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Intraspecific Hybrid Identification of Black Walnuts Via Marker Assisted Selection

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INTRASPECIFIC HYBRID IDENTIFICATION OF BLACK WALNUTS VIA MARKER ASSISTED SELECTION

A Master's Thesis

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Master of Science, Plant Science

By

Makenna Thompson

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ABSTRACT

The improvement of Eastern black walnut (*Juglans nigra* L.) cultivars could increase market expansion. Improved cultivars with superior qualities would increase profitability for both the seller and the buyer. Agronomically and economically important traits such as heavier nut weight, disease resistance, tree structure integrity, and yearly nut production can be efficiently improved by breeding black walnuts via marker-assisted selection. Microsatellite/simple sequence repeat markers were used in this research to determine potential intraspecific hybrids between 'Football' and 'Sparrow' cultivars. Intraspecific is defined as of the same genus and species. This quality makes it more difficult to identify hybrids because the parent plants DNA have similar base pair sizes. The two main goals for this research are to identify hybrid plants for the expansion of an existing mapping population and to optimize annealing temperatures of publicly available primer sets for the identification of additional polymorphic markers between 'Football' and 'Sparrow'. Of the 1,015 progeny plants tested, sixty-one were determined to be intraspecific hybrids. Those sixty-one new genotypes were added to the F_1 mapping population to a total of 237 plants at the Missouri State Fruit Experiment Station, Mountain Grove, MO 65711. Two hundred and eighty markers were tested for polymorphism and fifty-seven were detected as polymorphic for 'Football' and 'Sparrow' and kept for further assessment in future research.

KEYWORDS: marker-assisted selection, microsatellite, simple sequence repeat, black walnut, intraspecific, polymorphic

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Scientists are always looking for ways to improve crops. One-way scientists are trying to accomplish this improvement is plant hybridization. Hybridization of plant species for industrial purposes occurs all over the world. Hybridization has been purposed with increasing crop production, crop quality, plant vigor, increase economic value, and disease resistance (Thomas & Prindle, 2016). This experiment uses intraspecific hybridization for identification of 'Football' and 'Sparrow' progeny. Intraspecific hybridization is used between plants of the same genus and species (Schneider S.J., 2018). The idea of hybridization sounds simple, but putting the idea to practice can be difficult due to differing reproductive cycles of parent plants, pollination difficulties, unpredictable weather conditions, or species incompatibility.

Eastern black walnut (*Juglans nigra* L.) hybridization has piqued interest of black walnut growers, processors, and scientists as consumer demand for healthy snacks and fine wood quality have increased. Black walnuts have the highest valued lumber and can bring as much as $10,000 per tree (Mallik *et al.,* 2002; Nicolescu *et al*., 2021). They are also known for their unique bold nut flavor and nutritional nut meat.

Black walnut trees can reach commercial quality nut production by twelve years old, but peak quality nut production is reached at thirty years and can continue for another century (Schlesinger & Funk, 1977). The nut has been recently studied for its medicinal purposes and health benefits, specifically anti-inflammatory compounds, antioxidant properties, and antibacterial activity (Ho *et al*., 2019; Vu *et al*., 2020; Wenzel *et al.,* 2017). Many people pick up walnuts and take them to a hulling and processing center. Here they get paid after the nuts are hulled. Black walnut trees are native so the nuts can be harvested by the owner of the land the

tree is on (Rorabaugh *et al.,* 2011). Improved variety walnuts will bring more money because they are of high quality. Picking up walnuts is an easy way to make some money.

Black walnuts are important to Missouri, so researchers are searching for ways to improve black walnuts and their cultivars. But black walnut trees only grow in certain conditions, such as well drained soils, areas with limestone, with a warm and mild climate (Burns *et al.,* 1990). These conditions make Southwest Missouri adequate for black walnut production. Common diseases that attack black walnut trees are anthracnose, thousand cankers disease, and walnut twig beetle (Audley *et al.,* 2017). Thousand cankers disease has not yet been found in Missouri. Improving the overall quality of black walnut species will benefit industry and profit for localized places where the species grows. To improve the trees, plants can be bred or grafted to improve wood quality, nut quality, and disease resistance.

It is nearly impossible to crossbreed walnut trees by hand, so parent trees 'Football' and 'Sparrow' have rows parallel to each other where the seeds were collected. Black walnuts are monoecious plants which decreases the likelihood of self-pollination (Burns *et al.,* 1990). Therefore, we rely on the occurrence of natural hybridization between the parent species (Cseke *et al.,* 2021). Identification via DNA sampling would not be necessary if the species could be manually crossbred. But they are a wind pollinated species, making DNA extraction necessary. Because we are not pollinating the plants by hand, every new seedling will have a different physical and cellular composition (Crystal *et al*., 2016; Ebrahimi *et al.,* 2018).

In order to improve the hybrid black walnut, the hybrid cultivar must first be studied. This can be difficult due to the intraspecific species 'Football' and 'Sparrow'. Intraspecific means that these plants are of the same genus and species. Parental populations of black walnuts

vary from intraspecific hybrid populations in seed weight, yield, shell percentage, and days to flowering (Savaliya *et al.,* 2009).

Cultivars ‘Football’ and ‘Sparrow’ have shown promise in improving production. ‘Sparrow’ is selected for its resistance to anthracnose, thin and dry hull, annual nut production, and low kernel percentage (Schneider *et al.,* 2019; Land, 2019; Reid *et al.,* 2004, 2009). However, it does have a few setbacks. ‘Sparrow’ is known to be more susceptible to tree damage. This includes breaking of branches due to the weight of walnuts hanging from the limbs, frost damage, and lower structural integrity (Schneider S.J., 2018). Black walnut trees are most susceptible to weather damage when they are seedlings (Schlesinger & Funk, 1977). Favorable qualities of species ‘Football’ are strong limb and trunk strength as well as a heavier nut weight (Scheider *et al.,* 2019; Reid *et al.,* 2004, 2009). Less desirable qualities of ‘Football’ are alternate year harvest season, high susceptibility to anthracnose, thick and fleshy hull, and high kernel percentage (Warmund & Sambeek, 2014; Scheider *et al.,* 2019; Reid *et al.,* 2004, 2009). Black walnut species vary in nut producing schedules such as every year production, every other year, or no patterned production (Schlesinger & Funk, 1977; Hall *et al.,* 2019; McGranahan *et al*., 1988). The goal is to optimize crossing these two cultivars to create a higher yielding and an overall better performing tree.

In turn, the intraspecific markers and alleles are so closely related between ‘Football’ and ‘Sparrow’ that it can be hard to determine if the potential hybrid plant is a true hybrid or more similar to one of its parental plants (Contreras *et al.,* 2019; Darvhanker *et al.,* 2019). In this experiment we are hoping to identify true hybrid progeny from ‘Football’ and ‘Sparrow’ via marker assisted selection. The experiment is also looking at identifying more markers at

optimum annealing temperatures to aid in the hybrid identification. After identifying markers annealing temperatures, the markers will be tested for polymorphism.

Simple sequence repeat (SSR) markers allow for quick identification of hybrids because of the repetitive DNA sequence and have a one to six base pair repetitive sequence (Shailja *et al.,* 2020; Mason, 2015)(Appendix A). SSR markers have a high rate of polymorphism and can detect the changes in allele variation (Mason, 2015). Alleles are the variation of a gene. SSR is also a PCR (Polymerase Chain Reaction) based process. These qualities of SSR markers are why they are used for identification process in this black walnut hybrid identification research.

