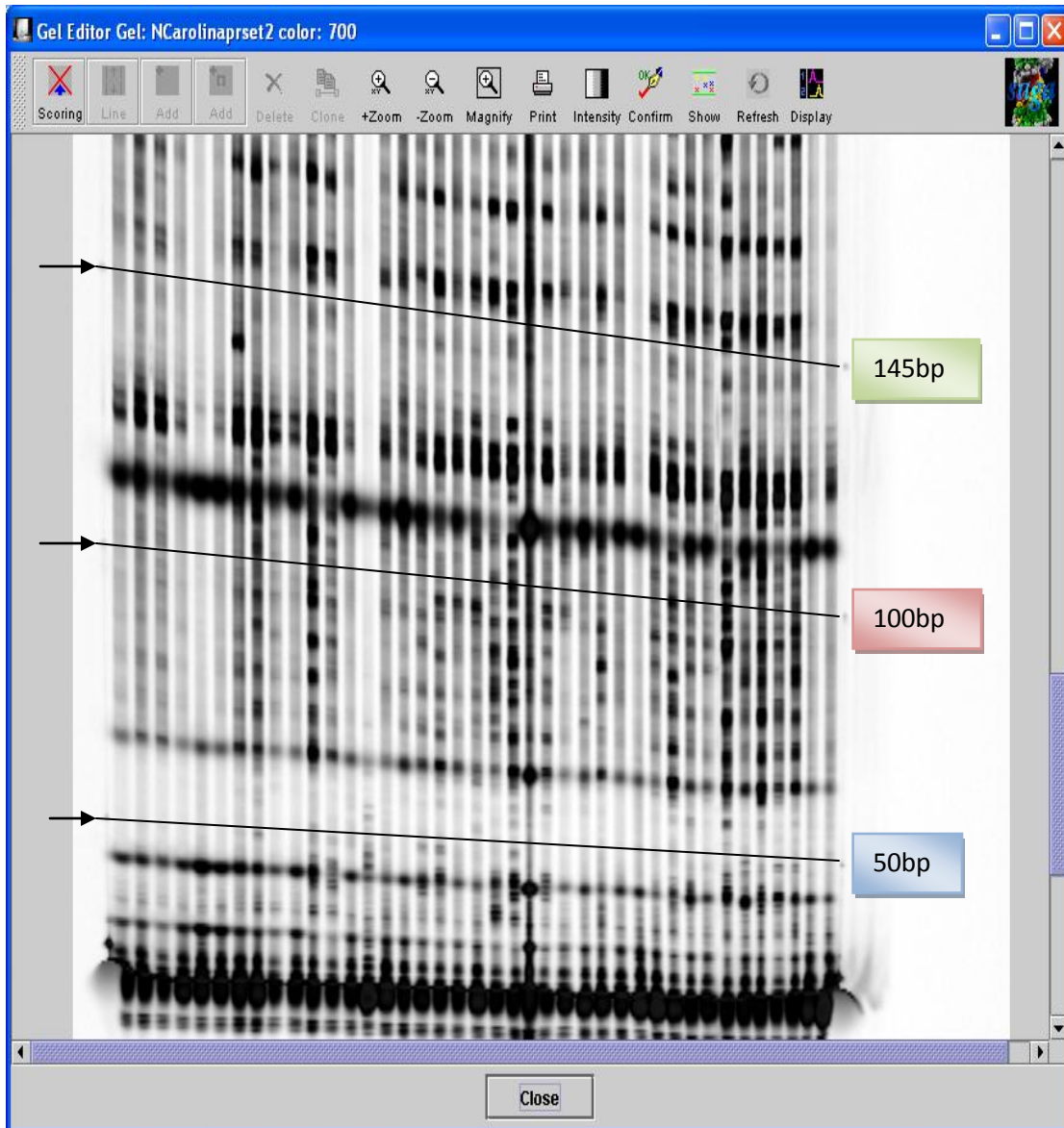
Fig. 21. AFLP markers generated using the primer combination M-CTA/E-AAC (primer set 2) from a sample collected in North Carolina.
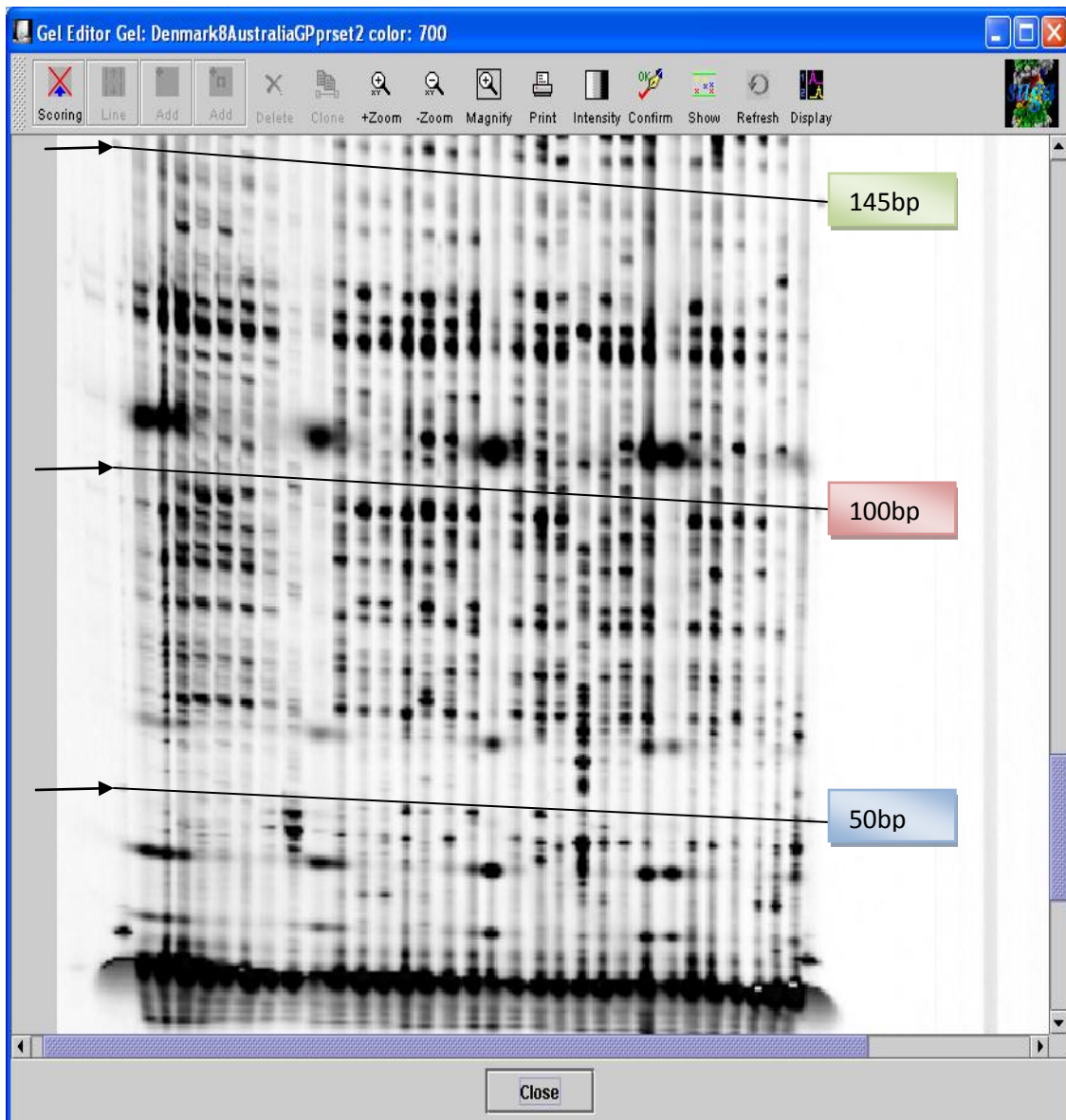
Fig. 22. AFLP markers generated using the primer combination M-CTA/E-AAC (primer set 2) from a sample collected in Denmark and Australia.
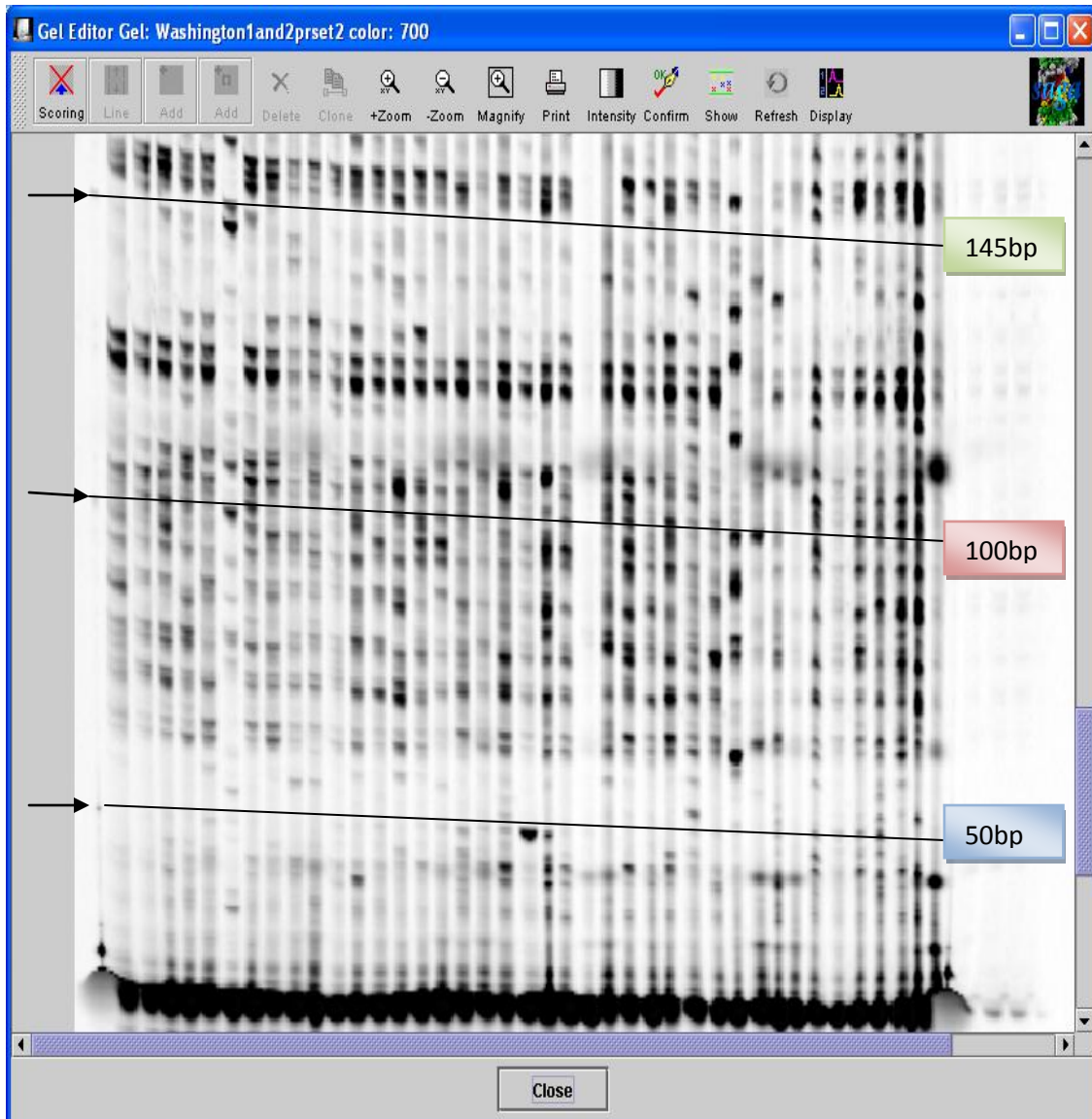
Fig. 23. AFLP markers generated using the primer combination M-CTA/E-AAC (primer set 2) from samples collected in Washington State.
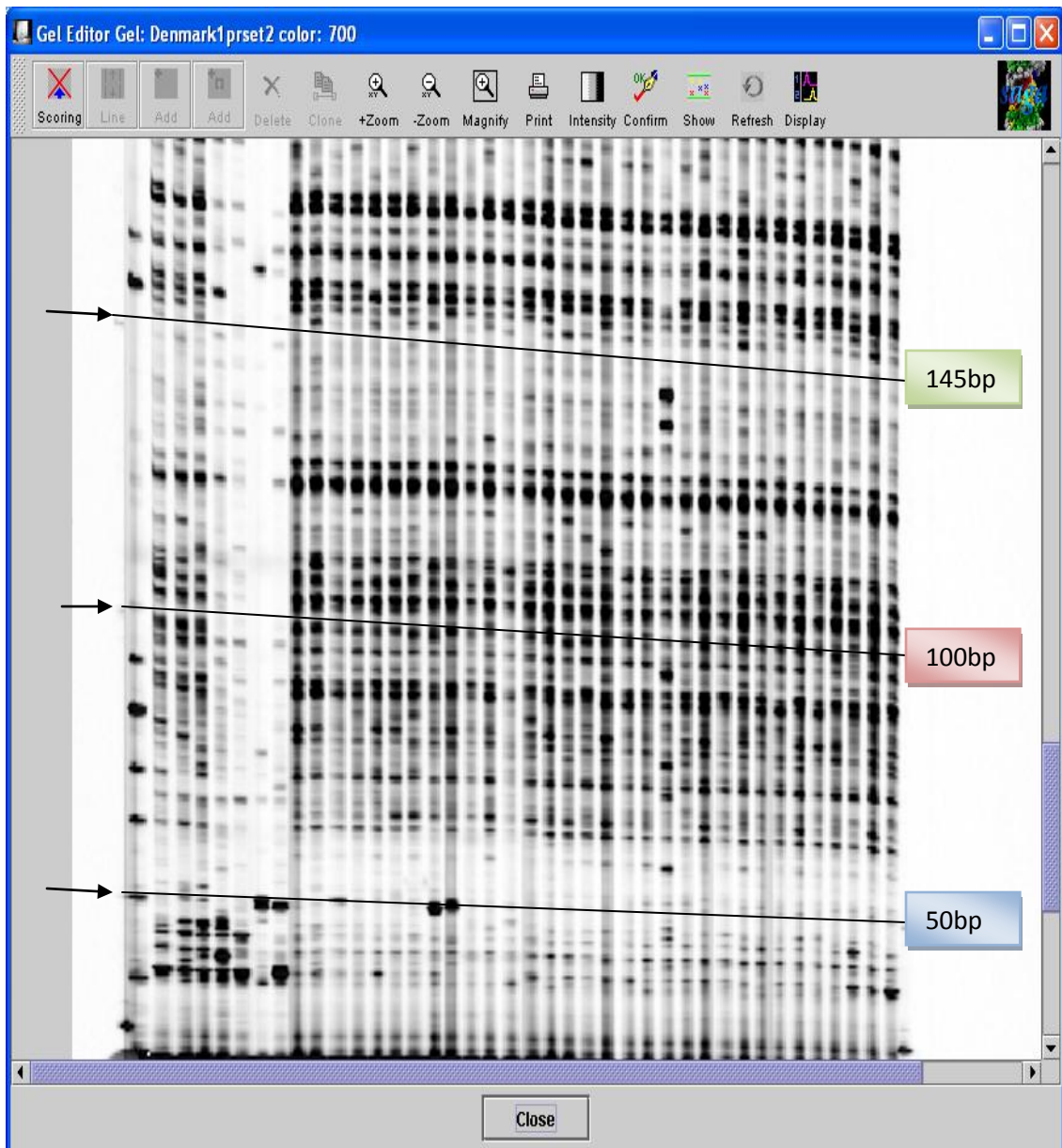
Fig. 24. AFLP markers generated using the primer combination M-CTA/E-AAC (primer set 2) from a sample collected in Denmark, designated Denmark 1.
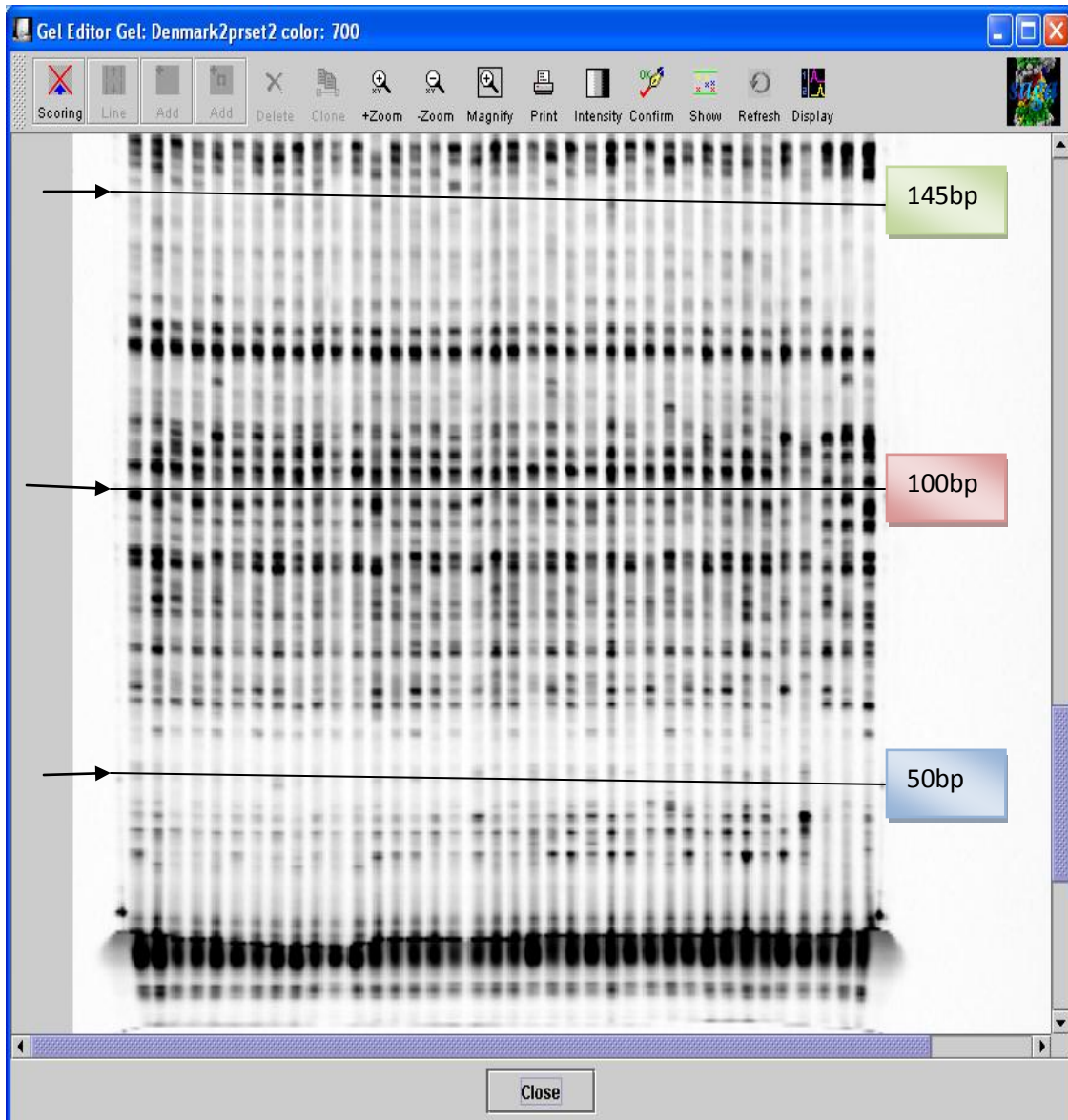
