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LINKAGE MAPPING AND PCR-BASED DNA FINGERPRINTING IN BLACK CHERRY (*PRUNUS SEROTINA*)

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ABSTRACT

Black cherry (*Prunus serotina*) is an important hardwood tree species in the United States of America because of its valuable timber; however it is a semi-invasive species in Europe. The *Prunus* species have been extensively studied with markers and linkage maps developed in many of the species. The main goal of our project is to identify QTLs related to response to ozone stress in black cherry. By identifying QTLs related to response to ozone stress, we can develop black cherry which are more resistant to ozone stress. This will help protect a valuable resource in the United States. To accomplish this, we needed to develop linkage maps based on full sibling populations of black cherry. PCR-based DNA fingerprinting was used to identify full siblings. QTLs can be located on the linkage maps.

We worked with open-pollinated populations of black cherry in this study. This meant that the crosses were not controlled, and so we had to perform whole population screening by using SSR markers to look for the full siblings in the populations needed to build linkage maps. These seedlings were obtained from black cherry which were first used by Penn State faculty members John Skelly and Kim Steiner, who studied black cherry for response to ozone stress. In this study we used three families of black cherry: MO-21 (most tolerant to ozone), R-14 (most sensitive to ozone), and R-12 (intermediate tolerance to ozone).

We extracted DNA from 600 samples from each seedling population of MO-21 and R-14. SSR markers developed from an initial EST black cherry library were identified and screened for polymorphism in the black cherry population. To date we have found 76 SSR markers to amplify and only two to be polymorphic. The screening is in process and as more markers will be found to be polymorphic they will be used for PCR to screen the families for full siblings. Linkage maps will be constructed from these data to identify QTLs related to ozone stress.

TABLE OF CONTENTS

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INTRODUCTION

Black cherry (*Prunus serotina* Ehrh.) is an important member of the Rosaceae plant family and has been studied because of its value as a hardwood tree in North America and semi-invasive species in Europe. The aim of our project on the black cherry is to screen a population for full siblings through DNA fingerprinting, and develop linkage maps to identify QTLs related to ozone stress. QTLs (quantitative trait loci) are a group of genes which are related to a phenotypic characteristic (such as ozone tolerance). These identified QTLs will be examined to see if there are any specific differences in the alleles or expression levels between trees tolerant to or sensitive to ozone. By locating QTLs, we can help create better trees and manage forests to survive ozone pollution. The ultimate goal of our project is to identify QTLs linked to ozone response, and use the linkage maps developed to identify QTLs for other characteristics in the future. The families we used in our study have been used in previous studies involved in ozone tolerance of black cherry.

The populations of black cherry used in our experiment were obtained from seedlings used in a study by Penn State University faculty members John Skelly and Kim Steiner. They studied the effect of ozone pollution on a variety of trees, including black cherry. Two families of black cherry were chosen from them for our project. These families were examined to see how sensitive they were to ozone. One group was found to be more tolerant to ozone (MO-21) and another group was found to be more sensitive to ozone (R-14) (Lee, et. al., 1999 and 2002). The black cherry used by Steiner and Skelly showed genetic variation in responses to stress from ozone pollution. These trees are open-pollinated (so most will probably be half-siblings) and we have to screen the population by using DNA fingerprinting to identify the full siblings necessary for developing linkage maps and locating QTLs. About 600 samples of MO-21 and 900 samples

of R-14 were used for our project. We expect 25% of the population to be full siblings, and about 200-300 will be used to develop linkage maps and for QTL analysis. There is a focus on helping these trees cope with ozone because of their importance to the economy of the United States.

The timber produced from black cherry is important in the eastern United States. It is a renewable resource important to the economy and is one of the highest valued timbers in the United States (Marquis, 1990; Haynes, 1990). However, high quality timber is produced only in certain areas around the world. Research has been done looking into the environmental and genetic causes which may make different quality black cherry (Dirlewanger, et. al., 2004). Black cherry trees found in Pennsylvania are very valuable, and keeping them alive and healthy is important (Jacobson, 2004). By locating genes and QTLs involved in black cherry trees' response to ozone stress, trees which are more tolerant of ozone can be developed and help them survive ozone pollution. Tropospheric ozone (O_3) is detrimental to black cherry (Chappelka, et. al., 1999). More O_3 means more oxygen radical stress. The loss of crops in the United States from O_3 stress costs billions of dollars. Black cherry is very sensitive to ozone, and because of this sensitivity it has been used as a bio-indicator for ozone pollution (Chappelka, 1999; Skelly, 2000). The survival of black cherry populations in the Allegheny Plateau region might be in danger because of a combination of stresses including high levels of anthropogenic ozone found to be present in Pennsylvania (Lee, et. al., 1999; Bennett, et. al., 1992; Neufeld, et. al., 1995). Penn State University is a great place to study the black cherry because of its close proximity to where most commercial black cherry production occurs.

The black cherry is part of the Rosaceae family which includes such fruits as apple, cherry, peach, plum, pear, apricot, almond, loquat, and quince. Rosaceae is an angiosperm

family with over 3,000 species and 110 genera throughout the world, and is made up of four subfamilies based on fruit type and chromosome number (Takhtajan 1997). One of these subfamilies is the Prunoidea subfamily with eight chromosomes (haploid number), and this subfamily contains the black cherry species (as well as peach, apricot, plum, and almond). Black cherry is believed to be a tetraploid species (meaning it has four copies of each chromosome) (Pairon and Jacquemart, 2005). These fruit trees all have advantages such as a small genome, long life, efficient methods of vegetative reproduction, and the ability to make interspecific crosses. There are questions surrounding black cherry's tetraploid inheritance.

Black cherry is thought to be allopolyploid (Pairon and Jacquemart, 2008). Allopolyploid is when an organism gets more than two genetically distinct sets of chromosomes. An allotetraploid would be diploid for two genomes with each genome coming from a different species. Usually an allopolyploid is from two different species interbreeding to create an individual with a haploid set of chromosomes from each parent which are then doubled. Some species could also be autopolyploid, which is when all the chromosome sets come from the same species. Polyploidy is usually the result of defects in meiosis. For example, if the spindle breaks down during meiosis, this can result in an uneven number of chromosomes moving towards each pole of the cell during cell division. Polyploidy is lethal in most species, except in plants. In plants, this causes speciation and diversification. Polyploidy can cause problems in determining the mode of inheritance and frequencies of alleles.

Black cherry could either have disomic or tetrasomic inheritance. Usually it is difficult to use microsatellite markers for a polyploidy species with disomic inheritance because it is difficult to determine the allele frequencies, and the inheritance patterns are very complex (Pairon and Jacquemart, 2008). It is difficult to use microsatellites in allopolyploid species

because there are more than two alleles at each locus and this creates a complex inheritance pattern. Allopolyploids usually have alleles present in more than one copy, but the allele dosage cannot be determined through PCR or gel electrophoresis.