To identify the intraspecific hybrids, polymorphic markers are needed to identify the correct parental DNA markers (Bos *et al.,* 2003). Polymorphism allows for markers to be identified at a marked location in a DNA sequence. Polymorphic markers of parental DNA are important to identifying intraspecific hybrids (Bos *et al.,* 2003). Polymorphic markers allow a DNA sequence to be linked to a specific phenotype. Phenotypes of black walnuts that may be linked to a specific marker in the DNA sequence could include nut weight, hull thickness, and moisture content.

The two main objectives of this study are to identify intraspecific hybrid seedling progeny of ‘Football’ and ‘Sparrow’ from seedlings and expand the hybrid mapping population, as well as optimize primer annealing temperatures and identify polymorphic markers. Annealing temperatures bind DNA strands together based on the melting temperature. Melting temperature is based on primer length and nucleotide composition. Publicly available markers are used to identify proper annealing temperature and polymorphic markers. The sequence of these markers is known by the annealing temperature is not recorded in the public record. Marker assisted selection and DNA amplification are used to aid in the identification of these hybrid plants (Pang

et al., 1970; Yıldız *et al.,* 2014). The newly identified optimum polymorphic microsatellites will be used as markers to create a mapping population of black walnuts (Woeste *et al*., 2002).

Developing a hybrid cross between these two parents could help solve production and disease problems. Harvest for both species is from October to November in Missouri. Identification of intraspecific hybrids and a larger marker identification range will enable further research of 'Sparrow' and 'Football'. A larger mapping population will be created with the identified hybrids, allowing for further research in areas such as yield, disease resistance, nut quality, tree quality, and overall plant health. The seedlings will be planted in an open field and monitored at the Missouri State Fruit Experiment Station.

The study is also purposed with furthering the research in intraspecific hybrids because this area has not been heavily studied. This experiment will serve as the baseline research for future projects relating to 'Football' and 'Sparrow' hybrid experiments. The combination of creating a mapping population of the identified hybrid plants and optimizing annealing temperatures for identifying polymorphic markers for the identifying of future black walnut 'Football' and 'Sparrow' hybrid plants creates a foundation for research on linkage mapping, disease resistance, nut production, etc.

METHODS AND MATERIALS

Nut Collection and Cold Treatment

Black walnut samples were collected under the female parent tree 'Football' of trees numbered 1,3, and 5 located at the University of Missouri Southwest research facility in Mount Vernon, Missouri, 65712, 37.08533 latitude, -93.86942 longitude (Figure 1) of 2021 and 2022. There are four rows of 'Sparrow' trees located to the West of the 'Football' trees and one row of 'Sparrow' located to the East. Approximately a total of 1,200 and 1,100 walnuts were collected in October 2021 and October 2022, respectively. Nuts were hulled on site of the collection, and they were then buried underground for stratification treatment.

A three-inch layer of nuts was placed in a partially buried tubs (9-12" underground) with soil layering the top and bottom of the nuts for the stratification process. Soil covered the bottom of the tubs before nuts were layered in the tub. A three-inch layer of nuts was placed on top of the soil. Each walnut layer was covered with soil. Each tub had two to three layers of walnuts.

The tubs have holes in the bottom to allow water to drain during the stratification process. The holes ensure that the walnuts are not sitting in water. Without the holes in the tubs, the walnuts could become rotten and molded, which may kill the nuts and decline germination rates.

The stratification process to break the dormancy cycle is particularly important because black walnuts will not produce a new plant from the seed without the cold treatment of stratification. Nuts were buried underground for approximately 100 days for cold stratification and were dug up from the tubs they were buried in. The black walnut seeds were allowed to dry out for a few days to get rid of any excess moisture.

Planting

Seeds were stored in Karls Hall Lab Missouri State University. Before planting, black walnut seeds were tested for viability by placing them in a bucket of water. If the seed sinks, the better chance of germination. When seeds were sorted, non-viable seeds were thrown away. Non-viable seeds were moldy, floated on top of the water when tested, had little to no weight, or were hollow.

The viable black walnuts were planted in 4" pots with potting soil in the greenhouse at Karls Hall Missouri State University. Jolly Gardener Pro-line C/25 growing mix and Jolly Gardener Pro-Line HFC/B HydraFiber advanced substrate growing mix was used as planting media in 2022 and 2023, respectively. Soil was wetted before filling pots to ensure there was enough soil to cover the seeds. When the soil was not wet, the dry soil would absorb the water and expose the seeds and the pots would have to be refilled with soil. The potting soil was filled a few inches above and below the seed.

After soil was added above the seed, the soil was packed down. The pots were not filled to the brim with soil but allowed a few inches for a water line. Seeds were watered daily while in the Karls Hall greenhouse. It took six to eight weeks for black walnut seedlings to germinate.

Leaf Sample Collection and DNA Extraction

Leaf samples were collected from germinated plants when there were at 4-5 leaf stage per plant. Leaflets were taken from the 2nd or 3rd leaflet, not the first leaflet. The 2nd or 3rd leaflet provides for higher quality deoxyribonucleic acid (DNA). Each leaf sample was marked with a tag to identify the sample taken. Tags were placed in the pots with the sample number, date, and experiment name for identification purposes. Tag numbers corresponded with sample numbers

written on a Ziplock bag containing the leaf sample. Sample numbers were not changed throughout the experiments and increased numerically.

Use OPS DIAGNOSTICS Protocol SYNERGY 2.0 Plant DNA Extraction Kit for DNA extraction (OPS DIAGNOSTICS). 50 milligram leaf tissue sample was added to the bead beater tubes along with 500 microliters of Plant Homogenization Buffer. Our bead beaters were set to 400 x 100 speed for 120 seconds for homogenization. 200 µl of supernatant was taken and transferred to a new microfuge tube. Before the DNA extraction begins, the incubator is set to 37°C. Setting the incubator before beginning the DNA extraction allows ample time for the incubator to warm up before it is needed. All other steps of protocol were followed as directed. After the extraction procedure was done, 1 µl of DNA sample was measured the concentration and the purity evaluated by the ratio 260/280 and 260/230 via Thermo Scientific Nanodrop Spectrophotometer 2000. These numbers 260, 280 and 230 correspond to the absorbance at the wavelengths 230 nm from salt and other contaminants, 260 nm from nucleic acid, and 280 nm from protein. The optimal 260/280 and 260/230 ratios are 1.8 and 2.0-2.2, respectively. The concentration of each DNA was normalized to 5 ng/µL for the PCR reaction.

Intraspecific Hybrid of 'Football' and 'Sparrow'

The true hybrid identification was determined via PCR Amplification and DNA fragment analysis. The PCR machine amplifies segments of DNA. Seven known polymorphic SSR markers of 'Football' and 'Sparrow' were used to test for true hybrids (Table 1 and Table 2). Each PCR reaction had a total volume of 9.2 µL including 2 µl of 5 ng/µL of the DNA, 0.8 µL of 2 µM WellRed M13 primer, 0.8 µL of 0.1 µM of forward primer and 0.8 µL of 2 µM of reverse primer, 0.2 µL of 25 mM $MgCl_2$ and 4.6 µL AmpliTaq GoldR 360 Master Mix buffer (Life

Technologies, Grand Island, NY). Each primer set was added with an 18-base pair of M-13 primer with D2, D3, or D4 WellRed fluorescent dyes (Table 2). D2 is represented as black peaks. D3 is represented as green peaks and D4 is represented as blue peaks in capillary electrophoresis. These different color dyes of M-13 WellRed primers added were used to facilitate the multiplexing of the PCR products for the DNA fragment analysis in capillary electrophoresis.