Fig. 25. AFLP markers generated using the primer combination M-CTA/E-AAC (primer set 2) from a sample collected in Denmark, designated Denmark 2.
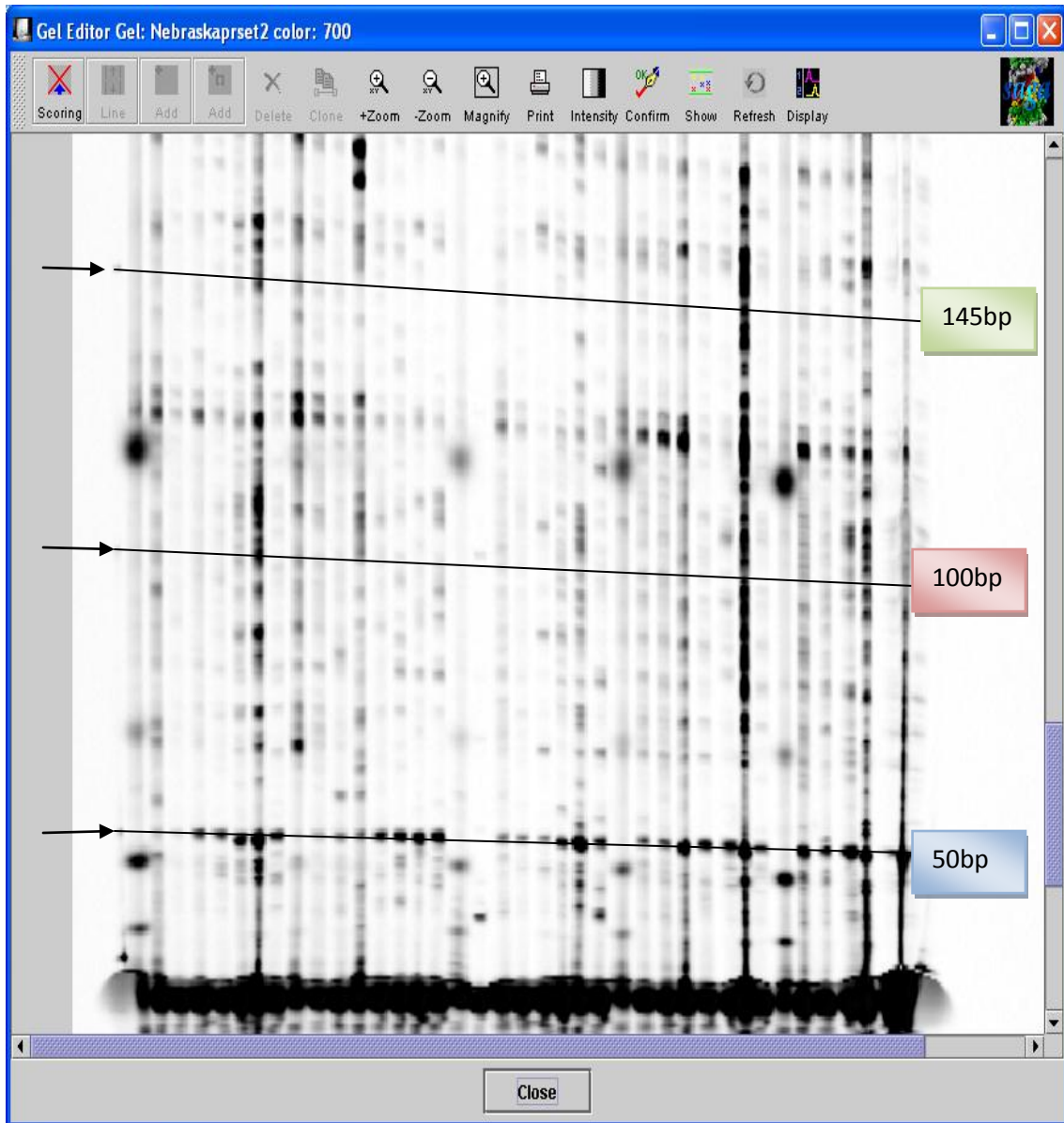
Fig. 26. AFLP markers generated using the primer combination M-CTA/E-AAC
(primer set 2) from a sample collected in Nebraska.
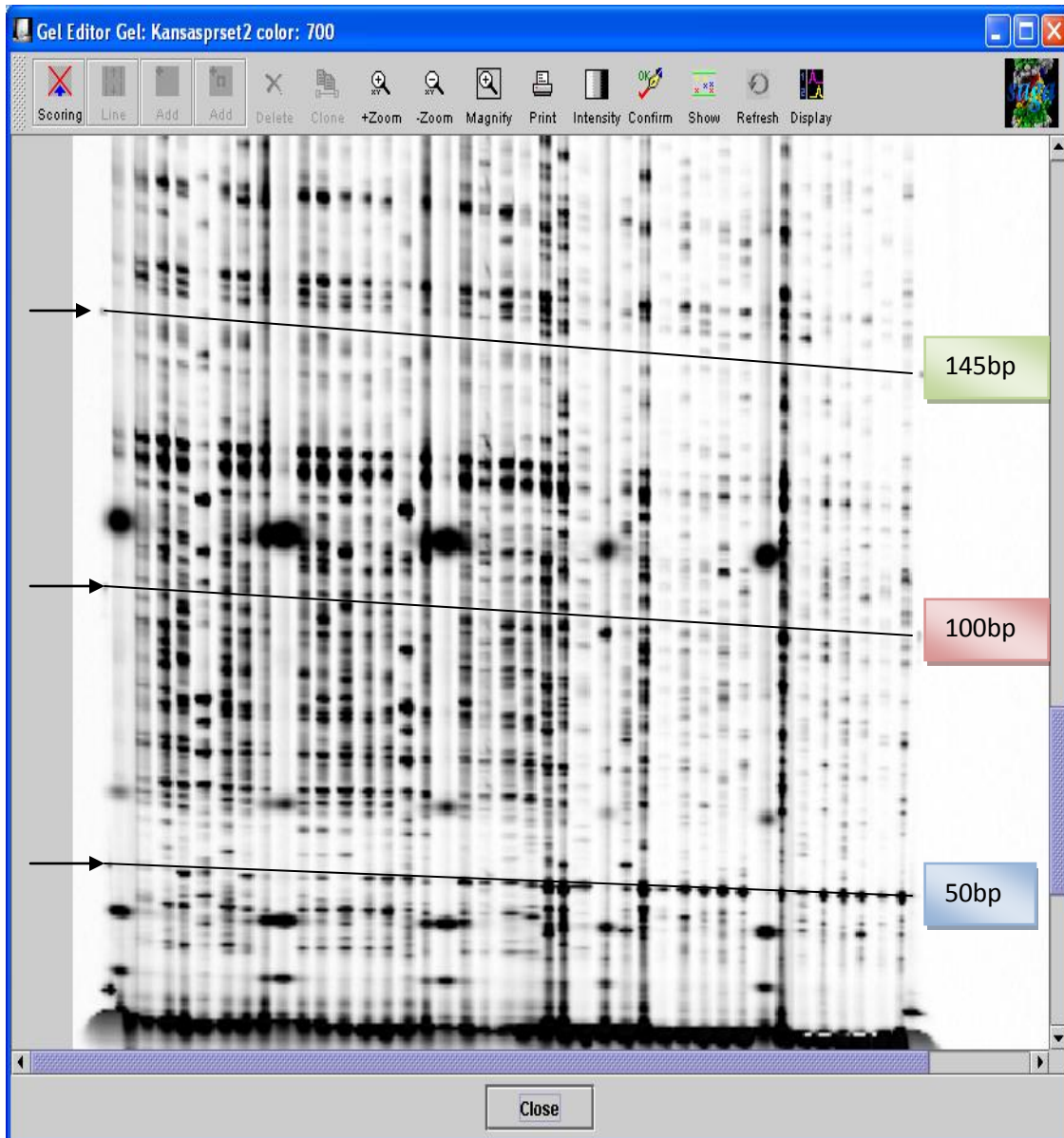
Fig. 27. AFLP markers generated using the primer combination M-CTA/E-AAC (primer set 2) from a sample collected in Kansas.
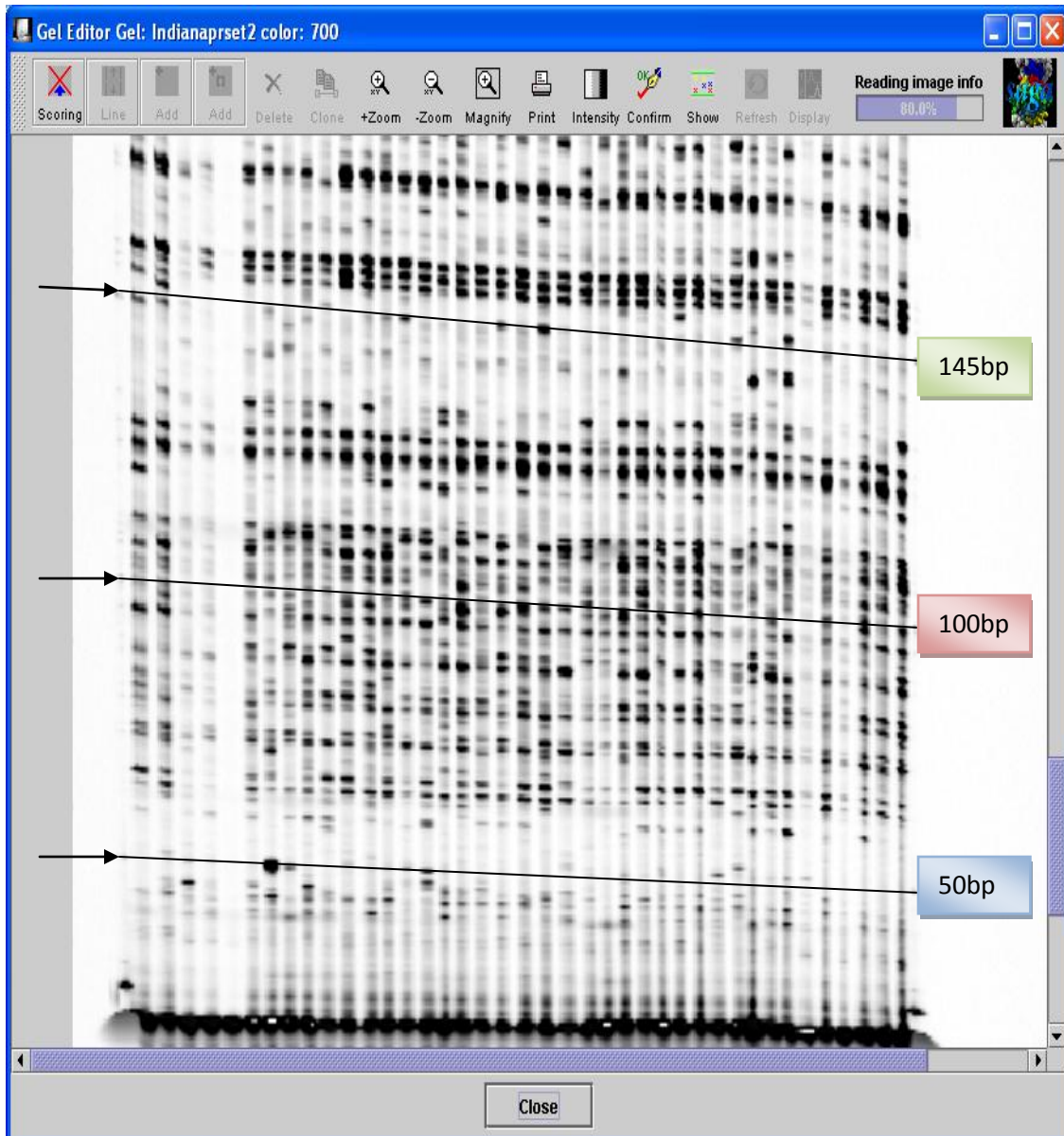
Fig. 28. AFLP markers generated using the primer combination M-CTA/E-AAC
(primer set 2) from a sample collected in Indiana.
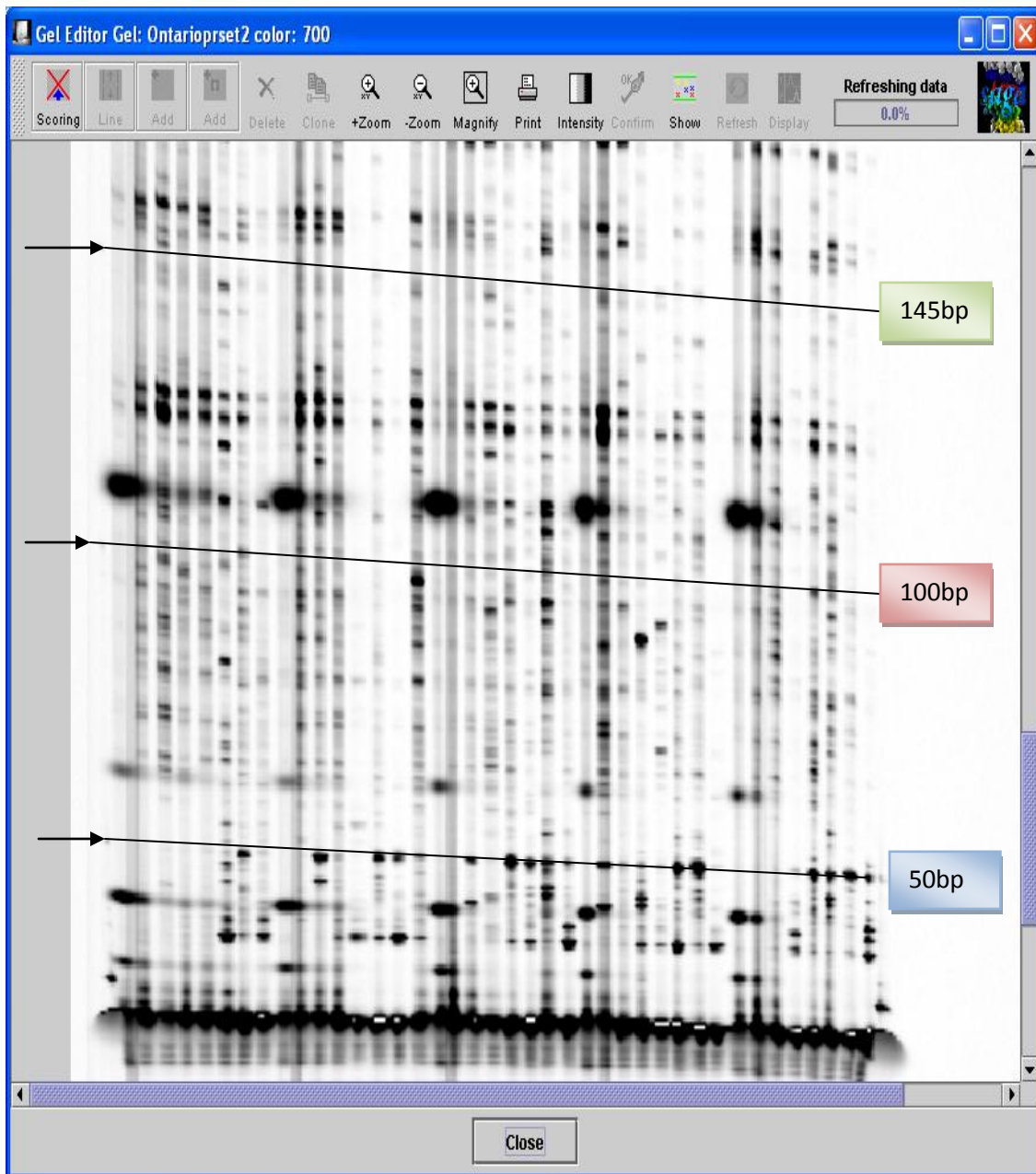
Fig. 29. AFLP markers generated using the primer combination M-CTA/E-AAC
(primer set 2) from a sample collected in Ontario.