Disomic inheritance is the natural, Mendelian form of inheritance (when there are two copies of a chromosome). This occurs when a single arrangement of homologous chromosomes is formed at meiosis. A tetraploid has two independently segregating genomes when disomic inheritance occurs (Pairon and Jacquemart, 2005). If the four chromosomes in tetraploids are randomly paired, then this produces tetrasomic inheritance (Pairon and Jacquemart, 2005). This can create an increase in the frequency of homozygous gametes compared to disomic inheritance (Pairon and Jacquemart, 2005). Tetrasomy is when there are four copies of one chromosome and two copies of all the others. To determine the pattern of inheritance, one can cross parents of known genotypes and analyze the frequencies of the progeny genotypes. Allopolyploids are formed when chromosomes double after hybridization and autopolyploids form when chromosomes of the same genome double (Pairon and Jacquemart, 2005). Autotetraploids have chromosomes with four homologous versions which pair randomly and leads to tetrasomic inheritance (which means all allelic combinations have equal frequencies) (Stift, et al., 2008). Allotetraploids are usually disomic for inheritance and have two homeologous sets with two homologous chromosomes each, and the chromosomes pair with their homologues (Stift, et al., 2008). Usually allopolyploids segregate disomically and autopolyploids segregate polysomically, but this is not always the case (Pairon and Jacquemart, 2005). The method of inheritance in the plant has a great effect on how alleles are segregated and genes are passed on. Tetrasomy is due to old duplications in the genomes, and can result in four peaks when tested for the number of alleles by PCR-based DNA fingerprinting. One peak from this analysis could

indicate homozygosity or the presence of a null allele formed by differences in the flanking sequences of the SSR the primers are based on (the primers can no longer attach to the flanking sequences because they have changed, so no SSR allele shows up in the results even though it may still be there).

One of the problems with microsatellite markers in polyploid species is that they do not reveal allele dosage. The presence of dominance in alleles can make genotypes indistinguishable and thus genotype frequencies cannot be translated into allele frequencies. Autopolyploids with polysomic inheritance usually have a higher frequency of homozygote individuals in the offspring (De Silva, H.N., et. al., 2005). An autotetraploid has up to four alleles present in one individual. They can form six different gamete combinations. An allotetraploid does not have random selection from four chromosomes to create gametes, but instead has the chromosomes divided into two homeologous loci (each with two chromosomes) and one chromosome from each locus is chosen to make the gametes (De Silva, H.N., et. al., 2005). Homeologous chromosomes are chromosomes only partially homologous (chromosome pairs with the same genes during meiosis) and are from ancestral genomes. Many polyploids have both polysomic and disomic inheritance patterns. Some studies have been conducted to create a method for estimating allele frequencies for a polyploid population with mixed mating and polysomic or disomic inheritance patterns (De Silva, et al., 2005). Other studies have developed a method to look at and analyze an intermediate inheritance between disomic and tetrasomic in specific species (Stift, et al., 2008). This study was done to see if segregation in tetraploids is due to disomic, tetrasomic, or intermediate inheritance.

One method used by Pairon and Jacquemart in 2008 to solve inheritance problems was by finding genome-specific primers for *Prunus serotina* Ehrh. Genome-specific primers are

specific for one of the genomes which helped create the species. Their study tested 67 microsatellite markers, found 26 with successful amplification, and five which were genome-specific primers. Pairon and Jacquemart showed it is possible to test differences between allopolyploid estimates of genetic differentiation and diversity. These genome-specific primers can be used to study the genetic structure of black cherry in its habitats, or model the establishment of black cherry in new habitats.

The method used to fingerprint the black cherry populations in our project is by using PCR (polymerase chain reaction). This is a simple and common molecular biology technique. Specific primers located along a sequence one wants to amplify are combined with the DNA. The primers will be based on flanking sequences of the SSRs (simple sequence repeats) we have identified and planned to use in this project. The DNA is heated so the strands separate, and then the temperature is cooled so the specific primers can attach to their DNA sequences. Then, the temperature is raised again to activate the primers (DNA polymerases) which amplify the specific DNA sequences they have attached to.

The results of the PCR are measured by inserting the DNA onto a gel and measuring it by gel electrophoresis. PCR amplification is successful when there is a sharp band located in the expected size range of the DNA fragment on the gel. The number of repeats of the SSR sequence causes the band size to be larger or smaller at the allele of each individual (each individual has two alleles at each locus). If the individual is heterozygous, it will show two different bands. If the individual is homozygous it will have one band. We used gels to determine amplifiable SSR, and GeneMapper® Software which is a genotyping software package that provides DNA sizing and quality allele calls for Applied Biosystems electrophoresis-based genotyping systems. The primers used for PCR are based on

microsatellites found in the black cherry cDNA sequence previously made by other members of the laboratory.

Microsatellite is the name for markers used in PCR to help fingerprint DNA and amplify specific regions. They are a repeated sequence of about 1-6 nucleotides, rare in coding sequences, and are usually evenly distributed along a chromosome. They are commonly used because of their high levels of polymorphism (the presence of two distinct genetic groups in a species) and codominant inheritance (Pairon and Jacquemart, 2008). Microsatellite markers have been very useful for analyzing genetic diversity and creating genetic linkage maps. Interpreting band patterns found by using microsatellite markers is made easier when the inheritance pathway in the species is known. Five microsatellite markers have been found in black cherry since 2008 (Downey and Iezzoni, 2000; Pairon and Jacquemart, 2005) and two of them are genome-specific (Pairon and Jacquemart, 2005).

Pairon and Jacquemart in 2005 used six microsatellite markers to test inheritance in black cherry and proved it to be disomic at all of these six locations. According to this study, the black cherry seems to be allotetraploid (Pairon and Jacquemart, 2005). There was a high number of alleles and observed heterozygosity seen in the progeny of this study which is usually indicative of allotetraploids (and disomic inheritance) (Pairon and Jacquemart, 2005).

In order to run PCR to screen the black cherry populations for full siblings, we need to identify primers to amplify specific regions of the black cherry DNA sequence. These primers were chosen from SSR-containing cDNA sequences of black cherry completed previously by other members of the laboratory. The primers we used in this study amplified SSR (simple sequence repeats) containing regions of genes. SSRs are a specific type of microsatellite. SSRs are short repeated sequence motifs found throughout eukaryotic genomes. SSRs are 2-6

nucleotide sequences repeated over and over again. Each DNA marker (SSR) corresponds to a particular sequence at a site (locus) in the genome and if more than one length of sequence is found at the site, then the marker is polymorphic (which means there are alleles at the locus that differ in molecular phenotype). SSR markers were created to help solve the problems of RFLPs. SSRs have high polymorphism, codominant inheritance (when the phenotypes of both alleles are shown), and are simple to develop (Aranzana, et. al., 2003). Thus SSRs are usually better than RFLPs in genetic studies. RFLPs required much larger amounts of DNA and time to use because they were assayed by Southern hybridization. PCR primers derive from cDNA or genetic flanking sequences. Flanking sequences (parts of the DNA sequence next to and on either side of the SSRs) are usually conserved in many species, allowing the same primers to be used in multiple species. More specific (and thus longer) primers result in fewer bands. In one study, Zietkiewicz, et al. looked at using primers for inter-SSR PCR of CA repeat loci (1994). They were able to use the same primers to amplify regions in different species' genomes. These SSR primers have intraspecies polymorphisms which reveal genetic differences specific to individuals within a species as revealed by PCR and DNA fingerprinting. Ideally, we would like to find 10 SSR markers which show amplification (bands) and polymorphism (more than one band, indicating the presence of multiple alleles) for the black cherry populations we are studying.