PCR reaction for this study used: phase one, one cycle of an initial denaturation stage at 95°C for 10 minutes, phase 2, with 40 cycles, denaturation stage at 95°C for 30 seconds, annealing stage at 60°C for 1 minute and Extension stage at 72°C for 1 minute; phase 3, 1 cycle of Extension stage at 72°C for 7 minutes.

Then the PCR product was verified via agarose gel electrophoresis to ensure PCR worked correctly. 1.5 percent of 150 milliliter agarose gel was prepared in the BioRad Sub-Cell GT machine. The agarose and TBE (Tris Borate EDTA) buffer were heated and then added Gel Red to the clear liquid mixture. Gel Red should be added when the temperature is around 55 °C. If the liquid is too hot, it will degrade the Gel Red and make it less reliable. Gel Red is a fluorescent nucleic acid stain.

Then we poured liquid into the gel bed with the well combs in place. After liquid solidified, we gently took out the well combs. We then added TBE into the electrophoresis bed and placed the gel inside the electrophoresis bed. Make sure the gel and wells were completely submerged in TBE before loading samples. DNA samples were loaded from left to right. We placed the DNA samples with the top loading wells closest to the negative, black colored end. The red end is positive. Since DNA is negatively charged, the gel will run towards the positive

red end. This experiment used a 100 bp DNA ladder to identify banding. We ran the gel in electrophoresis at 100 Voltz for 45-90 minutes.

After electrophoresis had run, we then placed the gel under the Gel Doc-It Imager. The VisionWorks program captured an image. We determined a rough estimate of base pairs retained from PCR based on the imaging. We were looking for bright bands on the gel with similar base pairs in both 'Football' and 'Sparrow'. The PCR concentration was estimated by the intensity of each band in the gel image, and the amount of samples were diluted with water based on the intensity for capillary electrophoresis.

After capillary electrophoresis was run, the allele sizes of the seven primer pairs were used to determine intraspecific hybrids (Table 1 and Figure 2). The aim is to have a perfect hybrid with seven matching marker set alleles in capillary electrophoresis, however, if more than five primer sets matched, the plants were kept. If the plant had less than five allele size pair matches, it was thrown out as well as its extracted DNA.

Primer Screening and Optimizing Annealing Temperatures for Identifying of Polymorphic SSR Markers of 'Football' and 'Sparrow'

One of the most powerful techniques applied in molecular biology is DNA amplification by PCR reaction. Optimization of PCR entails testing several variables. Optimizing the annealing temperatures of the primer is one of the effective approaches to increase PCR yield and specificity.

An annealing temperature is the temperature that the PCR primers bind to complementary template region of the target DNA. If the annealing temperature is too low, primer will anneal to sequence other than the intended target and this can lead to nonspecific PCR amplification.

However, if the annealing temperature is too high, there will be no primer binding to complementary DNA.

There are 939 SSR available from an open-source database from Hardwood Genomics Project https://www.hardwoodgenomics.org/organism/Juglans/nigra?tripal_pane=group_predicted_ssrs_genomic (Hardwood Genomics). 286 sets of these primers were screened.

The strategy used to determine the polymorphic SSR markers was adapting the PCR reaction condition by starting the annealing temperature at 60˚C to test both 'Football' and 'Sparrow'. The PCR products were visualized using gel electrophoresis, and clear bands for both parents were kept and continued for DNA fragment analysis via capillary electrophoresis to detect exact allele sizes (Figure 3). Those primers showed without clear or with multi band patterns on the gel were carried on for the next screening step.

For primer screening and optimizing annealing temperatures, the same PCR protocol was used for hybrid identification but adjusted annealing temperatures with veriflex steps. Veriflex is designed as a temperature gradient PCR block which can be run on many samples with various temperatures simultaneously in the same PCR machine. Every primer was testing annealing temperature range from 49-60 ˚C by using the gradient Veriti Thermal Cycler, a 96 well-fixed block PCR equipped with 6 VeriFlex zones for testing various annealing temperature (Thermo Fisher, Scientific).

Annealing temperature corresponding with the clearest band from gel electrophoresis was recorded regarding as optimum temperature for that marker. After optimum annealing temperatures were determined, primer sets were run through capillary electrophoresis to record the exact nucleotide allele size for the polymorphic marker set (Figure 4).

RESULTS

Intraspecific Hybrid Identification

One hundred and thirty and 885 seeds germinated from 2021 and 2022 planted black walnut in the Karls Hall green house, respectively. The germination rate was 12% for the year 2021 and 80% for the year 2022. Using the seven polymorphic SSR markers of 'Football' x 'Sparrow', there are 16 out of 130, 12%, and 45 out of 885, 5%, of germinated plants are intraspecific hybrids for the year of 2021 and 2022, separately. In total, 61 hybrids were identified for these two years. These intraspecific hybrids will be added to the population already planted in Mountain Grove. Hybrid plants were kept in the greenhouse and will later be planted at the Missouri State Fruit Experiment station, Mountain Grove, MO 65711, latitude 37.19749, -93.27925 longitude. These intraspecific hybrids will be added to the current mapping population of 176 plants in Mountain Grove.

Markers and Annealing Temperatures

Two hundred and eighty primer pairs from the Hardwood Genomics Project have been tested for optimum annealing temperatures, ranging from 49°C to 60°C. PCR results were visualized via gel electrophoresis imaging. Two hundred and seventy out of 280 primers demonstrated various band patterns in the gel image based on their annealing temperature settings. Each temperature that corresponded to the clearest band for both 'Football' and 'Sparrow' was regarded as the optimum annealing temperature for each of the individual primer sets. If only one band or no bands appeared on the gel for one of the cultivars, these primers were no longer evaluated.

Primer pairs with optimum annealing temperatures were run through capillary electrophoresis to determine the exact allele sizes for the markers. The optimal annealing temperatures and their base pair sizes of each primer were recorded and summarized (Appendix B).

Polymorphic Markers of 'Football' x 'Sparrow'

Fifty-seven out of 280 markers appeared with different allele sizes between 'Football' and 'Sparrow', and these were considered polymorphic markers. All allele sizes of the 280 markers were recorded (Appendix C-1). The identified polymorphic markers and their forward and reverse primer sequences were recorded (Appendix C-2). The genetic marker has two identical allele sizes called homozygous, and by contrast, the marker has different allele sizes called heterozygous. Among of these polymorphic markers, 17 are heterozygous for both 'Football' and 'Sparrow'. Twelve markers are homozygous for 'Football', but they are heterozygous for 'Sparrow'. Fifteen markers are homozygous for 'Sparrow', but heterozygous for 'Football'. Thirteen markers are homozygous for both 'Football' and 'Sparrow', but with different allele sizes for each parent. All of these markers will be considered for future use.

These newly detected polymorphic markers not only can be pooled to the current collection of markers for identification of 'Football' and 'Sparrow', but they will be, more importantly, the foundation material for linkage map construction.

DISCUSSION

Seedlings Germination

Seedling germination rates of nuts from the 2021 collection was twelve percent and the 2022 collection was eighty percent. The germination rates varied between these two years may be due to many potential reasons. One of the possibilities was the quality of seed production of 'Football', which produces every other year, and it could have had bad seed crop in 2021. Another potential reason for the low germination rate for the 2021 collection was that the seeds were in storage, 6 months, for too long. Instead, seeds collected in 2022 were planted the day after they were dug up from the stratification tubs in 2023. Different potting soils used could also cause the germinate rate differences. The Jolly Gardener Pro-line C/25 growing mix type of potting soil was used for 2021 which might be not optimal for walnut seedling growth; as opposed to, Jolly Gardener Pro-Line HFC/B HydraFiber advanced substrate growing mix potting soil was used for the year 2022 collection.