APPENDIX VIII

Dendrograms

All locations used in Arlequin
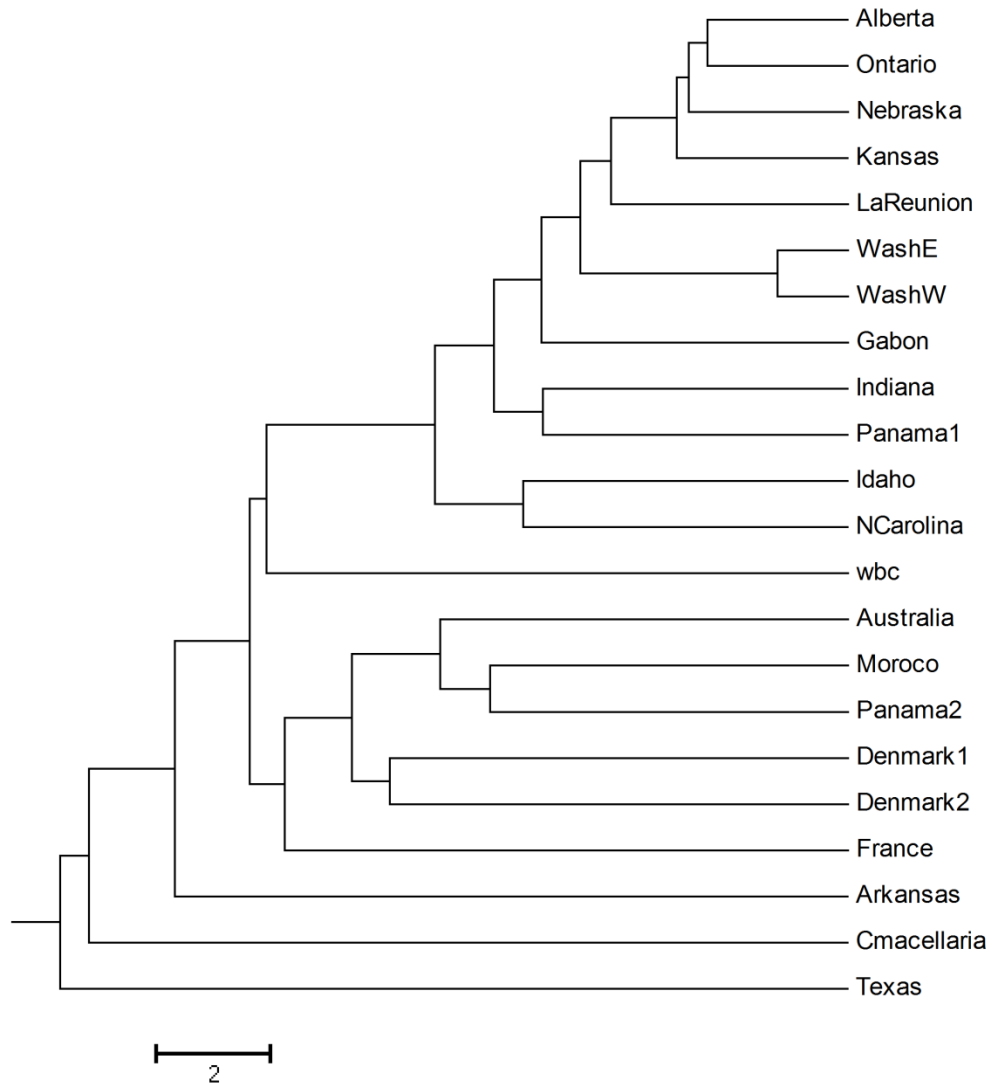
Fig. 30. Dendrogram generated in Popgene and Mega4 containing all samples but Montana. This tree is skewed, with the outliers mixing in with the stable fly samples. Even though the tree is skewed, some samples are still grouping together: the two Washington samples, the two Denmark samples, Nebraska and Kansas, Alberta and Ontario.

All locations used in Arlequin with no outliers
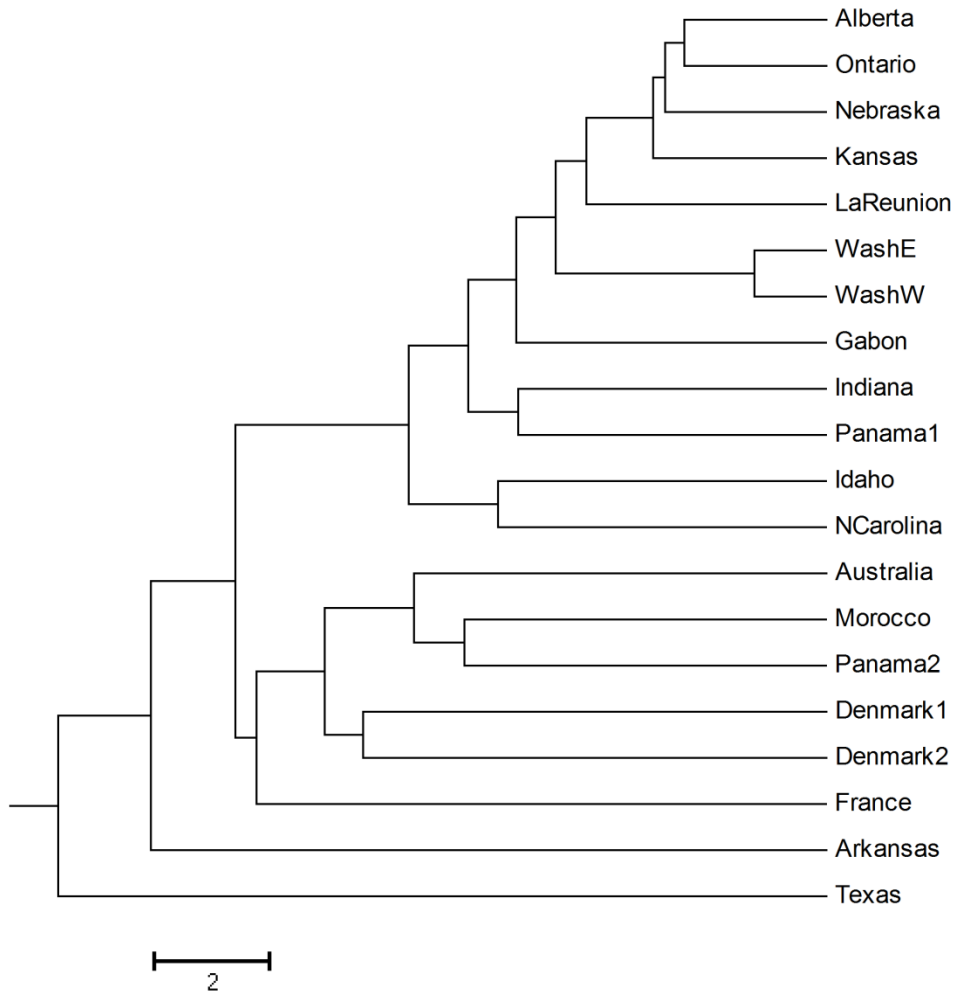
Fig 31. Dendrogram of all samples except Montana without the outliers. Montana was not used in these dendrograms because it was a possible cause of the skewedness. This dendrogram has not changed with the removal of the outliers.

North America used in Arlequin
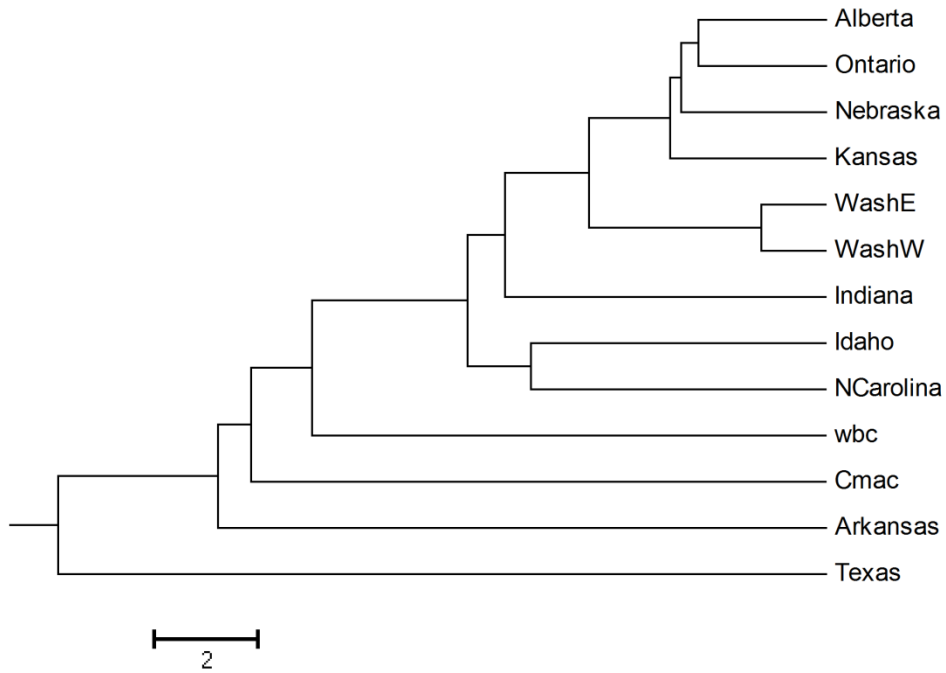
Fig. 32. Dendrogram of the North American samples used in Arlequin. Again the tree is skewed, with the outliers mixing in.