SSR primers have been used in other *Prunus* studies to fingerprint populations and identify genotypes. For example, Wunsch and Hormaza in 2004 used 12 SSR primers from peach to look at 42 alleles and identify 26 genotypes in sweet cherry plants.
They were also trying to analyze the S-allele to improve orchard management. Self-incompatibility (the inability to self-pollinate) is one characteristic selected for and is highly

polymorphic. An S-allele is a self-incompatibility allele. Markers to select for this characteristic have been created. They found three alleles in the traditional plants from the area they genotyped and some new alleles from new plant types added to the valley. This study can help to maintain the genotypes already in the plant population, as well as select new genotypes with better traits and avoid problems of incompatibility.

A similar study by Aranzana, et al. in 2003 developed a set of 24 SSRs to be used for a "genotyping set" for peach species. These are to be used as a reference for fingerprinting, pedigree, and genetic analyses in peach. The 24 SSRs are highly polymorphic and fall within 24 25-cM regions evenly spaced apart on the *Prunus* genome (Aranzana, et al., 2003). These markers were placed into the eight linkage groups of the *Prunus* TxE map (Aranzana, et al., 2003). This is a similar design and study to our project.

This project uses DNA fingerprinting to identify the full siblings in the populations of black cherry to be used for creating genetic linkage maps and identifying QTLs related to ozone stress. These full siblings must have one or two of the SSR alleles present in the female parent and the other allele will be from the male parent. We will use about ten microsatellite (SSR) markers. About 250 full siblings are expected for the creation of linkage maps. DNA polymorphisms will be used to differentiate, and thus fingerprint, each individual.

Once full siblings have been found in the populations of black cherry, linkage maps can be developed. A linkage map is a map of the relative positions of known genes or DNA markers on the chromosomes of a species. These maps can be used to identify QTLs (areas of the genome) which have a large effect on specific traits such as resistance to disease and quality of wood. Distances on a linkage map between genes are calculated by looking at recombination frequencies found in breeding crosses. These crosses are between parents differing in alleles of

two or more genes. Testcrosses (a cross between a homozygous recessive individual and the individual of interest) are conducted to determine which genes are linked together. If the progeny show the expected ratios for independent assortment, then the genes are probably linked. Linkage maps can be used to help with breeding by finding and selecting specific genes which give the species advantages. These maps show the arrangement of genes, location of genes, and distance between genes on statistical representations of the chromosomes.

Linkage maps can be used to identify QTLs. Some important characteristics in fruit trees act as major genes (QTLs), such as disease resistances, blooming, ripening and fruit traits (Dirlewanger, et. al., 2004). Some other traits relating to plant growth, yield, blooming, harvesting times, fruit quality, and plant architecture characteristics are being investigated to find QTLs. Linkage maps are used for mapping ESTs, QTLs, and cloning genes. Genetic linkage maps have become widespread and have been used in some of the Rosaceae family's species.

The reference linkage map for the *Prunus* species (the TxE map) was developed in 2004 by Dirlewanger, et al. This map was made by crossing the almond "Texas" with peach "Earlygold" to generate an F2 population with eight linkage groups and 185 SSRs (Dirlewanger, et al., 2004). This paper found the map position of 220 more markers on the TxE *Prunus* map (89 of them are SSRs) (Dirlewanger, et al., 2004). Dirlewanger's study mapped 28 major genes on the TxE map (Dirlewanger, et al., 2004). The markers in this reference map can be used in closely related species for genetic mapping (Sargent, et. al., 2004).

There are currently 15 genetic maps for members of the *Prunus* species, and existing ESTs from the *Prunus* species will be selected for markers in black cherry. Since 2008, 15 molecular genetic maps have been constructed for members of the *Prunus* species (Zhebentyayeva, et al., 2008). The diploid *Prunus* species are collinear in respect to their

linkage maps (Dirlewanger, et al., 2004). No major chromosomal rearrangements have been detected in these *Prunus* species to date (Dirlewanger, et al., 2004). There is a high level of synteny (two or more genes on the same chromosome) among the genomes of *Prunus* species (Dirlewanger, et al., 2004). *Prunus* has highly polymorphic populations for linkage studies, common linkage group terminology, transferable (anchor) markers to make other maps, QTL locations, and many DNA markers (Dirlewanger, et al., 2004).

A physical map of peach was made by using high-information content fingerprinting (HICF) and FPC software by Zhebentyayeva, et al. in 2008. A physical map is a map with all the major pieces of a chromosome labeled in order (such as open reading frames, restriction enzyme cutting sites, etc.). This physical map was made up of 2,138 contigs made of 15,655 clones from two complementary bacterial artificial chromosome libraries (Zhebentyayeva, et al., 2008). A contig is when fragments of cloned cDNA are overlapped on top of each other to provide a larger continuous piece of the sequence. The length of all of these contigs is about 303 Mb (104.5% of the peach genome) (Zhebentyayeva, et al., 2008). About 252 of these contigs were anchored to eight linkage groups of the *Prunus* reference map (Zhebentyayeva, et al., 2008). They found the HICF method to be more effective than using gels (Zhebentyayeva, et al., 2008). Peach is being developed as a model genome for the Rosaceae family.

DNA fingerprinting and the development of linkage maps has been used for various species. By using SSRs to identify full siblings in populations of ozone sensitive and ozone tolerant black cherry through DNA fingerprinting, we aim to build linkage maps to identify QTLs involved in response to ozone stress. By developing these genomic tools and resources for *Prunus serotina*, we will be able to identify QTLs for other characteristics of black cherry in the future. Knowing the location of QTLs for ozone stress response can help us propagate black

cherry that are more tolerant of ozone and help save one of the important hardwood tree species in the United States of America.

MATERIALS AND METHODS

Samples

About 2400 black cherry seedlings were grown in 2009 and screened for ozone tolerance or sensitivity. These seedlings in 2009 were treated to ozone delivered within CSTR chambers in sine-wave fashion (average 80 ppb) for eight hours per day, seven days per week with exposures beginning at 9:00 AM and ending at 4:59 PM.

The families, originally studied by John Skelly and Kim Steiner at Penn State, were examined to see how sensitive they were to ozone. One group was found to be tolerant to ozone (MO-21) and another group was found to be more sensitive to ozone (R-14), on average.