In addition, there was a fungus gnat problem with the potting soil in the Fall of 2022 that could have also contributed to the low germination rate. Although, other studies have shown a wide range of germination rates within the same group (McGranahan *et al*., 1988).

Identified Hybrid Plants

Low numbers of true intraspecific hybrid plants could be due to the unpredictability of wind pollination. The reproductive overlap between 'Football' and 'Sparrow' has only twelve to fourteen days between the male and female cycle (Reid *et al.,* 2004). The small window of

reproductive overlap does not allow for hand pollination, which is one reason for reliance on wind pollination.

The sixteen identified hybrid plants from the 2021 sample were kept in the Karls Hall greenhouse and repotted all plants to bigger pots months after they were identified as hybrids. During this time, some of the plants defoliated due to a pest infestation. The plants developed symptoms of pests during December 2022. The issue was identified as whiteflies and the insecticide Conserve was used to get rid of the pests on the remaining healthy plants. All the hybrid seedlings from the 2021 sample defoliated and it is unknown if the plants will reproduce leaves. Some of the defoliated plants are beginning to grow new buds.

The hybrid seedlings identified in 2023 were kept in the Karls Hall greenhouse. There was a smaller pest problem for the 2022 sample. Fugus gnats were still an issue but were less prominent in 2023 than they were in 2022. No other pests were identified.

Optimizing Annealing Temperatures

Optimizing the annealing temperatures of the publicly available markers was one of the effective approaches to increase PCR yield as well as specificity. The list of optimum annealing temperatures was created via PCR amplification at veriflex steps. Although, annealing temperature of primers can be estimated based on the length and composition of the primer sequence. The annealing temperature is also estimated to be 5°C lower than the primer melting temperature. However, after calculating the annealing temperature based on these parameters, it was concluded that the actual optimum annealing temperature and calculated annealing temperature did not match up for all the primers. The calculations for annealing temperature were an estimate and were not always exact. There were also some primers that worked at

multiple annealing temperatures. Therefore, screening several annealing temperatures of each primer via the PCR with VeriFlex Blocks helps to uncover more polymorphic markers for our study.

Future Goals

One future goal is to create a linkage map which shows the relative location of genetic markers on a chromosome and it can be a fundamental implement for genetic study. With the data collected, expanding the mapping population, and identifying more polymorphic markers, could be contributed to the linkage map construction. There is not yet a linkage map for black walnut species 'Football' and 'Sparrow'. One of the big challenges is that both parents are the same species, so many SSR markers show identical allele sizes, which hinders the ability to have informative polymorphic makers. New marker strategies, for example single nucleotide polymorphism (SNPs), may be used to help make these processes easier and more efficient.

Other future goals for this project include growing the hybrid mapping population and creating a linkage map for black walnut species 'Football' and 'Sparrow'. Insights to improve the next round of research is to plant black walnut seeds shortly after digging them up from stratification to ensure higher germination rates. Also, to increase germination rates, use Jolly Gardener Pro-Line HFC/B HydraFiber advanced substrate growing mix instead of Jolly Gardener Pro-line C/25 growing mix as soil media for potting seeds.

CONCLUSION

Hybridization is an important concept in crop improvement. Black walnut intraspecific hybridization could improve economic profitability, nut production, nut quality, disease resistance, and tree structural integrity. This experiment found sixty-one intraspecific hybrid seedlings from the collected samples. These seedlings will be planted in Mountain Grove, Missouri with the rest of the hybrid population and will be allowed to grow until they are mature enough for further research experiments. Fifty seven of the 280 primers have been identified as polymorphic primers for ‘Football’ and ‘Sparrow’ black walnut species.

The study was purposed with furthering the previous research in intraspecific hybrids because this area has not been heavily studied by the plant science community. The combination of expanding a mapping population and identifying polymorphic markers with optimum annealing temperatures for ‘Football’ and ‘Sparrow’ are an important asset for black walnut community. It will serve as a foundation for breeding research relating to ‘Football’ and ‘Sparrow’ species for many important traits such as disease resistance, nut production, and other traits of horticultural interest or significance.

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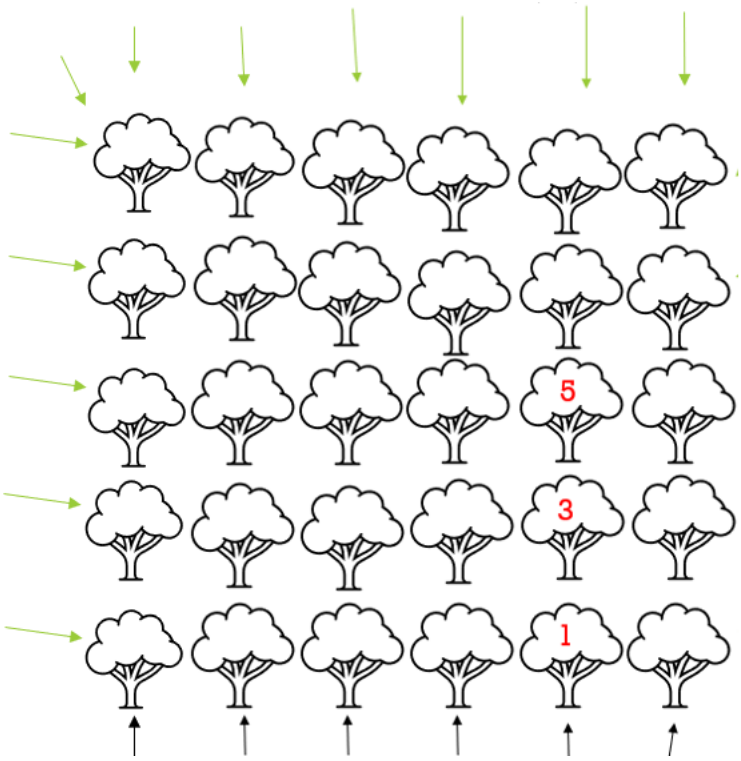
Table 1. Seven known SSR markers with allele sizes of 'Football' and 'Sparrow' listed. Each allele size with M13-tailed 18 base pairs is included.

WLLC	'FOOTBALL': ALLELE SIZE (BP)	'SPARROW': ALLELE SIZE (BP)	ORIGINAL RESOURCE
WLLC 605 + 606	148/150	152/152	Juglans_nigra_120313_comp29438_c0_seq2_ssr1687
WLLC 697 +698	184/184	181/181	Juglans_nigra_120313_comp26530_c0_seq2_ssr51
WLLC 933 +934	262/268	264/264	Juglans_nigra_120313_comp31676_c0_seq2_ssr1413
WLLC 1283 +1284	353/359	355/357	Juglans_nigra_120313_comp26249_c0_seq2_ssr361
WLLC 353 + 354	231/233	227/229	Juglans_nigra_120313_comp21665_c0_seq1
WLLC 497 + 498	282/284	278/280	Juglans_nigra_120313_comp32364_c0_seq3
WLLC 1305 +1306	344/344	350/350	Juglans_nigra_120313_comp13427_c0_seq1_ssr91

Table 2. Seven primers used for this experiment with their forward and reverse primer sequences as well as the original source for the primer sets.