North America no outliers with some removed
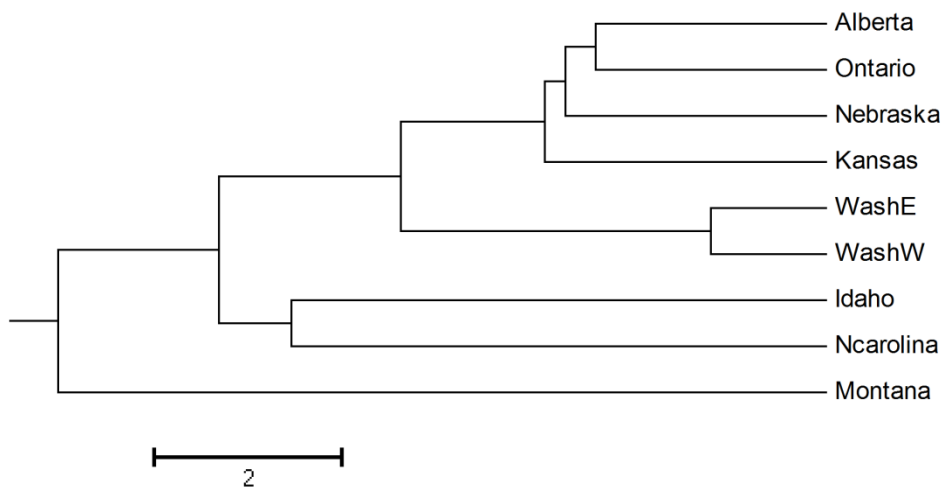
Fig. 33. Dendrogram of North American samples with no outliers and some of the locations removed. In this case there is a more logical grouping.

Old World with outliers
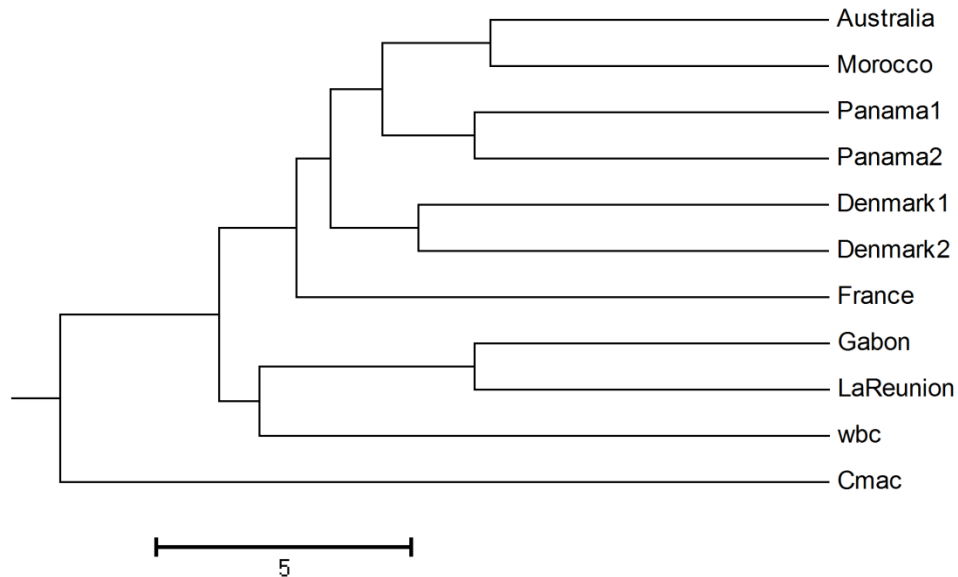
Fig 34. Dendrogram of the Old World samples with outliers included. The Old World stable flies group together in a much logical manner than North America, however although the outliers fall out of the stable fly groups, they are backwards, with the dipteran falling out by itself and the lepidopteran grouping more closely with the stable flies.

Old World with no outliers
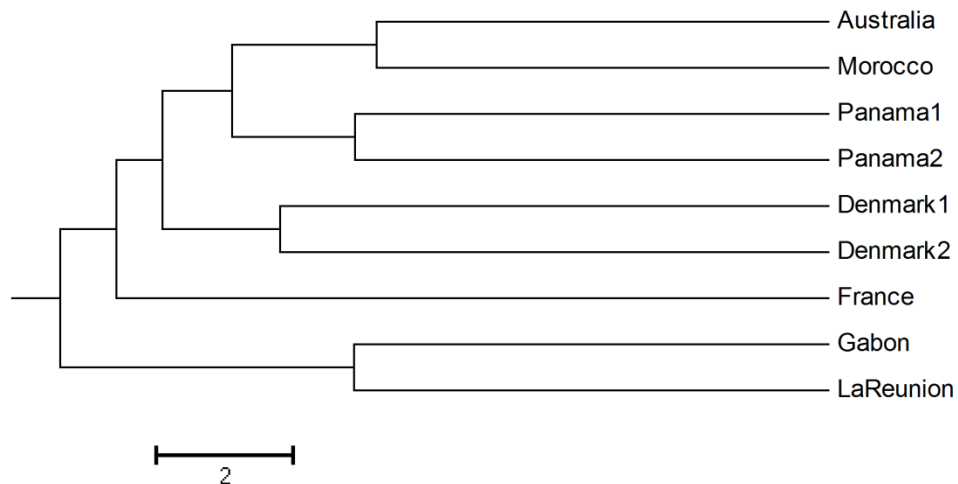
Fig. 35. Dendrogram of the Old World samples without outliers. The order is the same as it was with outliers, grouping together in a logical manner.

APPENDIX IX

Data Analysis

Several software packages are available for population genetic analysis. Some, such as Arlequin (Excoffier et al. 2005) and Popgene (Yeh and Boyle 1997), are free downloads from the internet. Others must be purchased. Most of the software still requires the use of a command line, which makes them very difficult to use if one is not familiar with computer language.

Many of the programs used in our lab are explained in detail in Krumm (2005). This project was analyzed using Arlequin and Popgene, so only these programs will be discussed here.

Popgene performs analyses such as Nei's (1973) gene diversity, F-statistics, test of homogeneity, genetic distance, gene flow, tests of neutrality, allele frequency, effective allele number, and it draws dendrograms. It analyzes haploid or diploid data, and dominant or codominant markers. This project used Popgene primarily for the dendrogram and gene diversity.

Operating Popgene

Before running Popgene, a data matrix must be created in a text file. Textpad is the best one to use for manipulating data, and cut-and-paste operations using a block mode. Following is an example of a data file.

Data matrix formatted for Popgene 3.1.

The first line must begin with a backslash, asterisk, the name of the project, an asterisk and a backslash. The number of populations and number of loci follow. After the locus name, all loci must be listed. Numbers can be used in place of names as it is less time consuming.

Example heading:
/*Stable flies*/
Number of populations = 10
Number of loci = 200
Loci name
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 (…)
until 200 is reached.

After the list of loci, leave one space and type:

Name = (name of population)
Fis = 0.0
(enter data matrix)

This is a fairly simple format for a data matrix, however any small mistakes will

prevent the program from running. Verify that all of the populations have the

same number of loci, and that the number of populations and loci match with the numbers listed in the heading. The symbol for missing data should be ".".

When Popgene is opened, a blank page will be displayed.
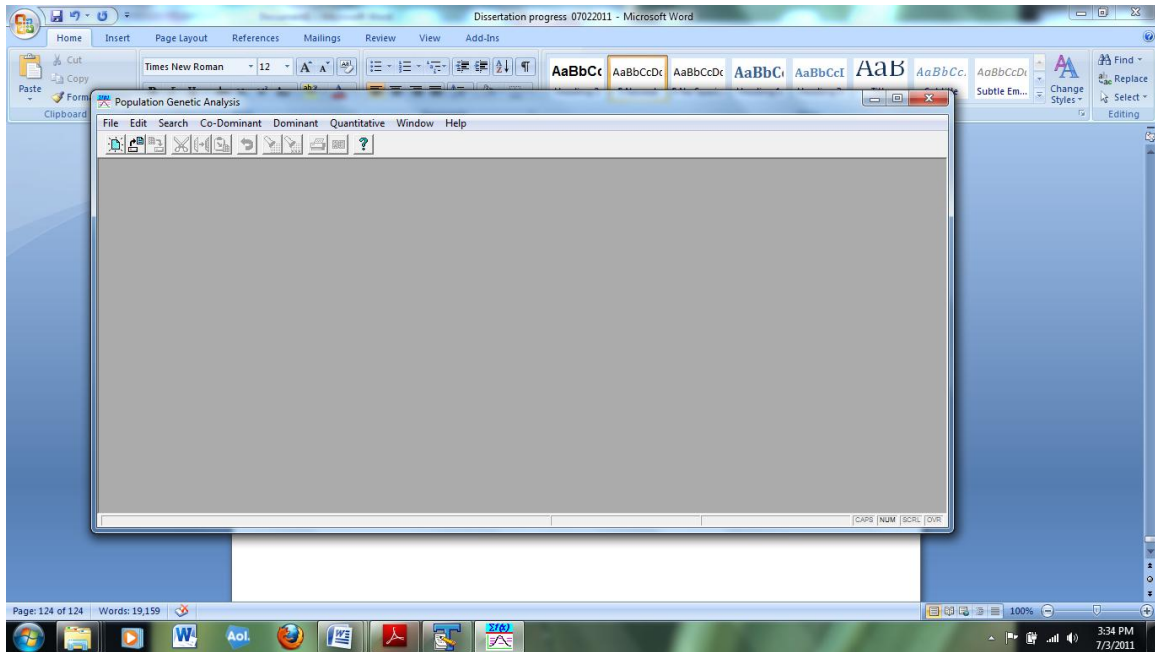
Popgene startup page.

Click on file > load data> dominant marker data. This will display a window in which the data file will be located and loaded into Popgene. When the data is loaded into Popgene, it will display in a second window. Now the type of data will be chosen. Click on the "dominant" button in the toolbar, and the choice of haploid or diploid data will be offered. Click on diploid. A window will be displayed offering the different analyses to be run. Choose the analyses needed, or click on "check all".