Seeds from open-pollinated black cherry were collected in 2008 by members of the Carlson laboratory from the Penn Nursery of Pennsylvania's Bureau of Forestry. These seeds were collected from three different families: M21 (ozone tolerant), R-14 (ozone sensitive), and R-12 (intermediate tolerance/sensitivity). About 2000 seeds from each family underwent dormancy at 4^{o}C in a moist medium for 90 days, and then were planted and grown under greenhouse conditions. At the end of ozone fumigations leaves from the seedlings were collected in the first year after being planted. They were stored at -20^{o}C to be extracted for DNA by the CTAB method later.

cDNA Sequence

A cDNA sequence was prepared previously by other members of the laboratory. They extracted RNA from the black cherry samples, assessed the RNA quality, isolated mRNA, and created the cDNA from this mRNA. From this cDNA a 454 library was constructed and sequenced by the 454 FLX pyrosequencing technology.

DNA Extractions

DNA was extracted from black cherry leaf samples according to a modified version of the CTAB (Cetyltrimethylammonium Bromide) method described by Doyle and Doyle in 1990.

CTAB solution for extraction was made by combining 2% CTAB, 100 mM Tris-HCl (pH of 8), 25 mM EDTA (pH of 8), 2 M NaCl, 2% PVP (added separately before each extraction experiment), and 2% β-mercaptoethanol (added separately before each extraction experiment). Table 1 shows the specific recipe for CTAB used for these extractions. Frozen, ground samples were mixed with CTAB solution, heated, centrifuged, mixed with phenolchloroform, centrifuged, mixed with 24:1 chloroform:isoamyl alcohol, centrifuged, precipitated with isopropanol, centrifuged, and the DNA pellets were cleaned and quantified.

Table 1: CTAB Solution Contents	
CTAB	4 g
PVP	4 g
1 M Tris-HCl	20 mL
0.5 M EDTA	25 mL
5 M NaCl	80 mL
H_2O	To 200 mL

This Table shows the solutions and chemicals used to make our CTAB mixture, along with how much of each was needed to make 200 mL of CTAB.

PCR

SSR primers used for PCR were selected by analyzing the black cherry cDNA sequence generated previously.

PCR was performed using the same set of DNA samples to screen for primers which would amplify. Table 2 shows the PCR Master Mix used for each well of the PCR plate. Eight primers were tested on each plate. Each well contained 10 μl of the primer and PCR Master Mix combination. Each well also contained 1 μl of the DNA template used (from the black cherry extracted DNA). About 20-50 ng of DNA was present in each well (reaction). The PCR plates were each run with the settings of 94^{o}C for 4 minutes, followed by 35 cycles of 94^{o}C for 30 seconds, 60^{o}C for 45 seconds, and 72^{o}C for 45 seconds, then after these cycles were complete the plate was left at 72^{o}C for 7 minutes. These PCR plates were run on a GeneAmp PCR System

9700 made by Applied Biosystems. After running the PCR cycles, the plate was tested on an agarose gel by using gel electrophoresis to detect amplification of the primer sequence. Then, the primers which amplified were radioactively labeled and examined to see if they are polymorphic. The polymorphic primers were used on all the extracted DNA samples for PCR to identify full siblings. These PCR plates were taken to the Schatz Center's automated ABI3700 96- capillary DNA analyzer for high throughput genotyping.

Table 2: PCR Master Mix	
DEPC H_2O	620 μl
5x Buffer	220 μl
25 mM $MgCl_2$	160 μl
10 mM of each dNTP	5 μl of each
Hot Taq	4.85 μl

This Table shows the chemicals and ingredients of the PCR master mix made for each set of eight primers tested on one PCR plate.

RESULTS

For this project, we divided the families between a few laboratory members to perform the extractions and primer testing for each family. My experiment focused on extracting DNA from 600 samples from the MO-21 population of black cherry. These samples have been used to screen 100 SSR primers, with 76 showing amplification. The primers which showed amplification were tested for polymorphisms, and two were polymorphic. These primers were then used to screen the MO-21 population to find full siblings and build linkage maps based on these full siblings. Table 3 shows a list of the SSR primers which amplified and were polymorphic.

Table 3: List of Amplifying and Polymorphic Primers

Name	Sequence
Con-131	CCCAGATTACATCAGCCACC
contig654	AAGAGCTCACTGCAGCTTCC

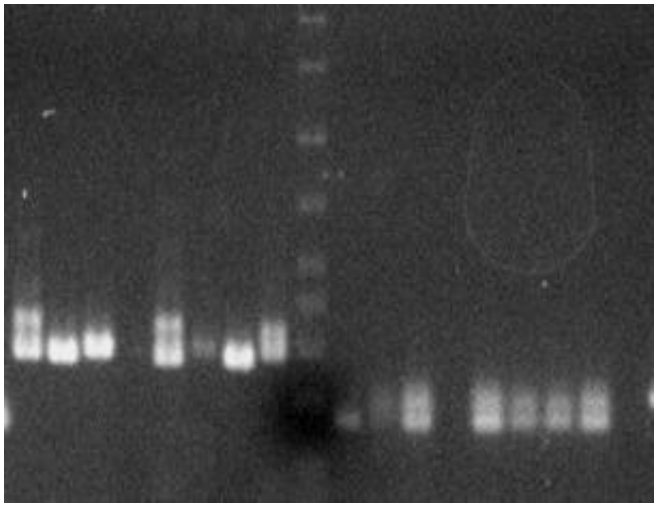
This Table shows the SSR primers used for PCR in this experiment to screen the population for full siblings. These primers amplified and were polymorphic in the black cherry population.

Myself and another Honors student, Daniel Devine, proceeded by testing 180 samples from the R-14 family by screening with SSR marker 131 to begin our full sibling and DNA fingerprinting analysis. Among these samples, 15% could not be identified because of low amplification and 10% were selves (identical to the mother). About 27% of the samples had the same profile. Three other pollinators had 14%, 10%, and 10% of the population identical to them. Three more pollinators had 5%, 6%, and 7% of the population identical to them. The rest of the pollinators had low percentages of the population identical to them.

Figure 1 shows the gel for marker 131, which is polymorphic. Figure 2 shows the GeneMapper analysis of this SSR marker and reveals seven different profiles (genotypes) found using this marker in the sample group used (180 individuals from R-14). The different band