Name in Lab	Forward Primer	Reverse Primer	Original Resource
WLLC 605 + 606	TTGTGTTTACGCAGCAAGGC	CGTCTTCCACTCTGCATTTGC	Juglans_nigra_120313_comp29438_c0_seq2_ssr1687
WLLC 697 +698	ACATGGATGAGGGCAAGAGC	AAACCCAAGACCCTCAGTGC	Juglans_nigra_120313_comp26530_c0_seq2_ssr51
WLLC 933 +934	AATTTGCCTTTGGATGGCGC	ATGCCACATGAGCCAAATCC	Juglans_nigra_120313_comp31676_c0_seq2_ssr1413
WLLC 1283 +1284	TCAACAGGCACAGGACTTCC	TAAGGAAGAGCAACGTCGGG	Juglans_nigra_120313_comp26249_c0_seq2_ssr361
WLLC 353 + 354	TGTGTGCGTACTTGATGGGC	ACTCCTCGGGAATTCTCAACC	Juglans_nigra_120313_comp21665_c0_seq1
WLLC 497 + 498	TGCTTTCTCAGAATCCAAACGC	TGTGAAGGCTTGTGGATGGG	Juglans_nigra_120313_comp32364_c0_seq3
WLLC 1305 +1306	ACTGGAACGTCGTTTCCTGC	ATTCGTTCTTGTTGCTGGCG	Juglans_nigra_120313_comp13427_c0_seq1_ssr91

Wind direction: N, NW, W



Sparrow Sparrow Sparrow Sparrow Football Sparrow

Figure 1. Black walnut collection site and parent cultivars. Nut samples were collected under parent tree ‘Football’ from trees 1, 3, and 5. Black arrows are pointing towards the parent trees ‘Football’ (F) and ‘Sparrow’ (S). The green arrows signify the wind direction, commonly coming from the North and West.

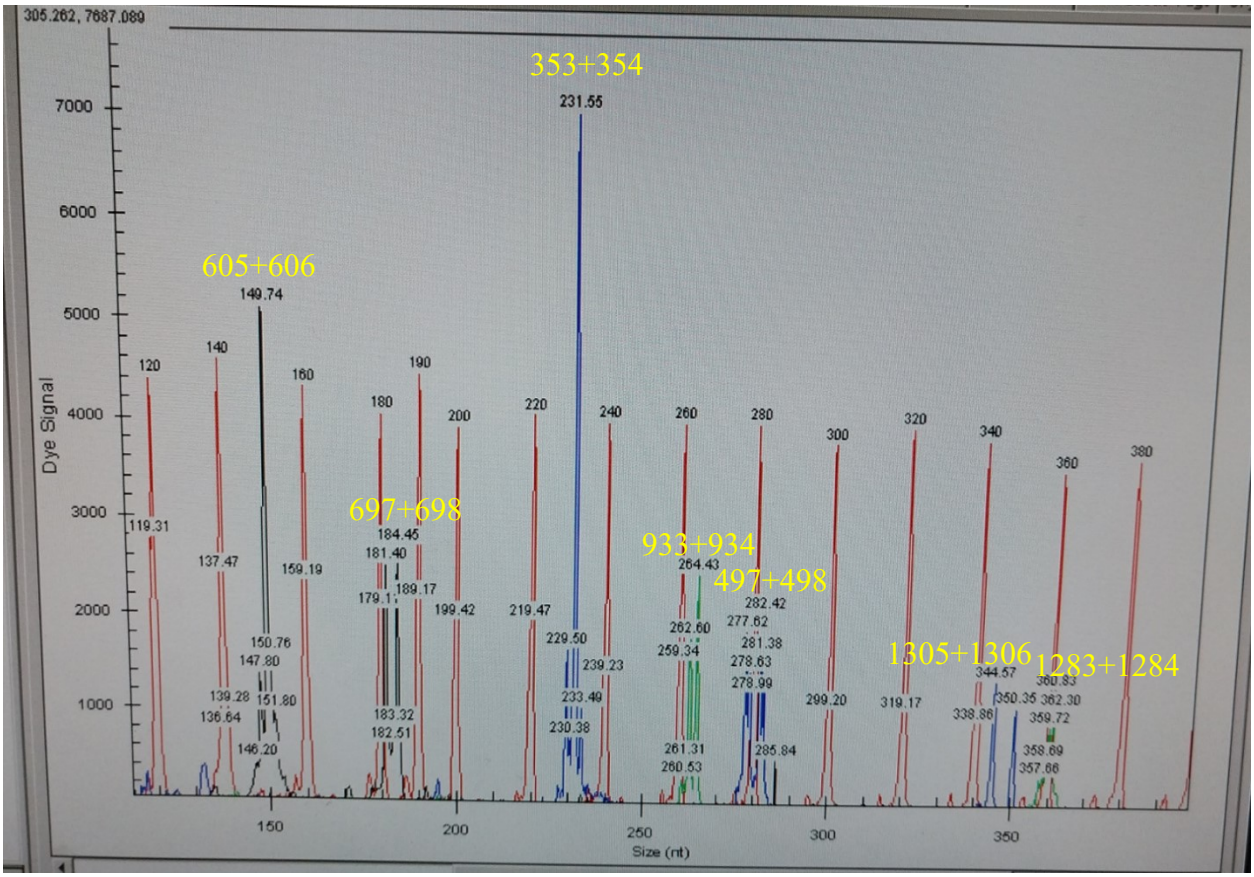


Figure 2. Image of capillary electrophoresis of a black walnut ‘Football’ x ‘Sparrow’ intraspecific hybrid plant. The y-axis measures the dye signal in capillary electrophoresis and the x-axis measures the allele size in nucleotides. The seven primer sets (in yellow) used for identification were used in capillary electrophoresis and the nucleotide size measured via capillary electrophoresis was recorded. This image shows all the peaks for the different primer sets and the nucleotide associated with each to identify this sample as an intraspecific hybrid.