Loading data into Popgene
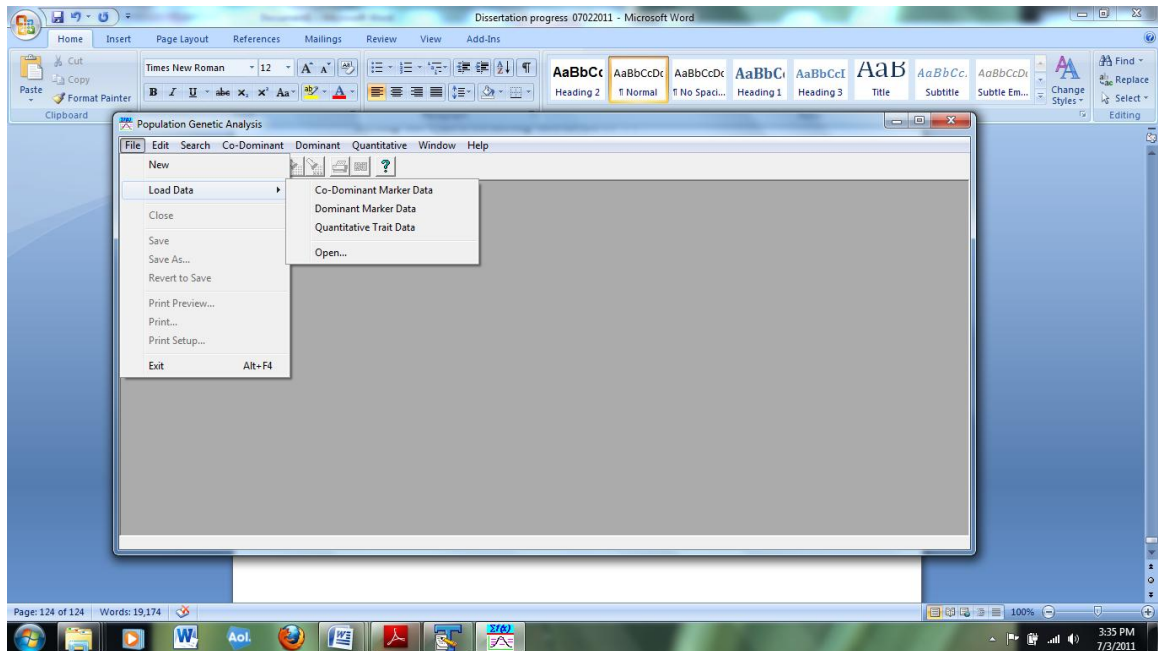
Loading data into Popgene.
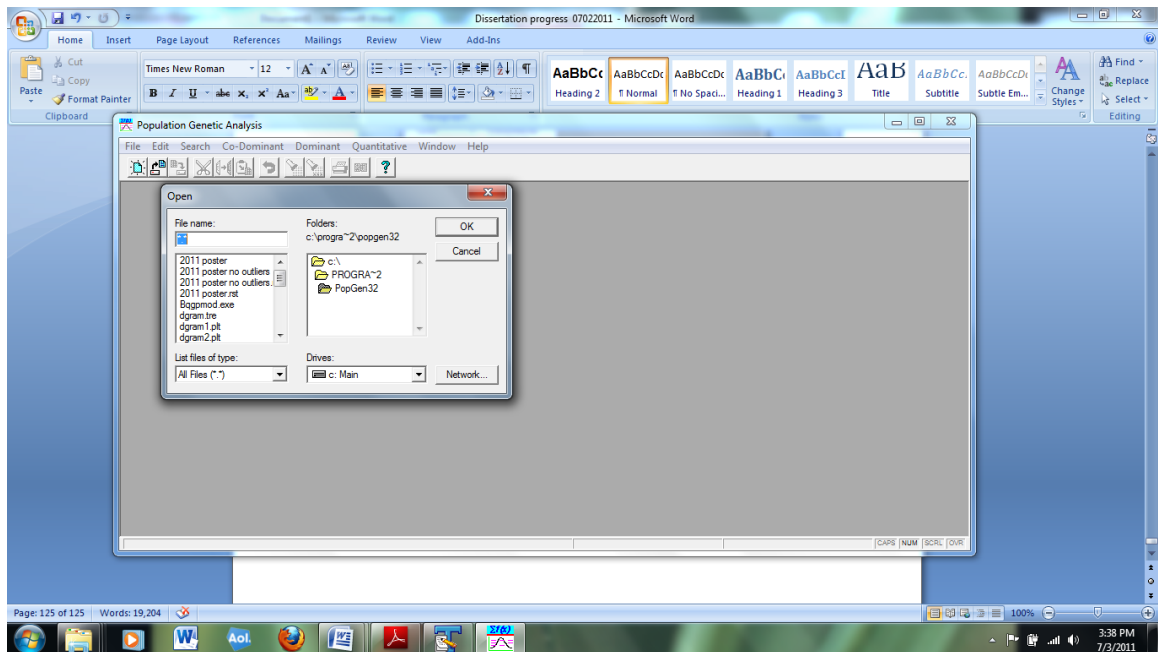
Loading data into Popgene.
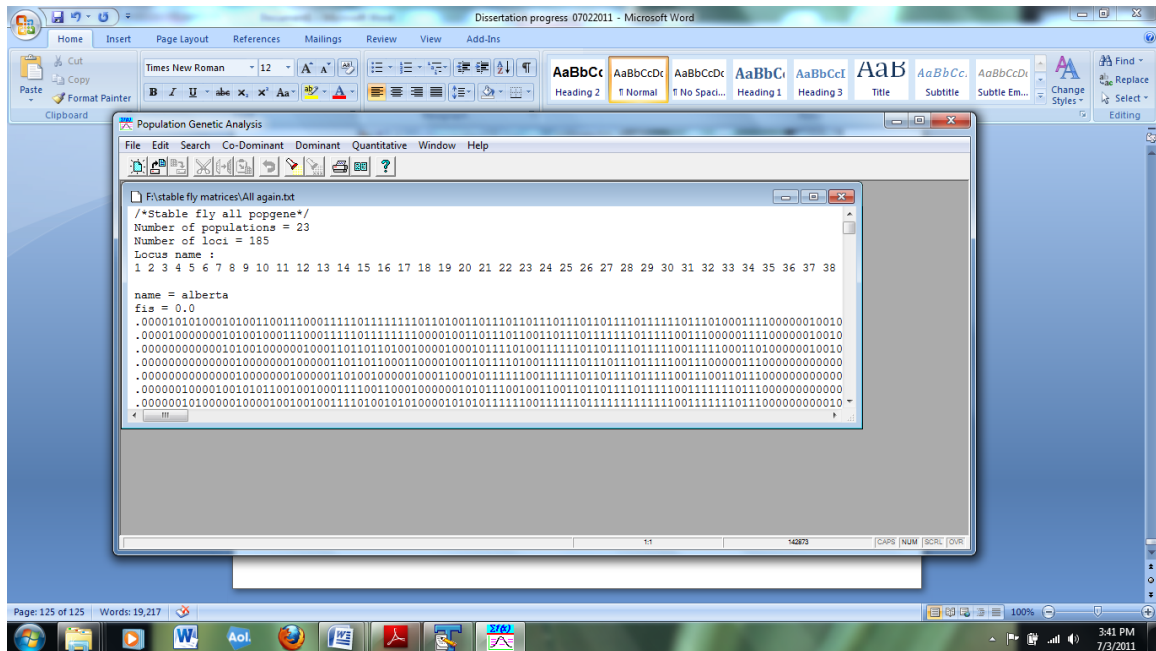
Fig. c. Loading data into Popgene.
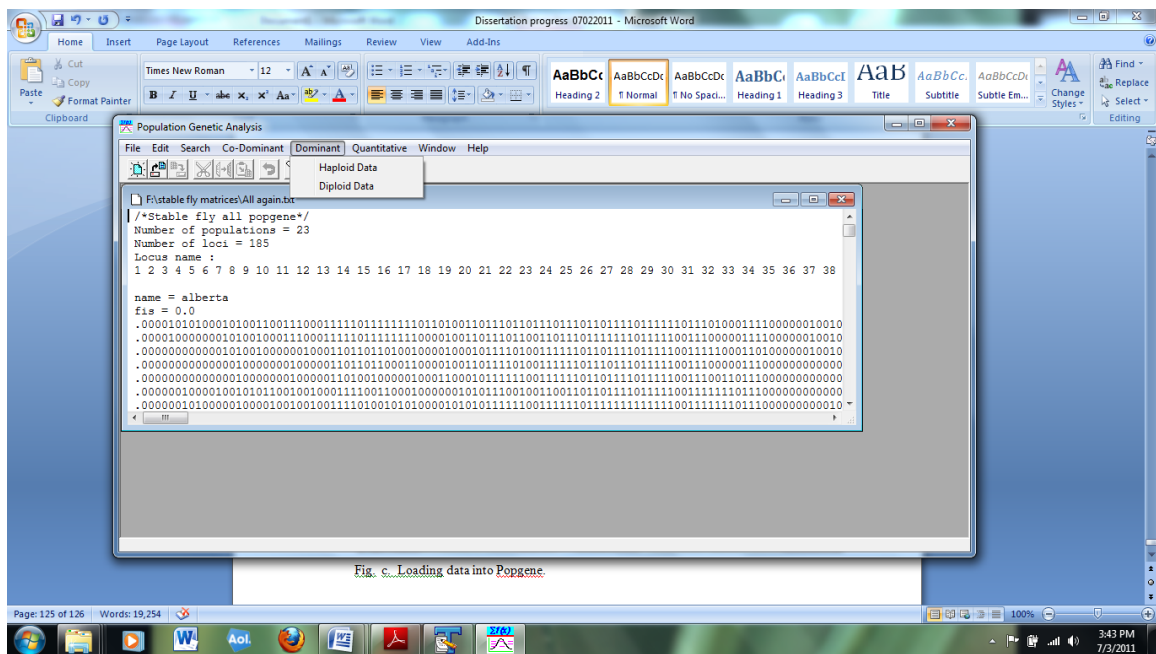
Choosing the type of data to be analyzed.
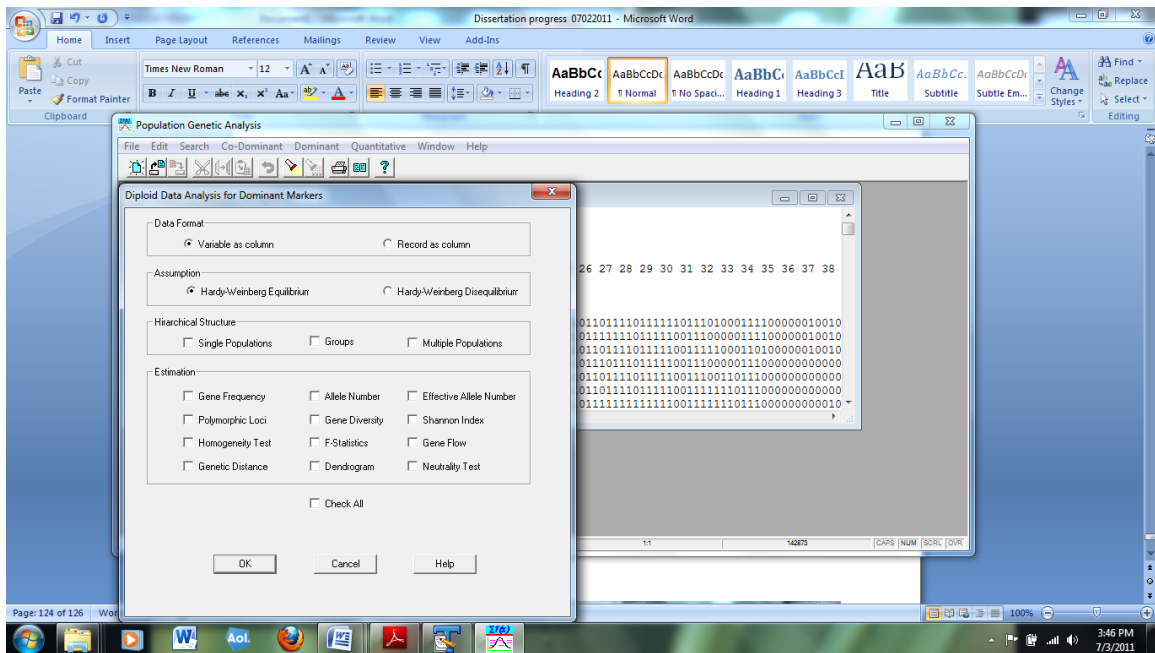
Choosing types of analysis to be performed.

Once the analyses have been chosen, click "okay". The following window will display.
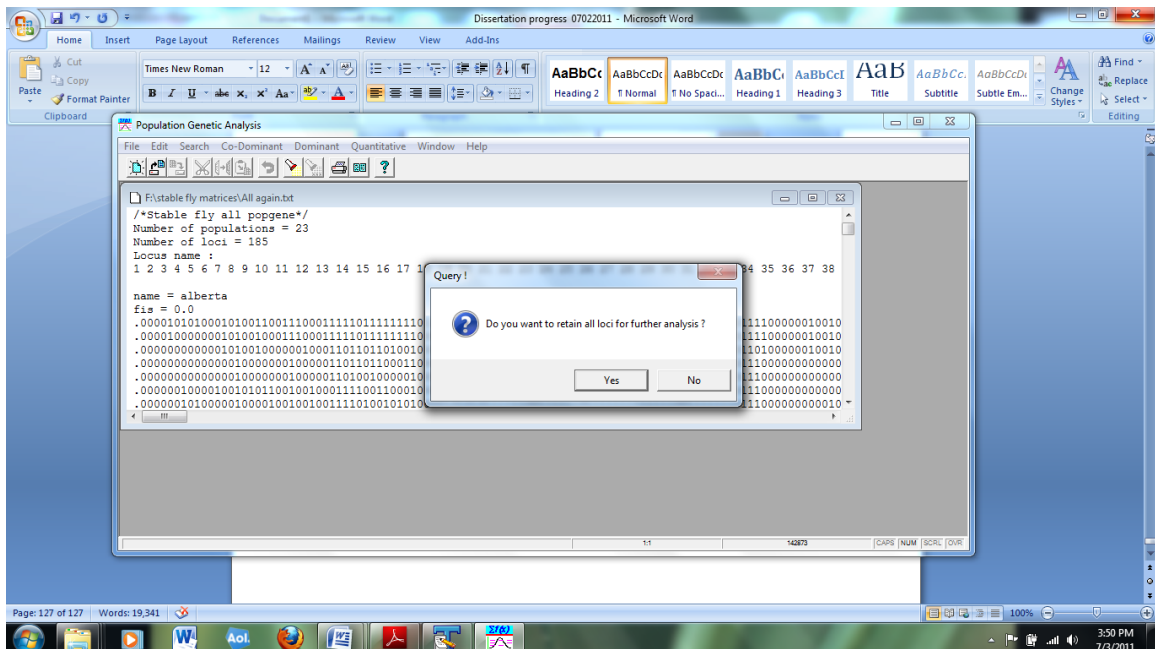
Choosing analyses to be performed.

If "no" is chosen in this query, a window will display where loci may be deleted.
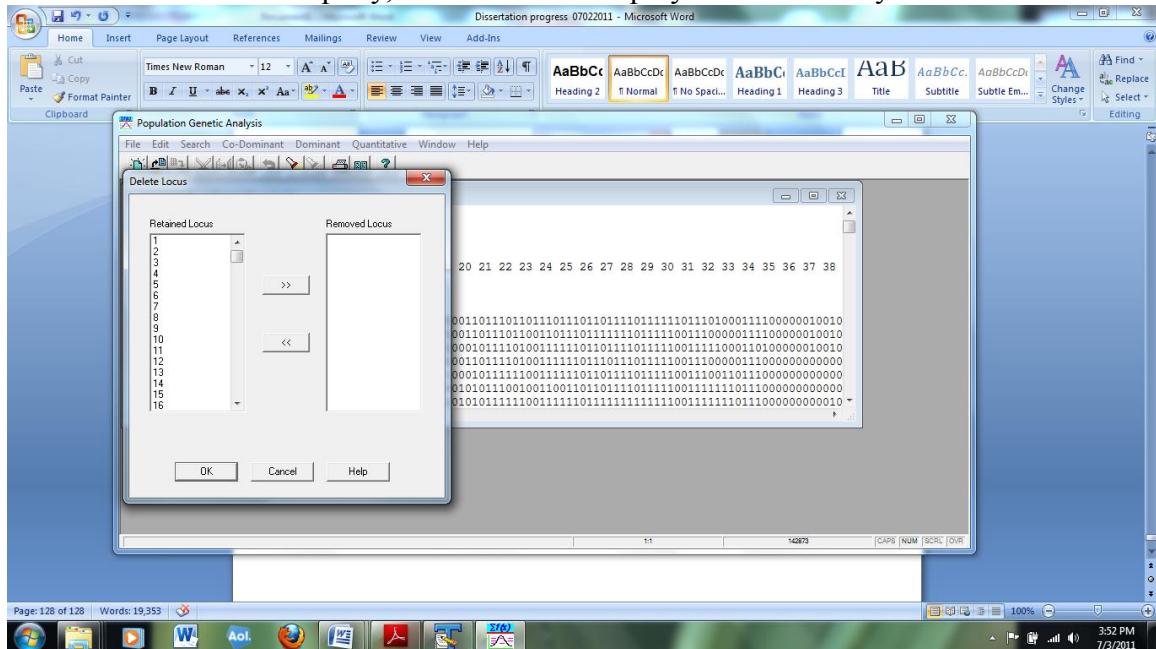
Option to delete loci.

If "yes" is chosen, another query will follow, asking if all populations should be retained for further analysis. If "yes" is chosen, the next step is to specify the number of groups to be used
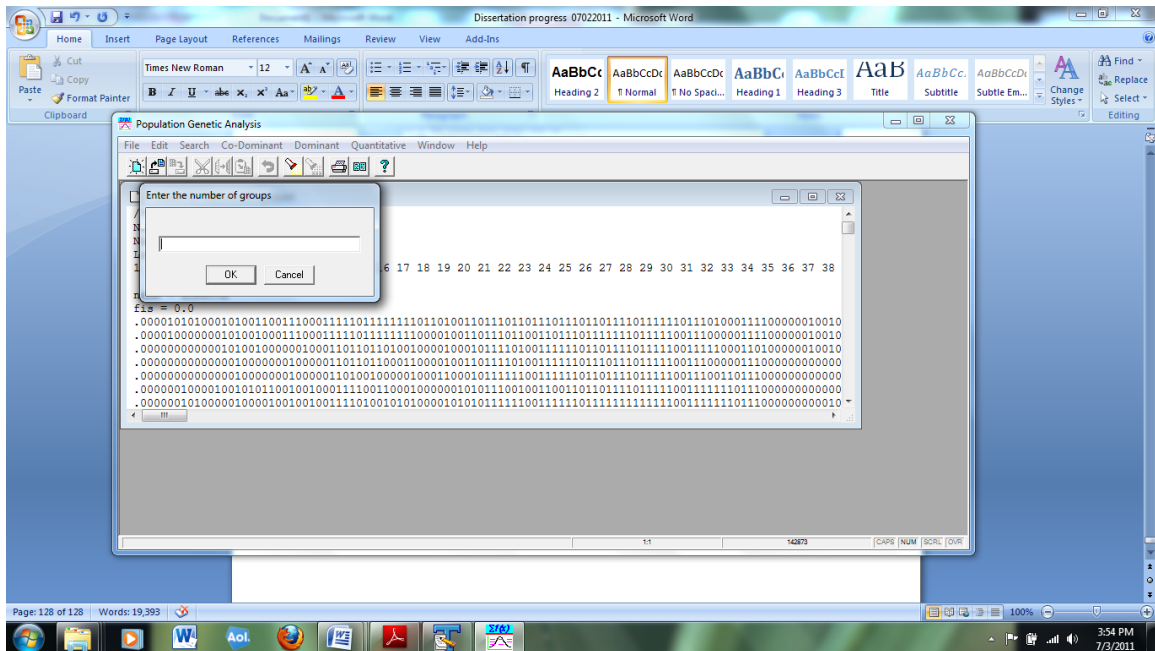
Specifying the number of groups.