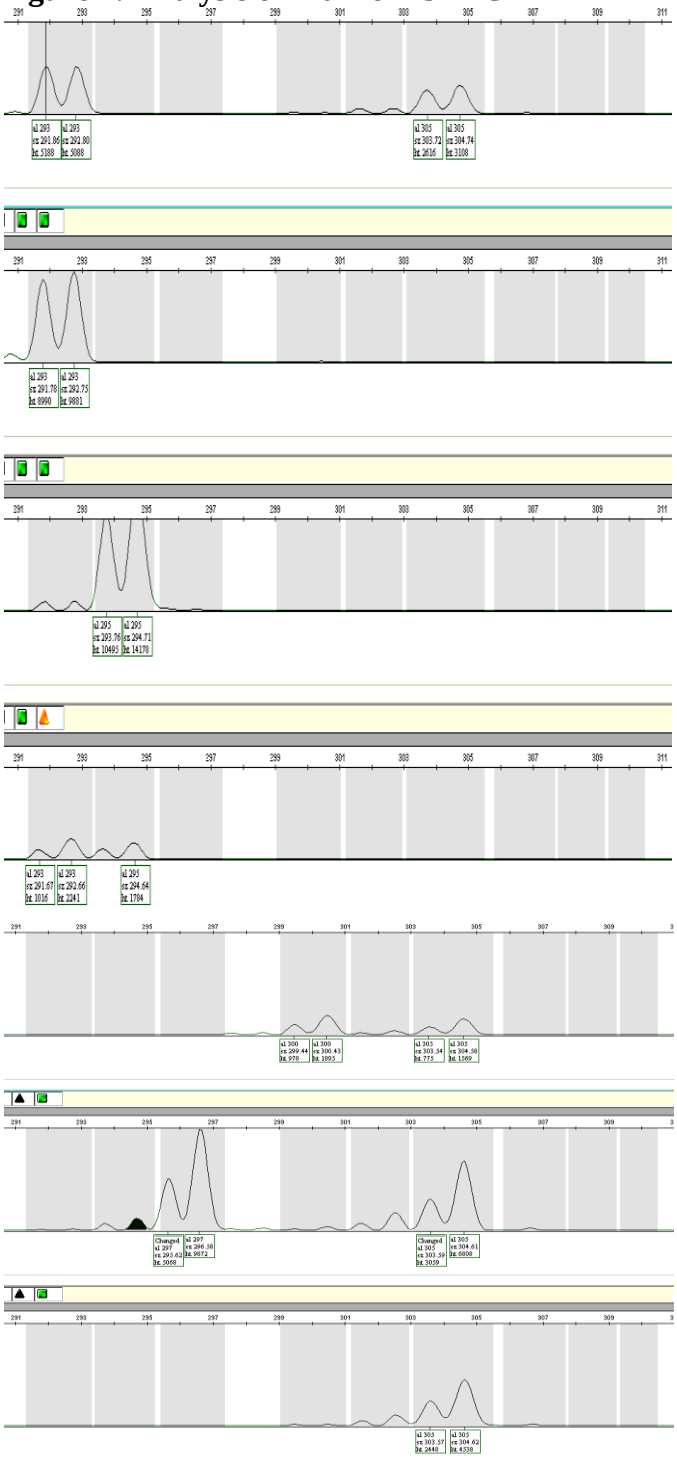
patterns (represented by peaks) reveal different genotypes. This analysis can be found in Figure 4 for another polymorphic marker, 654, showing it has six profiles (and its gel is shown in Figure 3). Figure 5 shows the gel for marker 1368 and Figure 6 shows the GeneMapper analysis revealing the same genotype in the sample group. Since the band patterns are all the same, the genotype is the same.

Figure 1: Gel electrophoresis of Marker 131 left and marker 855 right



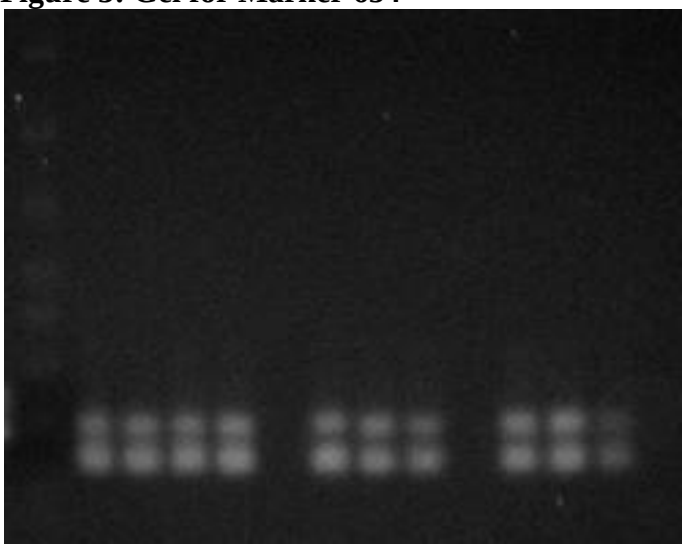
This figure shows the gel electrophoresis results of SSR marker 131 (on the left side) and 855 on the right side, both of which amplified and were polymorphic.

Figure 2: Analysis of Marker 131 PCR



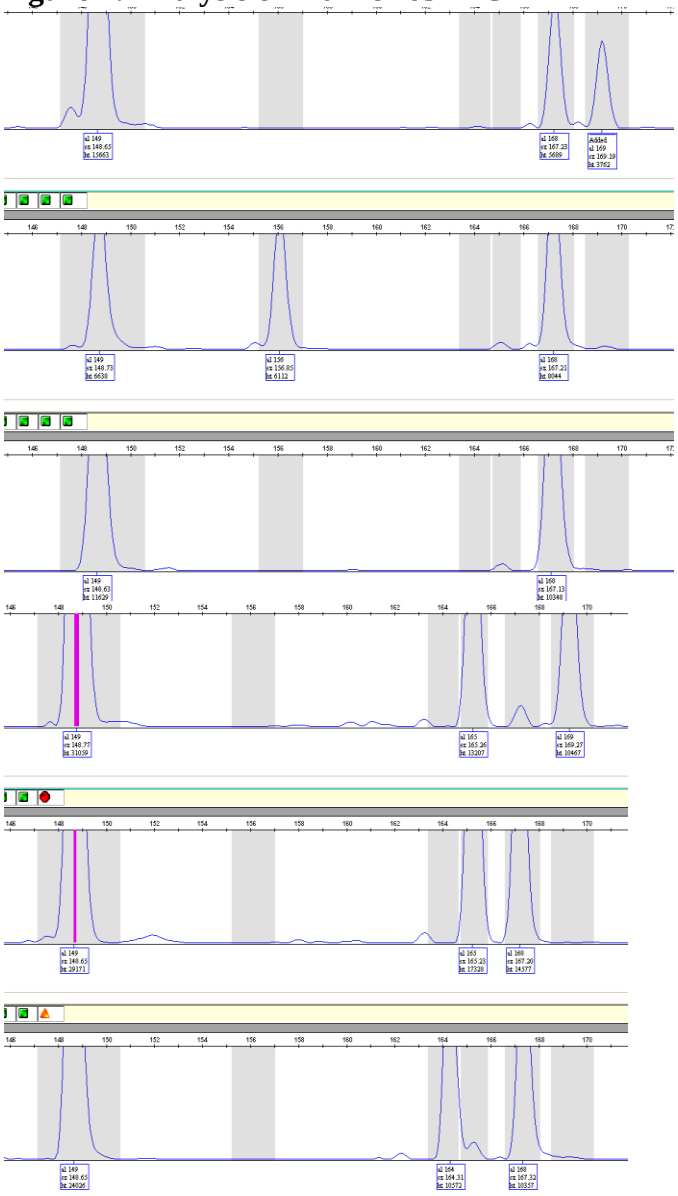
This figure shows the analysis from GeneMapper software showing seven different profiles and genotypes found in the population using SSR marker 131.