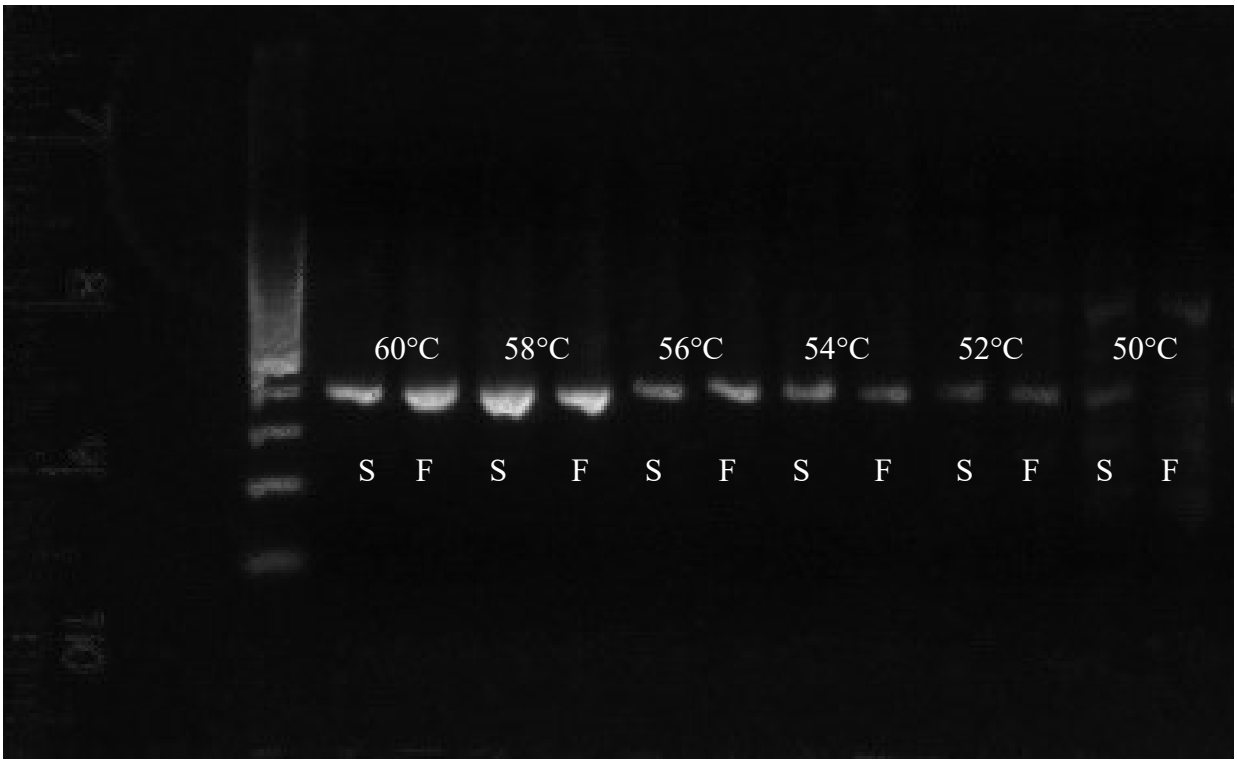
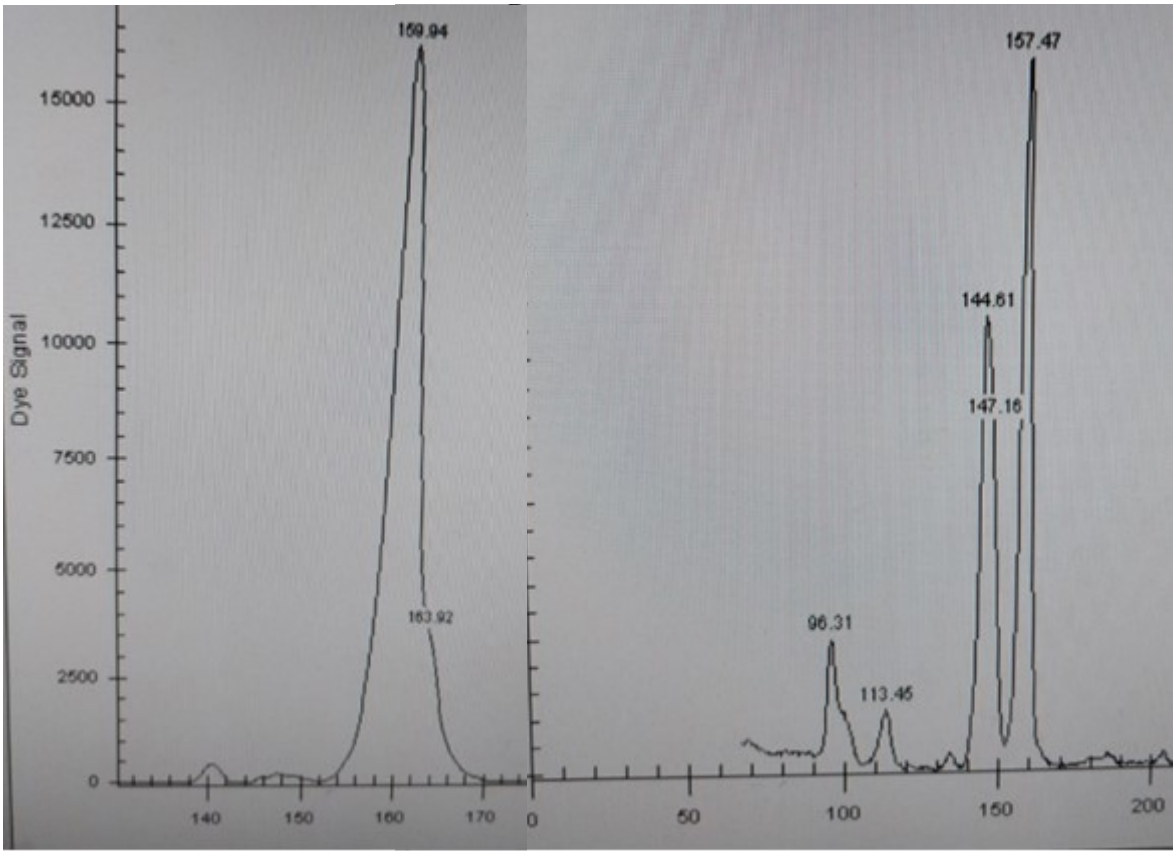


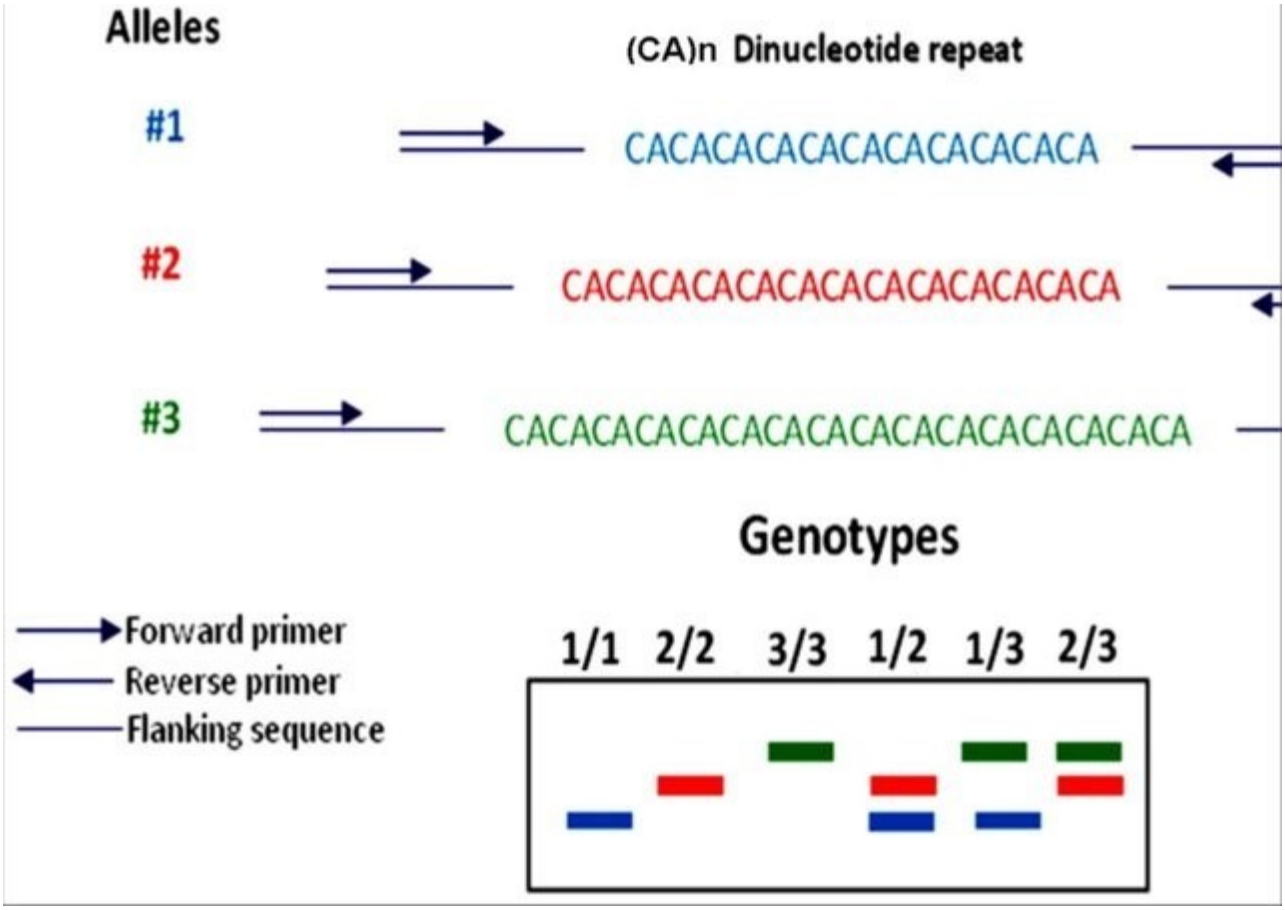
Figure 3. Gel electrophoresis imaging. After annealing temperature testing via PCR an agarose gel was run with 100 base pair DNA ladder as a reference. The brighter (more intense) the banding is on the gel, the more optimal the annealing temperature is. Each sample was tested with ‘Football’ and ‘Sparrow’ (F and S) DNA to determine optimum annealing temperature for both parents.