The groups will then have to be defined.
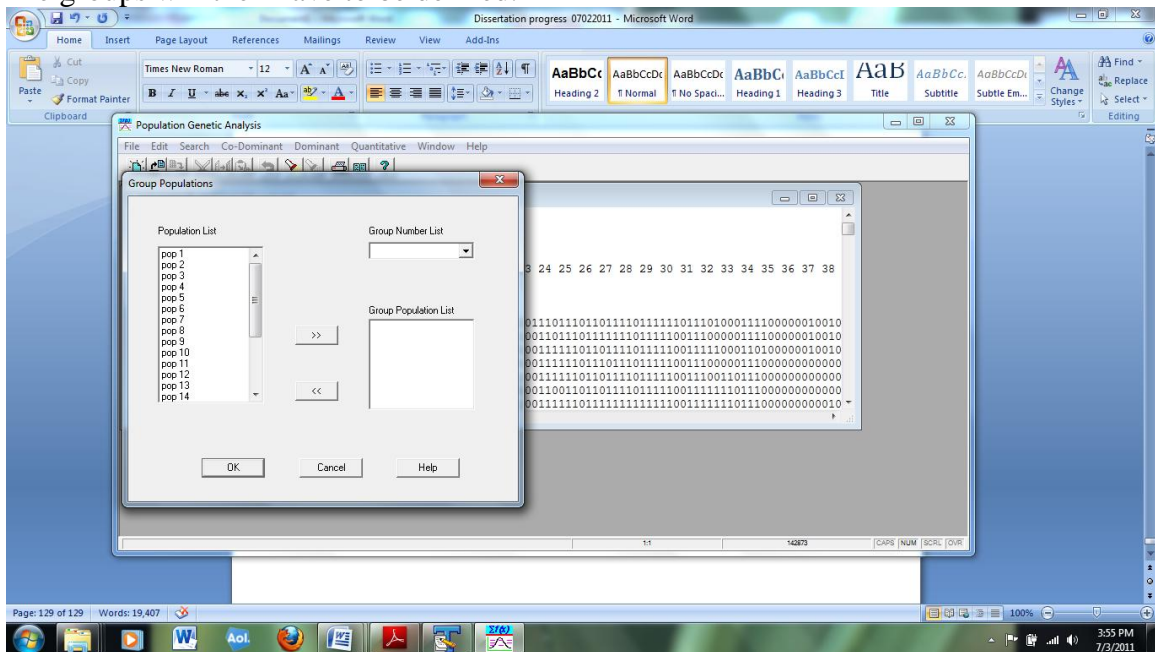
Defining the groups.

To define the groups, choose the populations on the left and the group from the

drop down menu on the top right. Click the right arrow and the chosen

populations will move to the window on the right. If a mistake is made and a

population must be removed from a group, simply click the left arrow. When all

the groups have been defined, click "okay".

The analysis will begin at this point, and the results will appear in a new window.

The results file can be very long depending on the analyses requested. Some

examples of the summarized results follow.
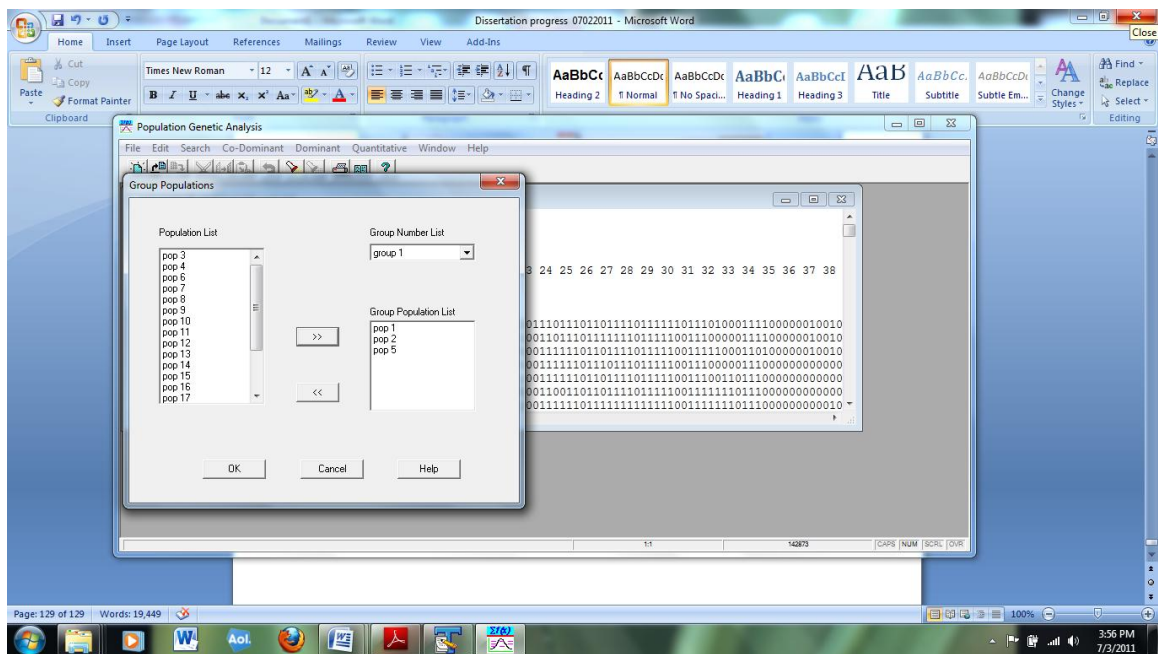
Defining the groups.
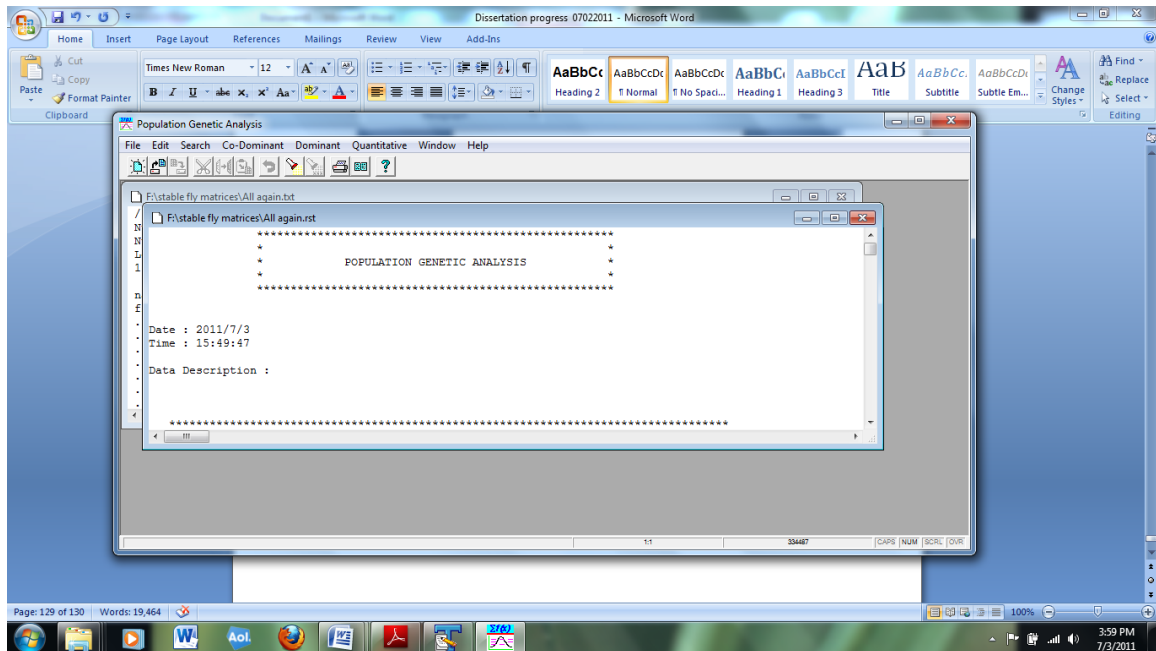
Results window.

Examples of results data from Popgene.

Nei's analysis of gene diversity in subdivided populations.

Population Genetic Analysis - [F:\stable fly matrices\All again.rst]

File Edit Search Co-Dominant Dominant Quantitative Window Help

```
                              +--5  +----------pop10
                              !  !  +---------------pop11
                              +--6
                              !  !        +-----pop20
                              !  !   +----1
                      +--7  +-------------
                      !  !              +----pop21
                      !  !  +--------------------pop7
                      +--10
                      !  !  !  +--------------------pop9
                      !  !  +--8
                +-12  !              +--------------------pop17
                !  !  !        +--------------------pop8
                !  +-----13
                !  !        +--------------------pop12
                !  !  +-16  +--9
                !  !              +--------------------pop14
                !  !        +--------------------pop3
                +-17  +----14
                !  !              +--------------------pop13
                !  !  +-11
                !  !              +--------------------pop18
        +------18  !  +--------------------pop6
        !  !     !  +--------------------pop23
  +-20  !  !
  !  !  !        +--------------------pop2
+---21  +--------------------
  !  !  !              +--------------------pop4
  !  !  !        +-------15
-22  +--------19  +--------------------pop5
  !  !     !
  !  !     +--------------------pop19
  !  |
                              +--------------------pop22
```

Dendrogram

Population Genetic Analysis - [F:\stable fly matrices\All again.rst]

File Edit Search Co-Dominant Dominant Quantitative Window Help

```
****************************************************************
**                                                            **
**      Nei's Unbiased Measures of Genetic Identity and Genetic distance    **
**              [See Nei (1978)  Genetics 89:583-590]          **
**                                                            **
****************************************************************
```

pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	****	0.7697	0.8502	0.7852	0.7406	0.8292	0.8993	0.8869	0.8856	0.9412	0.9138	0.8708	0.8816	0.8759	0.9489	0.9513
2	0.2617	****	0.8383	0.7451	0.7165	0.7470	0.7234	0.8077	0.7617	0.7852	0.7446	0.7504	0.7836	0.8001	0.7680	0.8015
3	0.1622	0.1763	****	0.8225	0.8109	0.8206	0.8151	0.7846	0.8723	0.8735	0.7891	0.8245	0.8748	0.7975	0.8307	0.8447
4	0.2419	0.2942	0.1954	****	0.8387	0.8156	0.7802	0.7752	0.8318	0.8170	0.7872	0.8101	0.8385	0.7664	0.7832	0.8046
5	0.3003	0.3334	0.2096	0.1759	****	0.7993	0.7165	0.6959	0.8233	0.7668	0.7105	0.7641	0.8441	0.7039	0.6950	0.7356
6	0.1873	0.2917	0.1977	0.2039	0.2240	****	0.7881	0.7798	0.8248	0.8209	0.7766	0.8035	0.8607	0.7737	0.7983	0.8172
7	0.1061	0.3238	0.2044	0.2483	0.3334	0.2381	****	0.8589	0.8771	0.9156	0.8714	0.8607	0.8554	0.8732	0.9084	0.9240
8	0.1200	0.2136	0.2426	0.2546	0.3625	0.2488	0.1521	****	0.8281	0.8976	0.8652	0.8565	0.8348	0.8776	0.9037	0.9088
9	0.1215	0.2721	0.1366	0.1842	0.1945	0.1927	0.1311	0.1886	****	0.9111	0.8327	0.8455	0.8874	0.8383	0.8613	0.8787
10	0.0606	0.2419	0.1352	0.2021	0.2656	0.1974	0.0882	0.1080	0.0931	****	0.9098	0.8912	0.8893	0.8906	0.9443	0.9368
11	0.0902	0.2948	0.2368	0.2393	0.3418	0.2528	0.1376	0.1448	0.1831	0.0945	****	0.8414	0.8517	0.8380	0.9198	0.9324
12	0.1383	0.2871	0.1930	0.2106	0.2690	0.2188	0.1500	0.1550	0.1678	0.1152	0.1726	****	0.8529	0.8870	0.8883	0.8724
13	0.1260	0.2439	0.1338	0.1761	0.1695	0.1500	0.1561	0.1806	0.1195	0.1173	0.1606	0.1591	****	0.8264	0.8554	0.8868
14	0.1325	0.2230	0.2262	0.2660	0.3511	0.2565	0.1356	0.1306	0.1764	0.1159	0.1767	0.1199	0.1907	****	0.9037	0.8859
15	0.0524	0.2639	0.1855	0.2444	0.3639	0.2252	0.0961	0.1013	0.1493	0.0573	0.0836	0.1184	0.1562	0.1012	****	0.9423
16	0.0500	0.2212	0.1688	0.2174	0.3071	0.2019	0.0790	0.0956	0.1293	0.0653	0.0700	0.1365	0.1201	0.1211	0.0594	****
17	0.1058	0.2909	0.1834	0.1957	0.2579	0.1956	0.1399	0.1614	0.1072	0.1079	0.1503	0.1539	0.1460	0.1556	0.1107	0.0954
18	0.1960	0.2346	0.1584	0.1909	0.1876	0.1930	0.2588	0.2444	0.1466	0.1991	0.2578	0.2358	0.1317	0.2656	0.2458	0.1953
19	0.3595	0.3691	0.2531	0.2216	0.2218	0.3084	0.3796	0.3356	0.2421	0.3047	0.4155	0.2825	0.2608	0.3742	0.3877	0.3488
20	0.1150	0.2786	0.1528	0.1568	0.2280	0.1892	0.1272	0.1972	0.1222	0.1003	0.1312	0.1521	0.1213	0.1673	0.1323	0.0882
21	0.0781	0.2568	0.1409	0.1508	0.2414	0.2024	0.1012	0.1460	0.1147	0.0682	0.0855	0.1280	0.1147	0.1362	0.0959	0.0588
22	0.2340	0.4462	0.3333	0.3471	0.4358	0.3395	0.2568	0.2705	0.2842	0.2348	0.2519	0.2331	0.2856	0.2539	0.2096	0.2552
23	0.1892	0.2624	0.2167	0.2732	0.3708	0.2772	0.2077	0.2528	0.2191	0.1741	0.2138	0.2086	0.2038	0.2055	0.2026	0.1960

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Distance matrix based on Nei's unbiased measures of genetic identity and genetic distance. Nei (1978).

Operating Arlequin

Arlequin performs operations such as linkage disequilibrium, Hardy-Weinberg equilibrium, As in Popgene, a data matrix must first be set up in textpad. However, Arlequin will build an outline for the data using the "project wizard" if preferred. With an outline, only the data needs to be pasted into the matrix. Following is an example of an outline produced by Arlequin and a completed data matrix. Using the project wizard will be discussed shortly.

Arlequin is not a free style format. Every character must be positioned perfectly so that Arlequin can read the file. Preparing the data matrix carefully can save hours of searching for problems later. If an error is encountered, Arlequin will produce an error log which may be of assistance.

Outline for a data matrix produced by Arlequin's project wizard.

Data matrix produced by project wizard with data entered.

When Arlequin is opened, the following screen will appear. If it has just been

downloaded, the Arlequin configuration must be specified.