Figure 3: Gel for Marker 654



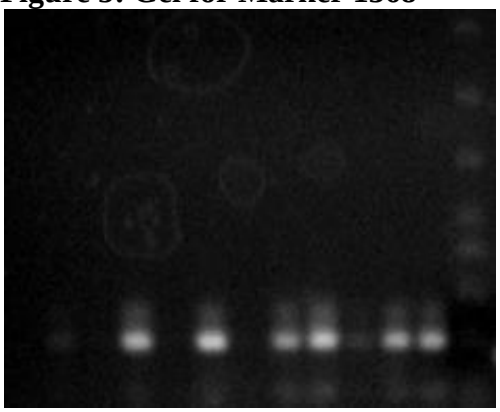
This figure shows the gel electrophoresis results of SSR marker 654 which amplified and is polymorphic.

Figure 4: Analysis of Marker 654 PCR



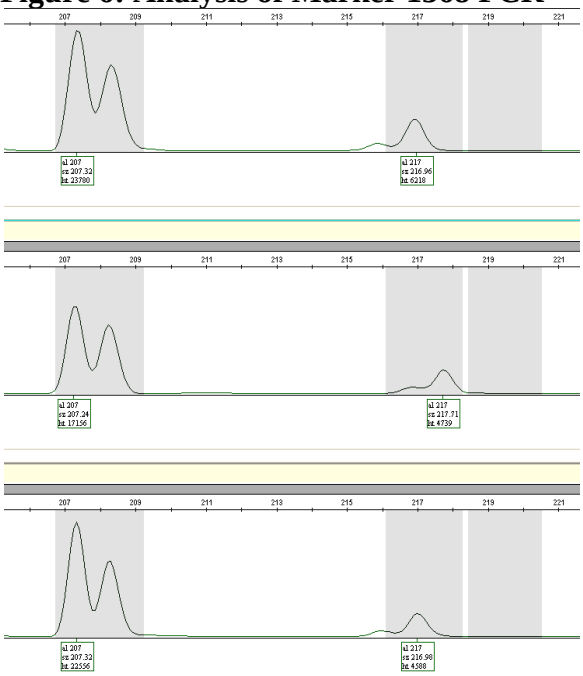
This figure shows the analysis from GeneMapper software showing six different profiles and genotypes found in the population using SSR marker 654.

Figure 5: Gel for Marker 1368



This figure shows the gel electrophoresis results of SSR marker 1368 which amplified and is not polymorphic.

Figure 6: Analysis of Marker 1368 PCR



This figure shows the analysis from GeneMapper software showing the same profile and genotype found in the population using SSR primer 1368.

DISCUSSION

Having identified these polymorphic SSR markers for our black cherry populations, we can continue screening the population to identify full siblings. With these full siblings, we will be able to develop linkage maps and use these maps to identify QTLs related to ozone stress in the *Prunus serotina* species. The QTLs we identify can be used to screen black cherry populations looking for individuals with genotypes similar to those we have found to be related to ozone tolerance. These genotypes can be used to grow more forests of black cherry with increased resistance to ozone stress and help sustain this tree species. Or, trees can be created with these genes and QTLs inserted to generate more viable trees.

Although this project has not yielded much in terms of results, we do have some to offer. We have been able to identify two polymorphic SSR markers to use to screen the black cherry population for full siblings (although we need four to eight more polymorphic markers to complete this part). Our test of 180 samples from the R-14 family by screening with SSR marker 131 showed 27% of the samples having the same profile, which means there is a preferred pollinator (father) in this part of the black cherry population. This was the highest percentage of samples having the same profile. Unfortunately, this does not tell us much because it is only one marker used on 15% of the population we hope to test. However, it could point to the major source of pollination for our population. As we find more SSR markers, we will be able to have a more informative and supported view of the full siblings and pollinations of this group of *Prunus serotina*.

Hopefully our results will not be hurt too much by poor DNA quality or concentrations. We encountered some trouble early on in our DNA extractions with consistently poor results (especially after Christmas break). However, after some research into DNA extractions, we

modified our protocol to keep PVP out of our CTAB mixture until the day we used the CTAB because of PVP's short bench life in solutions. This modification, as well as our increased experience, paid immediate dividends with high quality DNA and high concentrations. We have also not been able to find polymorphic primers as rapidly as we expected. There are a lot of markers which are yet to be tested for polymorphism, so hopefully ones we selected with similar repeats to the two polymorphic primers we already found will be successful as well.

By the end of this project, many genomics tools and resources for *Prunus serotina* will have been developed. New SSR markers for *Prunus serotina* will be developed which can be used in other *Prunus* species as well. The creation of linkage maps will be able to help other researchers and scientists identify and locate specific genes. Other scientists can use the tools generated by this project to look at the genome for black cherry to investigate QTLs related to other important characteristics (such as disease resistances, ripening, and plant growth). These tools can be used to continue studies on the black cherry and help protect it for the future.

Marker assisted selection (MAS) tools will be created from this project and can be useful for improving black cherry or selecting better seedlings. Other traits will be analyzed for QTLs, such as growth rates, adaptation to the environment, and wood quality. New genes for ozone response will be looked for by creating and using a *Prunus* microarray based on either the peach genome sequence or our *Prunus serotina* cDNAs. These new genes will be evaluated by using transgenic plants (with the specific genes identified) in the future to see if the genes are able to confer ozone tolerance to sensitive genotypes.

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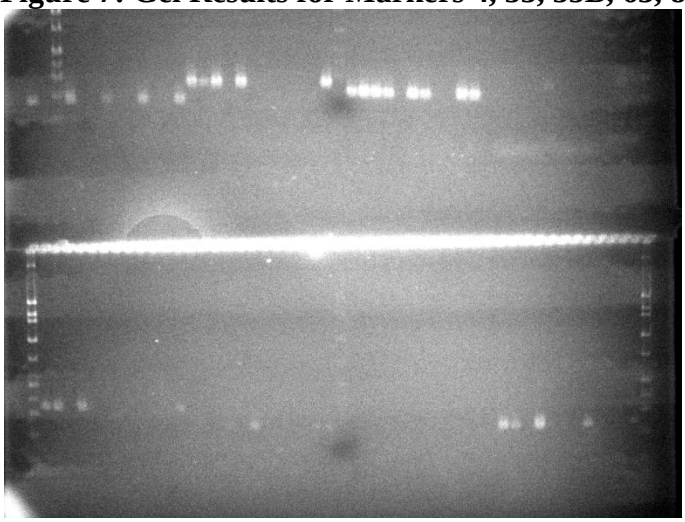
APPENDIX A