Capillary electrophoresis image of an identified polymorphic marker. This polymorphic marker set is 635+636 with an annealing temperature of 52°C. The left image is of parent 'Football' and the right image is of parent 'Sparrow'. The y-axis measures the dye signal in capillary electrophoresis and the x-axis measures the allele size in nucleotides.

APPENDICES

Appendix A: Simple sequence repeats (SSR)



A visual explanation of the principle of SSR marker identification. Variations of a gene are called alleles. The genotypes table at bottom shows the gel pattern of the markers after DNA extraction, PCR amplification, and base pair imaging via gel electrophoresis. (Alzohairy *et al*., 2015) Polymorphic SSR markers are based on length variation in sequence repeat number.

Appendix B: Raw data from optimizing annealing temperatures. PCR annealing temperatures ranged from 49°C- 60°C. Approximate base pair size estimated via gel electrophoresis. A cell marked 'x' did not have a clear band at that corresponding temperature. 280 primer pairs were tested from primers WLLC 107-1358.

Annealing Temp.: °C **Primer Lab Name**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
107+108		x		x		x		300		x		x
111+112		300		x		x		x		x		x
113+114		x		x		x		150		150		150
117+118		225		x		220		250		225		280
119+120		220		x		x		x		x		x
121+122		x		120		120		120		150		x
125+126		270		x		x		x		280		280
127+128		180		180		180		180		200		170
133+134				180								
137+138		x		x		x		x		x		200
143+144		230		190		190		x		180		170
145+146		x		x		x		x		x		x
149+150		150		170		150		170		150		150
151+152		250		225		225		x		230		225
157+158						250						
163+164		180		200		180		200		220		200
171+172		200		200		180		230		200		180
175+176		x		170		x		x		160		180
183+184		150		170		150		150		150		150
185+186		175		175		175		175		185		185
191+192		x		x		300		300		250		x
195+196		x		x		x		x		x		180
199+200		250		250		250		270		270		290
201+202		x		x		x		x		x		150
203+204		x		x		x		x		300		300
219+220		350		350		350		350		350		350
229+230		120		120		120		100		x		120
231+232		300		300		x		350		250		280
233+234		x		x		x		x		220		x
257+258		150		120		120		120		120		120
263+264		x		x		x		x		x		120
273+274		x		x		x		x		x		220
289+290		300		250		x		x		x		x
291+292		170		150		x		x		x		x
319+320		x		x		x		x		x		x
323+324						350						

Appendix B: Raw data from optimizing annealing temperatures- continued. PCR annealing temperatures ranged from 49°C- 60°C. Approximate base pair size estimated via gel electrophoresis. A cell marked 'x' did not have a clear band at that corresponding temperature. 280 primer pairs were tested from primers WLLC 107-1358.

Annealing Temp.: °C Primers: Lab name	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
323+324						350						
339+340		x		x		x		x		x		x
353+354		x		x		x		x		x		x
355+356		x		x		x		x		220		220
359+360		250		250		x		x		x		x
361+362						275						
363+364		x		270		270		250		x		280
367+368		x		x		x		350		x		x
369+370		200		200		x		x		180		x
371+372		x		350		300		x		300		300
375+376	x	x	x	x	x	x	x	x	x	x	x	x
377+378		150		150		150		150		170		170
379+380		250		250		x		x		250		250
383+384		200		x		x		x		200		x
385+386		300		300		x		300		x		x
387+388						250						
397+398		220		200		220		200		200		200
401+402		300		300		300		300		300		300
405+406	x	x	x	x	x	x	x	x	x	x	x	x
407+408		150		x		150		x		x		x
409+410		180		180		180		180		180		160
413+414		150		150		150		150		150		120
415+416		x		x		x		x		300		300
417+418		200		200		200		200		180		180
419+420		x		200		200		200		200		200
427+428		x		380		x		380		400		270
429+430		300		x		x		x		x		300
431+432		x		200		200		200		200		200
433+434		x		x		280		280		280		x
435+436		300										
439+440		x		200		x		x		x		x
441+442		x		x		x		x		220		x
445+446		x		x		x		x		300		x
447+448		x		220		x		x		x		x
449+450		x		x		x		150		x		x
455+456		x		x		x		x		320		350

Appendix B: Raw data from optimizing annealing temperatures- continued. PCR annealing temperatures ranged from 49°C- 60°C. Approximate base pair size estimated via gel electrophoresis. A cell marked 'x' did not have a clear band at that corresponding temperature. 280 primer pairs were tested from primers WLLC 107-1358.

Annealing Temp.: °C Primers: Lab name	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
457+458		x		x		x		x		x		x
459+460		x		x		x		x		380		380
463+464		x		x		x		x		x		x
465+466		x		x		x		x		x		x
469+470		350		x		x		x		x		x
477+478		x		150		x		170		x		x
483+484		200		200		200		200		200		200
487+488		x		x		x		x		x		220
489+490		180		180		x		180		x		180
493+494		x		220		x		x		x		x
495+496		300		300		x		220		220		220
497+498		x		x		x		x		x		x
499+500		x		x		x		x		300		300
501+502		300		300		280		280		300		300
503+504		x		x		x		x		x		x
505+506		x		x		x		280		x		x
507+508		400		350		x		x		x		x
511+512		x		x		x		x		x		x
513+514		280		270		250		250		220		220
525+526		x		x		x		300		300		300
529+530	x		x		x		x		x	x	350	
531+532		x		x		x		x		180		x
535+536		120		120		120		120		x		x
541+542		x		200		x		x		220		x
543+544		x		x		x		x		x		220
545+546		x		x		x		x		x		x
549+550		x		300s		300s		300s		200s		200s
553+554		x		x		x		x		x		x
559+560		280										
561+562		120		120		120		120		120		120
563+564		x		280		x		280		270		300
567+568		x		x		x		x		x		120
573+574		180										
577+578		x		x		x		x		x		x
579+580		x		x		x		x		x		x
581+582		x		x		x		x		x		x

Appendix B: Raw data from optimizing annealing temperatures- continued. PCR annealing temperatures ranged from 49°C- 60°C. Approximate base pair size estimated via gel electrophoresis. A cell marked 'x' did not have a clear band at that corresponding temperature. 280 primer pairs were tested from primers WLLC 107-1358.