Arlequin start page.
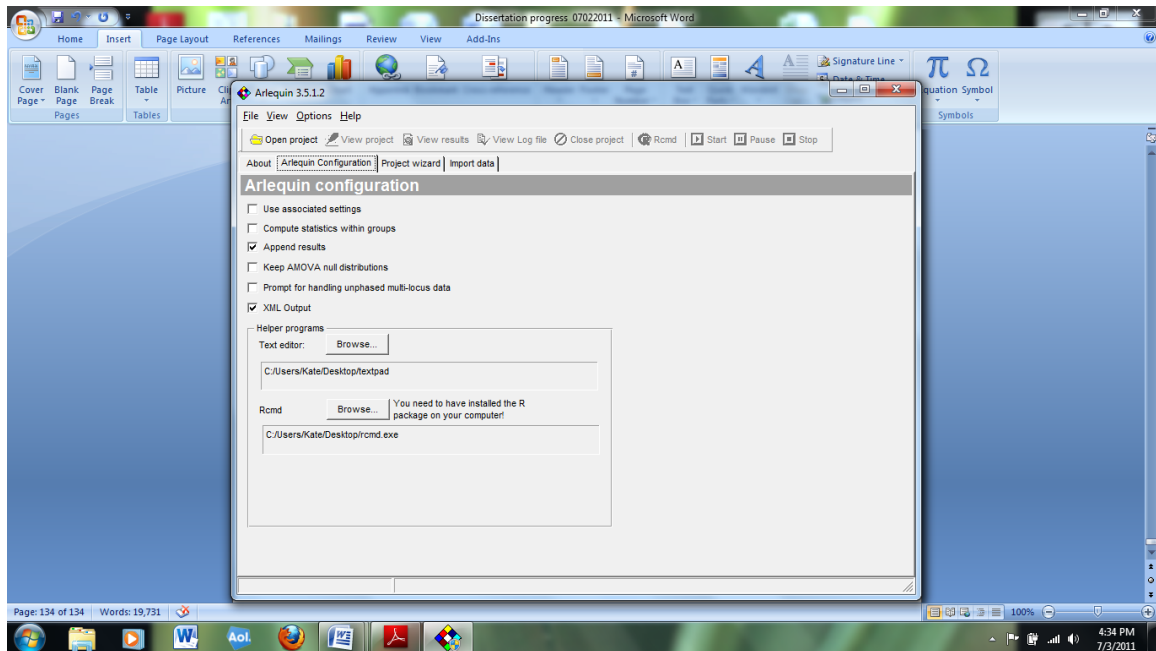
Arlequin configuration window.

In the configuration window, specify desired items in the check boxes. Then a text editor must be specified. Text pad is probably the best choice. Arlequin version 3.5 can now connect to the R-project to create graphics. If "R" has been downloaded, specify the "Rcmd.exe" to enable output in XLS file format.

Click on the "project wizard" box to create the outline for a data matrix. In the first window, the parameters of the data must be chosen.
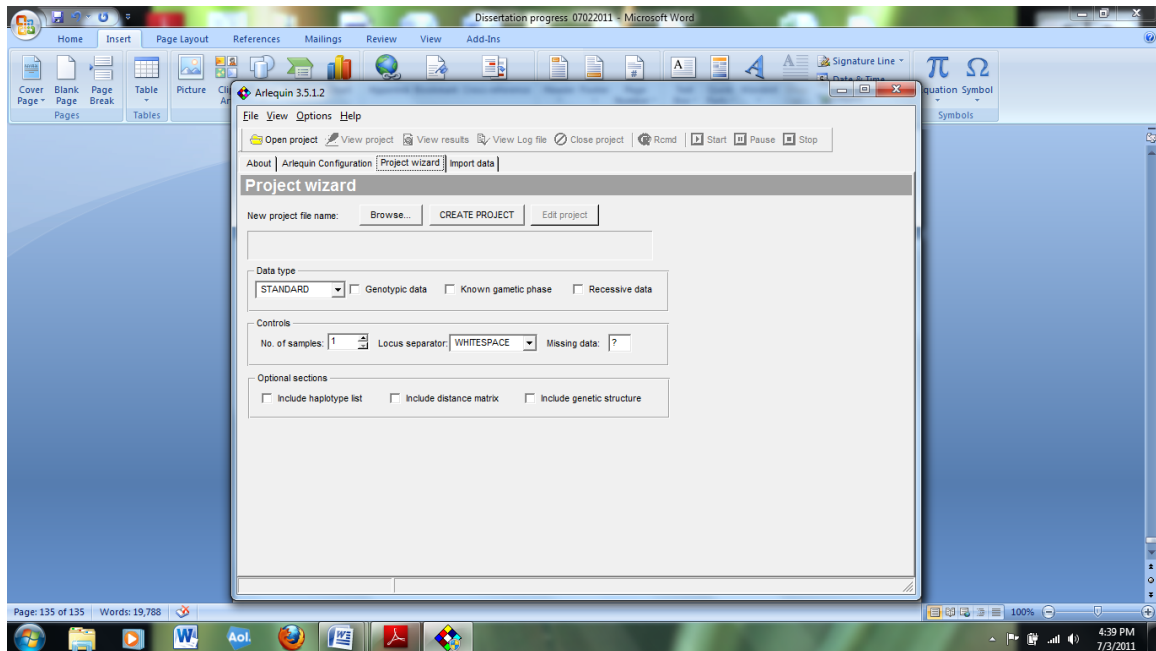
Project wizard window.

In the project wizard window, choose the type of data (Standard, DNA, Frequency, Microsatellite or RFLP). AFLP data falls under the RFLP category. Choose between genotypic data, known gametic phase, recessive data, or leave these blank. With AFLP data they can be blank. The "number of samples" box refers to the number of populations in the data set, not the number of individuals. Scroll to the number of populations. Choose indicators for locus separators (whitespace, tab or none) and missing data. Characters used for missing data include "?", "9" or "." Finally, options are offered to include a haplotype list, a distance matrix, and genetic structure.

A file must be created for the outline. Specify a file name and include ".arp" at the end of the name. For example, open a blank text pad page, and save it as "stablefly.arp". The outline will be created in this file. When the above tasks are

completed, click on "create project". The outline will be created. Set up the data

matrix in the outline before continuing.

When the data matrix is complete, click on "open project" on the toolbar

or in the "file" button. Open the file containing the data matrix. Next, go to

"import data". The following window will appear.
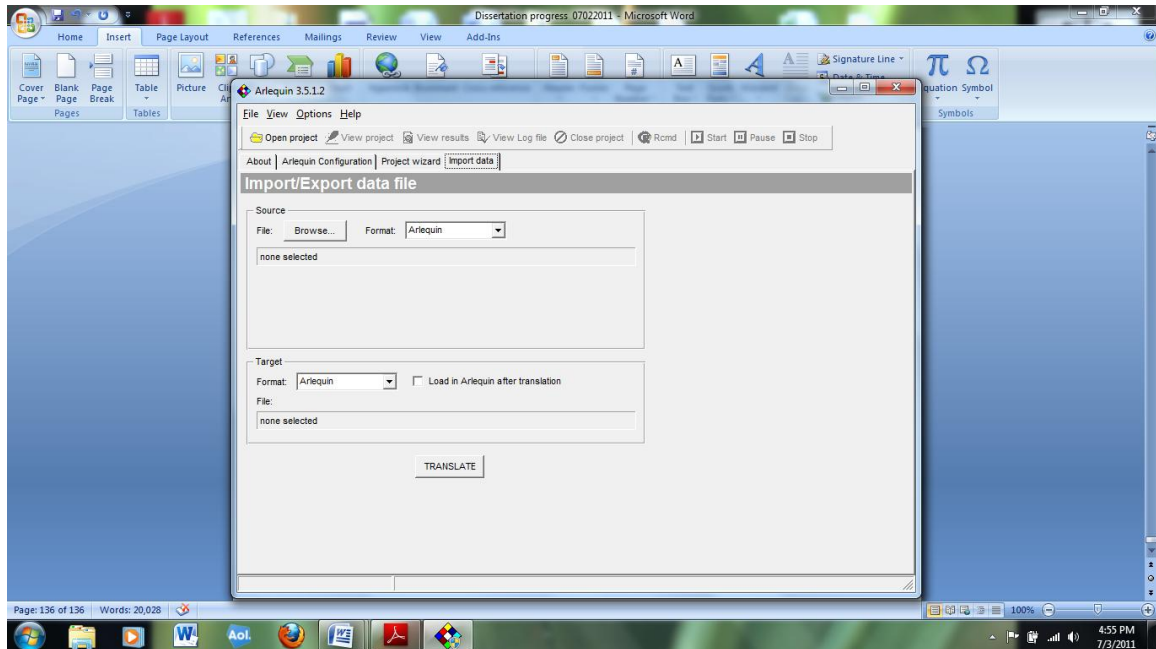
Importing data into Arlequin.

Click "browse" and load your data file. Since ".arp" was added to the file name,

both the source and target will be "Arlequin". Click "load in Arlequin afer

translation" and "translate". If all is well with the data matrix, the populations

and groups that have been specified will show up in the left pane. Click "start" to

begin analysis.
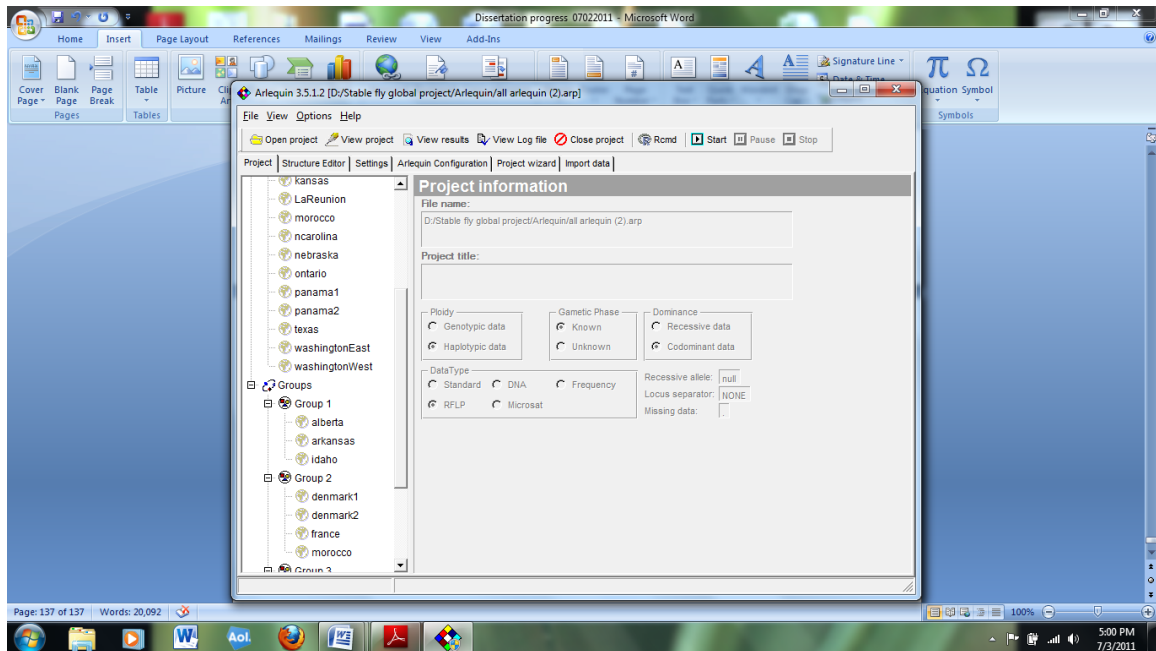
Project successfully entered into Arlequin.

While Arlequin is analyzing the data, a task bar at the bottom of the screen will indicate the percent of analyses completed and the current analysis being performed. Analyses can be paused or stopped by using the buttons next to the start button

Examples of Arlequin output

The Analysis of Molecular Variance (AMOVA).

Pairwise F_{st}s: Distance matrix.

Population average pairwise differences

APPENDIX X

Lab Supply List

GENERAL MERCHANDISE

Aluminum foil
Bleach
Bottle brushes
Dish soap
Garbage bags
Hand soap
Paper towels
Plastic wrap
Pledge furniture polish (for AFLP plates)
Scrub pads
Wax paper

LOCAL COMPANIES:

LICOR BIOSCIENCES:
402-467-0700

IRDye700 sizing standard	Item #4000-45	50-700bp	$100
Polyacrylamide gel	Item #827-05669 ……KB Plus 6.5%		$99
Polyacrylamide gel + TBE	Item #827-05669		$119
Pre-amp primer mix	Item #829-06193		$85

MIDLAND MEDICAL:

Autoclave tape
Labeling tape
Latex gloves, Lg
Latex gloves, Sm
Kimwipes
Nitrile gloves, Lg
Nitrile gloves, Sm

APPLIED BIOSYSTEMS:

AmpliTaq 360	100U	$62.50
	250U	$146.00
	1000U	$516.00

BIOEXPRESS

Agar	Item #J637-1000G	1kg	$97.00
Ammonium persulfate tablets	Item #K833-100	100mg	$24.50
Centrifuge tubes 15Ml	Item #C-3394-I	10 racks	$61.00
Centrifuge tubes 50mL	Item #C-3394-4	case of 500	$69.00
Chloroform	Item #0757-950ML	950mL	$38.50
Chloroform:Isoamyl 24:1	Item #X205-950ML	950mL	$40.30
Ethidium bromide	Item #E406-5ML	5mL	$17.50
Gloves, latex	Item #G-4040-S (or L)		$79.00
Gloves, nitrile	Item #G-4091-S (or L)	case	$78.50
Glycerol	Item #0854-4L	4L	$130.00
Isoamyl alcohol	Item #0944-1L	1L	$28.90
Microcentrifuge tubes	.Item #C-3260-I	.500/pk	$19.40
PCR tubes w/attached caps	Item #T-3035-I	120 strips	$72.00
PCR tubes caps incl	Item #T-3014-I	125 strips	$72.00
Petri dishes	Item #T-2883-12	case of 420	$59.97
Phenol:chloroform:isoamyl	Item #0883-400ML	400mL	$32.90
Pipettors	Item #P-3960-(size)		
$189.00			
Proteinase K	Item #0706-100MG	100mg	$76.60
Styrofoam freezer boxes	Item #R-8000-NL	case of 20	$35.00
Tris (base or HCl?)	Item #0497-5KG	5kg	$207.10
Tube racks 80-place	Item #R-792-2	case of 6	$24.97
Tube racks 96-place	Item #R-4910-2	case of 6	$39.97

BIOLOGIX:
913-648-8578

Centrifuge tubes 15mL	Item #10-0151	case of 500	$50.00
Centrifuge tubes 50mL	Item #10-9501	case of 500	$75.00
Microcentrifuge tubes (1.7mL)	Item #80-1500	case of 5000	$50.00
Microcentrifuge tube racks	80 place	case of 20	$29.00
Freezer boxes plastic	Item #90-9100	case of 20	$59.00
Petri dishes 100x15mm	Item #66-1501	case of 500	$54.00
Pipette tips Small…(10μL)	Item #20-0010	case	$40
Med…(200μL)	Item #20-0200	case	$50
Large..(1000μL)	Item #20-1000	case	$95
Solution basins (boats)	Item #25-0051	case of 50	$19.00

GE HEALTHCARE (AMERSHAM)

Bind silane	25mL		$105.00

INTEGRATED DNA TECHNOLOGIES (IDT):

Adapters	$52.00
E-primers (IRDye-labeled)	
$132.00	
M-primers	$52.00
Primers +1 for pre-amp mix	

INVITROGEN:

Pre-amp primer mix	Item # 10792-018	1mL	$93.75

MIDWEST SCIENTIFIC:
800-227-9997

Bullseye Taq Polymerase 500U	Item #BE110203		$119.00
Bullseye Taq Polymerase 1000U	Item #BE110204		$179.00
10μL tips	Item #AVR11	(1000/bag)	$16.84.
200μL tips	Item #40200C	(1000/bag)	$14.85
1000 μL tips	Item #AVR4	(1000/bag)	$19.31
Ammonium persulfate (100g)	Item #IB70080		$23.00
Boric Acid	Item #IB70096	(2.5kg)	$95.00
Bromophenol blue (25g)	Item #IB74040		$58.00
Chloroform (500mL)	Item #IB05040		$29.00
dNTPs	Item #BE502004		$154.00
EDTA .5M soln	Item #IB70185	(400mL)	$90.00
Ethanol 200 proof	Item #IB15721		$46.00
Ethidium bromide soln (10mL)	Item #IB40075		$33.00
Gloves, latex			$7.6015.10/box
Gloves, nitrile			$8.40-15.00/box
Glycerol 1L	Item #IB15762		$56.00
Isopropanol 99%	Item #IB15735	(1L)	$29.00
Labeling tape	Item#ST-12-1 (2, 3, 4, 5, 6) 1 roll		$3.68
Microcentrifuge tubes 1.7mL	Item #AVSS1700	(500)	$14.88
Parafilm	Item #HS234526C		$61.00
PCR Tubes w/caps	Item #AVSST		$97.41
PCR Tubes w/o caps	Item # AVST		$67.00
Caps for PCR Tubes	Item #AVSTC-N		$17.00
Proteinase K..(100mg)	Item #IB05400		$85.00
Sodium chloride (1kg)	Item #IB07071		$32.00
Temed (50mL)	Item #IB70120		$27.00
Tris HCl(500g)	Item#IB70162		$59.00

NEW ENGLAND BIOLABS:
978-927-5054

EcoRI restriction enzyme	Item #R0101L	$212.00
MseI restriction enzyme	Item #R0525L	$244.00
T4 DNA ligase	Item #M0202L	$252.00

SIGMA-ALDRICH:
866-266-2015

Absolute ethanol	Item # 459844-4L	$225.00
Acetic acid	Item # 320099-500mL	$46.90
Agarose	Item # A9539-500g	$890.00
Ammonium persulfate	Item #A3678-100G	$37.20
B-mercaptoethanol	Item #M3148-250ML	$61.60
Boric acid	Item #B7901-1KG	$75.90
Chloroform	Item #C2432-6X500ML	$213.00
CTAB	Item #H6269-500G	$164.00
EDTA	Item #E9884-1KG	$119.00
Ethidium bromide	Item # E1510-10ML	$47.60
Glycerol	Item #G5516-1L	$85.90
Isopropanol	Item #34965-2.5L	$116.00
Isoamyl alcohol		
Proteinase K	Item # P2308-100MG	
$104.00		
RNaseA	Item #R6513-250MG	$358.60
Sodium chloride	Item #S3014-1KG	$51.00
Temed	Item #T9281-100ML	$103.00
Tris Base	Item #T1503-5KG	$102.00
Tris HCl	Item #T3253-500G	$106.50

APPENDIX XI

Troubleshooting

Analyzing DNA from insects can be a rewarding experience when things go smoothly. However, projects are seldom completed without some troubles along the way, especially when the object of the research (DNA) is not visible. One can only speculate as to the problem, and work through it by the process of elimination. From DNA extraction, through PCR, gel electrophoresis and data analysis, any number of mistakes can occur, reagents can be damaged and equipment can malfunction. It can be a frustrating experience trying to determine the cause of a problem, especially if no one has encountered it previously.

This section was written, not to solve every issue that may occur in the lab, but to list those issues and offer suggestions for determining a solution. Many of these issues   occurred during the course of my project and the projects of others in our lab, and the cause has not yet been discovered. Hopefully it will be of use to other students entering the field of genetics to know that problems they are faced with have been encountered by others.

DNA Extraction

Problem: Poor quality DNA.

Suggestions:

- The guts of the specimens may not have been removed completely, leaving extraneous materials in the sample that would not be removed by the protocol.

- Samples were not homogenized enough. If the cells are not lysed the DNA is not extracted from them.

- RNaseA or ProteinaseK steps may not have been performed properly, or the enzymes are not working due to careless handling such as being left on the counter for long periods. This would leave more RNA or proteins in the sample.

- In the chloroform step, the aqueous layer was not carefully removed from the chloroform:isoamyl layer. There is usually "junk" between the layers that should not be transferred with the aqueous layer.

Problem: No DNA

Suggestions:

- Dissect the specimens under a microscope to make sure you are not scraping away the muscles as well as the gut.

- Make sure the DNA pellet is not being poured off with the alcohol.

- When air drying the samples, make sure all of the ethanol has evaporated before adding TE buffer. This can be tested simply by flicking the tube. If any ethanol remains it will splash onto the side of the tube.

PCR

Good DNA does not always result in good gels. A problem will not be evident, however, until the gel is run, so I will discuss PCR problems in the context of the gel results. It is helpful to run the DNA on an agarose gel to test the quality before proceeding to the AFLP steps.

Problem: The sample forms streaks and is too dark.

Gel with dark streaks. The sizing standards are not visible.

Suggestions:

- The DNA could be degraded. This can only be determined by agarose gel electrophoresis. The Nanodrop® does not diagnose this problem.

- Too much DNA in the sample. Run the 20µl dilutions on the Nanodrop® to verify the concentration.

- Too much primer. Lower the amount of primers stepwise and compare the results.

Problem: The gel doesn't run far enough.

Suggestions:

- Not enough dNTPs. Perform a stepwise test for amount of dNTPs to use for your samples.

- Not enough DNA. It could have had a low concentration after extraction or it could have been diluted too much during AFLP step 4 or step 6.

- Not enough primers. Do a stepwise primer test.

- Not enough taq polymerase. Do a stepwise test for amount of taq.

Problem: Huge black blobs occur on the gel. This is one we have not yet solved. See fig.

Suggestions:

We tested just about everything for this one. It could be degraded DNA or a combination of factors that would not occur when reagents are tested singly.

Problem: Nothing except the sizing standard appears on the gel.

Suggestions:

This is probably a malfunction of the thermal cycler. It happened to me while working on this project. All reagents were recently purchased. The selective mix had been optimized. My DNA came out very good. The gels became worse as I progressed in my research, but I would randomly get very good gels. Near the end of my research one of the thermal cyclers stopped working completely.
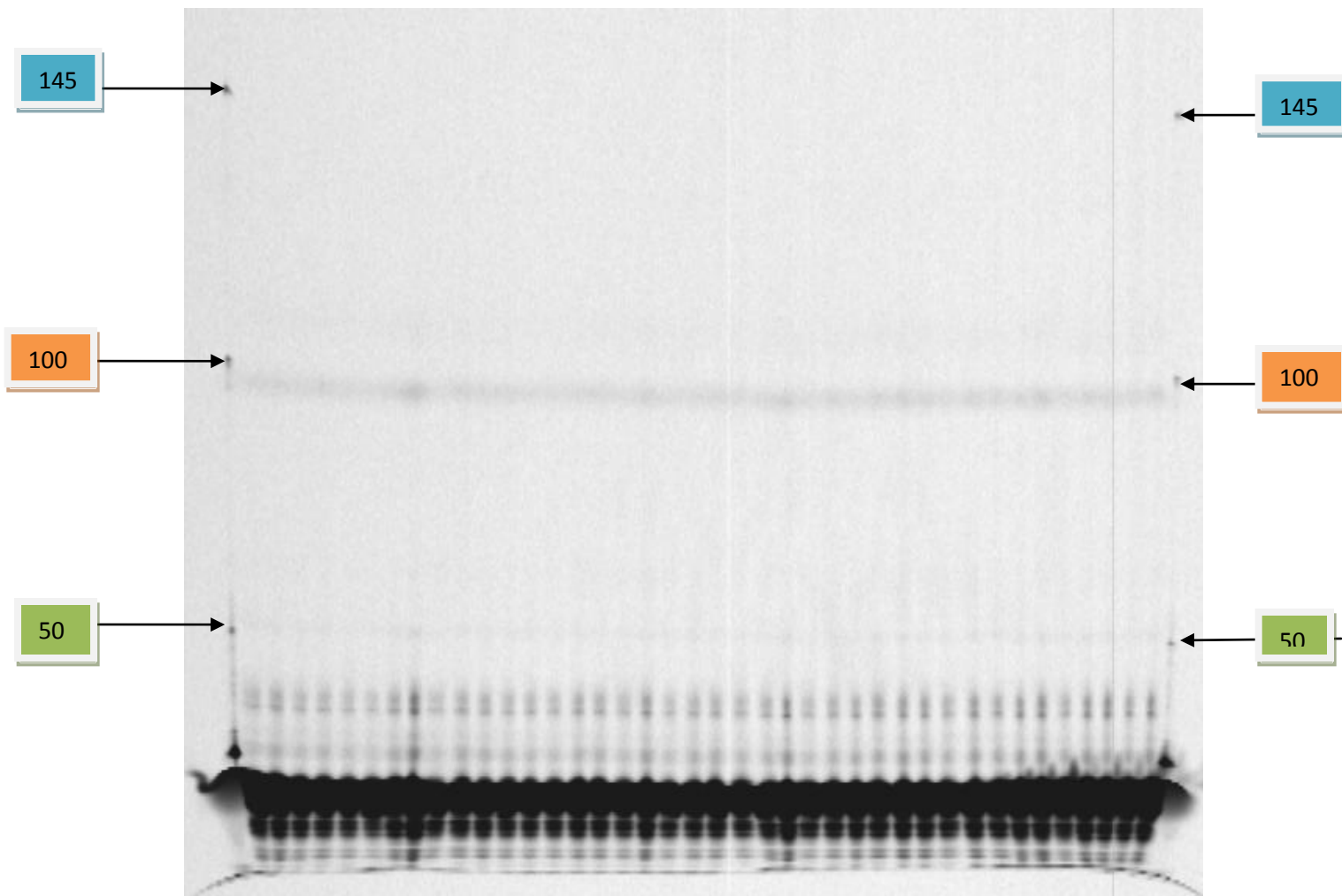
This happened with ~50% of my gels. The sizing standard and primer front are visible but no bands.
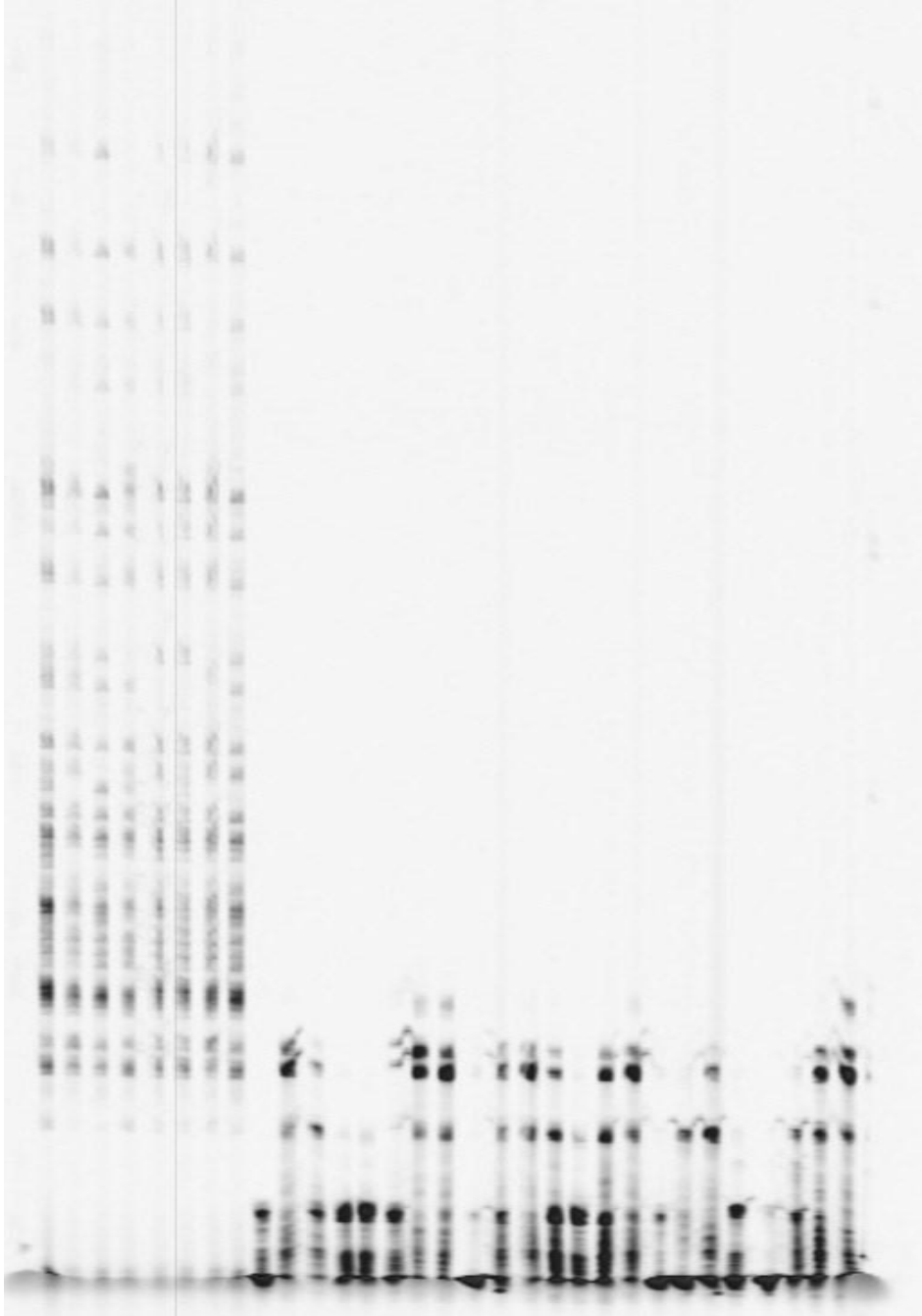
Gel that did not run far enough. This gel was from a primer test between Licor and IDT primers. At the time of the test we did not know that IDT primers lacked dNTPs.

Electrophoresis

Problem: The pre-run stops and the error message indicates an open circuit.

Suggestions:

- The obvious solution is to check that the lower and upper buffer tanks and the power cord are plugged in properly.

- This error will also occur if there is not enough buffer in the upper tank.

  Problem: The pre-run stops and the error message indicates a gel "leak".

  Suggestions:

- Make sure the gel is allowed to set for at least 1.5hr.

- Check the position of the gel in the machine. The arms may not be set properly into the hooks.

- The gel is actually leaking. Make a new gel and start over.

Data analysis

There are far too many problems with data analysis and use of the software to address here. My suggestion for this section is to be meticulous in your setting up of the data, from SAGA to Arlequin and Popgene. There is nothing like spending 3 days trying to make a program work when the problem could be as insignificant as a misplaced semi-colon.