Table 4: List of SSR Primers that Amplified in Black Cherry	
Locus Name	Sequence
contig00004	AACCTAATTCCATGGCTCAAA
contig00011	CTCGAGCTCAACCTCAGACC
contig00033	AATGGTTTCAGTACCTGCGG
contig00035	CTCGGAACACCAATAGGCAT
contig00035b	CGACCGTGGAATTTCTTTGT
contig00084	ATTCCCCAACTTCACAGTGC
contig00113	AGCCAATAGACGACCACCAC
contig00131	CCCAGATTACATCAGCCACC
contig00168	ATTGAAGCCTTGTGCCTCAT
contig00371	ACAAGAACAAACAAACGCCC
contig00387	AGTCCTCGGTGGAGGAATTT
contig00412	AAAGGGCAAGCAAGTAGCAA
contig00424	GCCGTGGAGATGACGTAGTT
contig00497	TTCTGTTTGTTGGGTTTGTGA
contig00563	TTCGGATTGCCTAGTATTCCA
contig00654	AAGAGCTCACTGCAGCTTCC
contig00706	GCACAGTGACTTTTGATCAGC
contig00814	TTTCCCACTCGTCCAGAATC
contig00827	AAGGAAGACGCATCACCATC
contig00828	TGATTGGCCGTGTATTAGCA
contig00855	AGTTGAGGGGTGGGAATAGC
contig00855b	TGGTTCCTGGCACATGAGTA
contig00967	ATGCCCTCAAAACCTGACTG
contig01083	GCAGAGCAGTGATGCATTGT
contig01111	TTTTTCTCTGCCCCAAACAC
contig01201	TCTCTCTCCTCCCTCCTTCC
contig01272	GCTGAAGACGACGATGATGA
contig01308a	TGGAAGTGGTTGGAGGATTG
contig01368	CGGATTTGAACCGATGACTT
contig01373	GAGTTCGCAACCGAGTCTTC
contig01379	ATCTCCCTCCTGGGTGAAGT
contig01414	GAAAGCAAGCTTAGAGGCACA
contig01582b	CAACCTCCCCCTTCTCTCTC
contig01646	AATGGGTAGGCATTGCAGAG
contig01761	TGATGATTCTGAGGACATGGA
contig01772	AAGGGACGTTATCACGAACG
contig01920	AGGATGACGACGACGATACC
contig02161	ACCCACCCACCTCCTACATT
contig02167	GGCATGCAGAACAAAGGATT
contig02528	ACAAGGCTTGGGAGGTTTTT
contig02547	TTTTGTTTCTGGGTCCTGCT

contig02841	CAGCAATGGGATGACAACAG
contig02953	CCTCCAACCATTTGAGAGGA
contig03039	GTTTTTCCCAATCCTCCCAT
contig03204	TACCTCATCAGCAAATGCCA
contig03707	TTGGATTGCCAATAACCCAT
contig03780	TCCACATCTGTCCTGTCCTG
contig04043	GGGTTGTGAAACCCAAACTG
contig04321	TTCAGAAAGAAAATCAAGCGA
contig04518	GACCAACCCATTGAGCCTAA
contig04630	ACGCATACACACGCAGAGTC
contig04848	CCCTATAAGGGGTGTCCCTC
contig04899	AGCTGCCCAAACACTGAAAG
contig05045	GAGCTACCAAGGTCCAGTGC
contig05075	CCGTCAGCAAAAACTACAAGC
contig05302	GTCTGTCTGTGCTGTGGGTG
contig05317	TCACGCTGTGAAAAAGATCG
contig06758	AAAGGGAGGAGGTGCAGAGT
contig07005	GCCCACAACACAAGCCTTTA
contig07362	AAAAGTTCAAACCCGAAACG
contig07501	TCAATTCTTCCCCTCTGCCT
contig08061	CCAAAGGGGAATTACAGCAA
contig08280	CCCGCAGAGAGAGACATTG
contig08295	CAATTCTCTCTCCCAAGCCA
contig08518	CTGCTTCTCTCACACCTCCC
contig09884	CGAGAAGGACGAGGAAGTTG
contig10076	CTCGCTGAGGTTGGCTTAAC
contig10103	CCCTACCCTACCCCCTTTAAT
contig10841	ATATGTTTGCTTCTTGGCCG
contig10918	GGCTGATGGTAAAGCTGCTC
contig10924	CATCAGGAAGTGCACGAGAA
contig11204	ATTTCCATCTCCCCCATTTC
contig11232	GGGGCAGTATGTGCTTGAAT
contig11351	ATGGTCCAAAGCAAAGCATC
contig11605	GACCGATCTTTTTCATCCGA
contig11610	GGGAAGGCTGGTGATTATGA

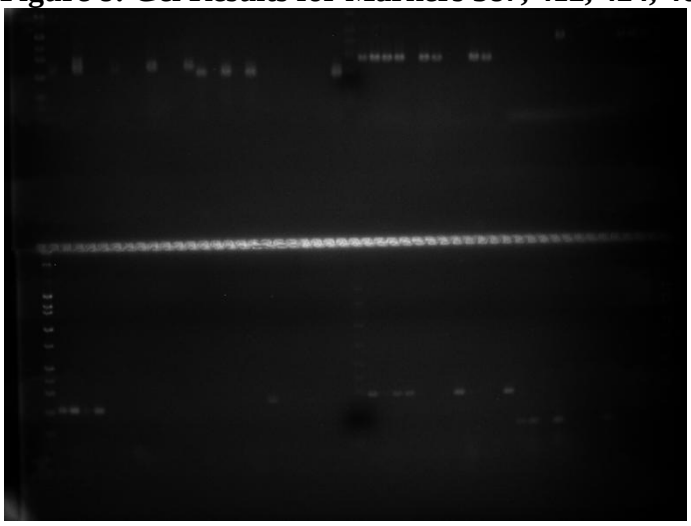
APPENDIX B

Figure 7: Gel Results for Markers 4, 33, 35B, 63, 84, 158, 316, 371



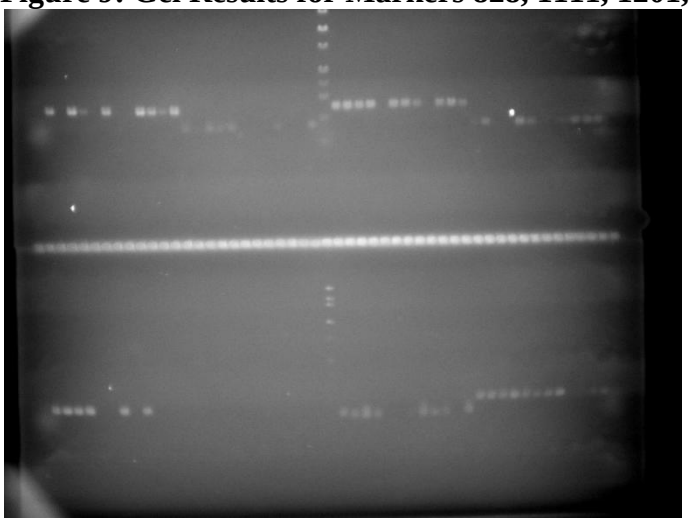
This gel shows the results of the PCR amplification for markers 4, 33, 35B, 63 (top row, left to right) and 84, 158, 316, and 371 (bottom row, left to right). The bands amplified at the expected size for markers 4, 33, 35B, 84, and 371.

Figure 8: Gel Results for Markers 387, 412, 424, 461, 497, 511, 706, 1308B



This gel shows the results of the PCR amplification for the markers 387, 412, 424, 461 (top row, left to right) and 497, 511, 706, and 1308B (bottom row, left to right). The bands amplified at the expected size for markers 387, 412, 424, 497, and 706.

Figure 9: Gel Results for Markers 828, 1111, 1201, 1308A, 1414, 1582A, 1582B, 1646

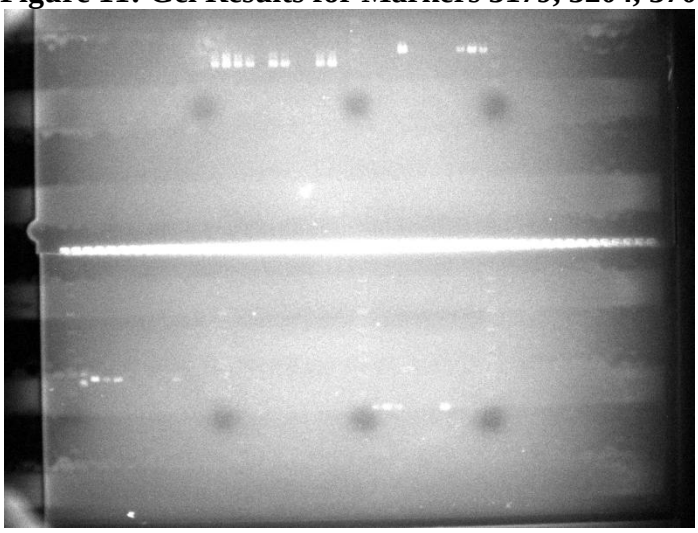


This gel shows the results of the PCR amplification for the markers 828, 1111, 1201, 1308A (top row, left to right) and 1414, 1582A, 1582B, and 1646 (bottom row, left to right). The bands amplified at the expected size for markers 828, 1111, 1201, 1308A, 1414, 1582B, and 1646.

Figure 10: Gel Results for Markers 1772, 1920, 2161, 2175, 2549, 2621, 2673, 2793

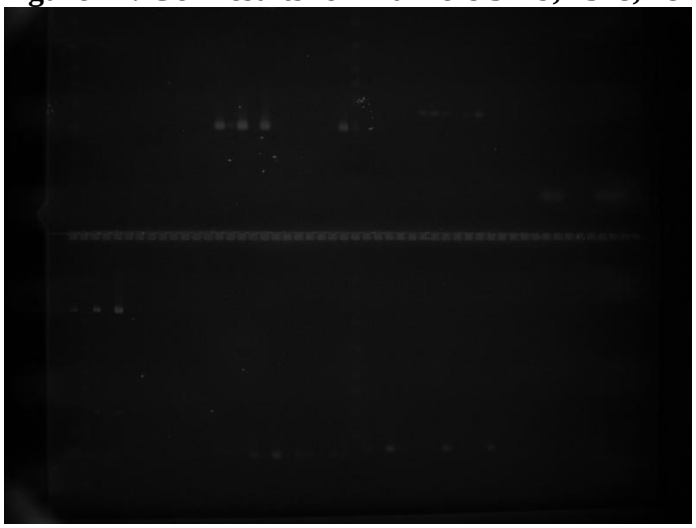
This gel shows the results of the PCR amplification for the markers 1772, 1920, 2161, 2175 (top row, left to right) and 2549, 2621, 2673, and 2793 (bottom row, left to right). The bands amplified at the expected size for markers 1772, 1920, and 2161.

Figure 11: Gel Results for Markers 3179, 3204, 3707, 3875, 4043, 4142, 4321, 4518



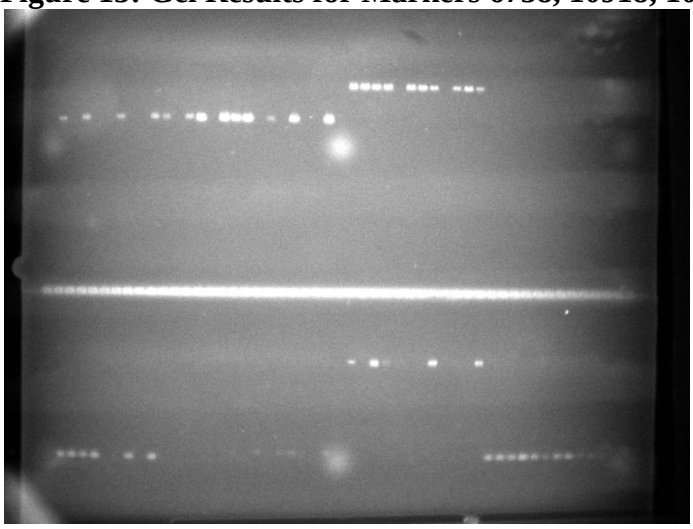
This gel shows the results of the PCR amplification for the markers 3179, 3204, 3707, 3875 (top row, left to right) and 4043, 4142, 4321, and 4518 (bottom row, left to right). The bands amplified at the expected size for markers 3204, 3707, 4043, 4321, and 4518.

Figure 12: Gel Results for Markers 3179, 4518, 4848, 5045, 5075, 5302, 5317, 5682



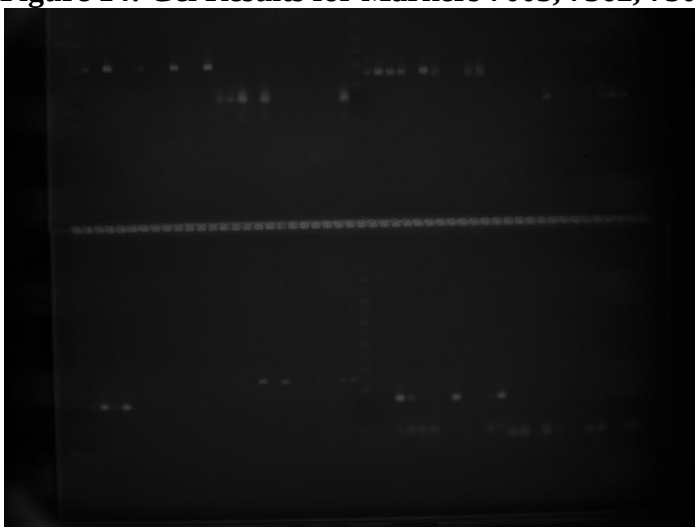
This gel shows the results of the PCR amplification for the markers 3179, 4518, 4848, 5045 (top row, left to right) and 5075, 5302, 5317, and 5682 (bottom row, left to right). The bands amplified at the expected size for markers 4518, 4848, 5045, 5075, 5302, and 5317.

Figure 13: Gel Results for Markers 6758, 10918, 10924, 11278, 11351, 11204, 11232, 11351



This gel shows the results of the PCR amplification for the markers 6758, 10918, 10924, 11278 (top row, left to right) and 11351, 11204, 11232, and 11351 (bottom row, left to right). The bands amplified at the expected size for markers 6758, 10918, 10924, 11351, 11204, and 11232.

Figure 14: Gel Results for Markers 7005, 7362, 7501, 7823, 8061, 8280, 8295, 8518



This gel shows the results of the PCR amplification for the markers 7005, 7362, 7501, 7823 (top row, left to right) and 8061, 8280, 8295, and 8518 (bottom row, left to right). The bands amplified at the expected size for markers 7005, 7362, 7501, 8061, 8280, 8295, and 8518.

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