Annealing Temp.: °C Primers: Lab name	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
585+586		x		x		x		300		300		x
591+592		120		120		120		120		120		120
595+596		x		x		x		x		x		x
597+598		x		x		x		x		x		400
599+600		x		150		x		x		150		x
601+602		x		x		x		x		x		x
605+606		x		x		120		120		120		120
607+608		x		170		170		170		170		x
609+610		120		120		x		x		x		x
621+622		x		x		x		150		x		x
623+624		x		x		x		x		x		x
625+626		x		x		x		x		x		150
629+630		x		x		x		x		170		170
635+636		170		170		170		170		170		170
637+638		x		x		x		x		x		x
639+640		150		x		150		150		150		150
641+642		x		x		x		x		x		400
643+644		x		150		x		x		150		150
645+646		x		x		x		x		x		x
649+650		x		x		x		x		x		x
651+652		x		x		x		x		x		x
653+654		180		x		x		x		180		x
657+658		x		x		x		x		320		300
659+660		x		x		x		180		x		x
661+662		x		x		x		x		x		320
665+666		x		x		x		x		x		x
669+670		x		x		x		x		300		300
671+672		x		x		x		x		x		300
673+674		x		x		x		x		x		280
675+676		x		x		x		x		x		400
681+682		x		150		150		x		160		160
687+688		x		x		x		x		x		x
689+690		x		x		x		x		x		400
691+692		170		170		x		x		x		x
693+694		x		x		x		x		x		x
695+696		180		170		x		180		x		x

Appendix B: Raw data from optimizing annealing temperatures- continued. PCR annealing temperatures ranged from 49°C- 60°C. Approximate base pair size estimated via gel electrophoresis. A cell marked 'x' did not have a clear band at that corresponding temperature. 280 primer pairs were tested from primers WLLC 107-1358.

Annealing Temp.: °C Primers: Lab name	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
697+698		x		x		x		x		x		x
699+700		x		x		x		x		x		x
701+702		x		x		x		x		x		300
705+706		x		x		x		x		x		x
711+712		x		x		x		x		x		x
713+714		x		x		x		x		x		x
715+716		x		x		x		x		x		x
717+718		x		x		x		x		x		x
719+720		x		x		x		x		x		x
723+724		x		x		x		x		x		x
725+726		200		150		150		200		150		200
727+728		150		x		150		150		150		x
729+730		x		x		x		x		x		x
733+734		x		150		x		x		150		150
735+736		180		x		x		x		180		x
737+738		x		x		x		400		x		300
739+740		x		x		x		x		x		x
749+750		x		170		170		x		170		170
759+760						100						
765+766		160		160		160		160		160		x
771+772		180		170		x		180		x		x
773+774		x		x		x		x		180		x
777+778		x		x		x		x		x		x
783+784		x		x		x		x		x		x
785+786		200		200		200		x		x		x
797+798		200		200		200		200		200		200
801+802		200		200		200		200		200		x
803+804		200		200		180		170		x		x
809+810		210		200		180		180		x		200
819+820		x		x		x		x		x		x
821+822		x		x		x		x		x		x
829+830						280						
831+832		x		x		x		x		x		x
847+848		220		180		180		180		180		x
855+856				200		x		220		x		400
869+870						250						

Appendix B: Raw data from optimizing annealing temperatures- continued. PCR annealing temperatures ranged from 49°C- 60°C. Approximate base pair size estimated via gel electrophoresis. A cell marked 'x' did not have a clear band at that corresponding temperature. 280 primer pairs were tested from primers WLLC 107-1358.

Annealing Temp.: °C Primers: Lab name	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
875+876		x		x		x		x		x		x
881+882	x		x		x		x		x		350	
891+892		x		x		x		x		x		200
895+896		x		x		x		x		x		x
897+898		x		x		x		x		x		200
901+902						150						
911+912		x		x		x		x		x		x
915+916		x		x		x		x		x		200
923+924		190										
929+930		200		x		200		x		x		x
931+932		x		300		200		x		x		x
933+934		x		x		x		x		x		x
937+938		x		x		x		200		200		200
943+944		210		220		220		220		x		200
945+946		x		x		x		x		x		x
951+952		x		x		200		200		180		200
959+960		x		x		x		200		200		210
965+966		x		x		x		300		300		280
969+970		x		x		260		260		260		x
975+976		x		x		x		x		350		x
977+978		x		x		x		x		x		210
979+980		x		200		200		200		200		x
981+982		x		x		x		x		x		x
985+986		x		x		x		x		x		220
989+990		300		x		280		280		230		230
991+992		x		200		200		190		180		200
993+994		x		x		x		230		x		x
995+996						350						
997+998								220				
999+1000		x		x		x		x		x		x
1001+1002						300						
1003+1004				300								
1009+1010		x		200		200		x		x		x
1027+1028		x		x		x		x		x		x
1035+1036		x		x		x		x		250		250
1041+1042		x		x		x		x		x		400

Appendix B: Raw data from optimizing annealing temperatures- continued. PCR annealing temperatures ranged from 49°C- 60°C. Approximate base pair size estimated via gel electrophoresis. A cell marked 'x' did not have a clear band at that corresponding temperature. 280 primer pairs were tested from primers WLLC 107-1358.

Annealing Temp.: °C Primers: Lab name	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
1049+1050		270		250		200		x		x		x
1051+1052		x		x		x		x		x		x
1055+1056		250		250		210		210		220		x
1057+1058		x		x		250		250		300		x
1061+1062		x		x		x		x		x		x
1063+1064		x		x		x		x		x		x
1067+1068		x		x		x		220		220		220
1073+1074		320										
1077+1078		x		250		250		x		x		x
1089+1090		300		280		270		260		270		x
1091+1092		300		270		250		250		250		320
1093+1094		x		x		x		x		300		300
1101+1102		x		x		x		300		250		250
1103+1104				300								
1105+1106		x		x		280		250		x		x
1111+1112		x		x		300		280		280		280
1115+1116		x		x		x		x		x		x
1119+1120		x		x		x		x		x		x
1121+1122		x		x		250		x		x		x
1123+1124		x		250		x		x		x		x
1133+1134		x		x		x		x		250		250
1139+1140		250		250		250		250		300		300
1145+1146		300		x		250		x		x		x
1147+1148		350		300		250		250		250		x
1151+1152		x		x		x		x		x		x
1155+1156		x		x		x		x		x		x
1165+1166		x		x		x		x		250		250
1169+1170		x		x		x		x		x		x
1183+1184		x		250		250		250		250		250
1187+1188		x		250		x		250		250		250
1189+1190				200								
1199+1200		x		x		x		x		x		250
1201+1202		250		200		x		x		x		x
1219+1220				100								
1221+1222		x		x		x		x		300		300
1235+1236		x		x		270		250		x		x

Appendix B: Raw data from optimizing annealing temperatures- continued. PCR annealing temperatures ranged from 49°C- 60°C. Approximate base pair size estimated via gel electrophoresis. A cell marked 'x' did not have a clear band at that corresponding temperature. 280 primer pairs were tested from primers WLLC 107-1358.

Annealing Temp.: °C Primers: Lab name	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
1245+1246		x		x		x		x		x		x
1247+1248				120								
1251+1252		x		300		300		300		x		x
1263+1264		x		350		x		x		x		x
1267+1268		x		x		x		x		x		x
1269+1270		x		x		x		300		300		x
1277+1278	x		x		x		x		x		x	
1281+1282		x		x		x		x		300		300
1283+1284		x		x		x		x		x		x
1285+1286		x		x		x		x		x		250
1297+1298		400		x		x		350		350		x
1299+1300		x		x		350		350		350		x
1303+1304		x		x		x		x		x		x
1305+1306		x		x		x		x		x		x
1313+1314		x		x		x		x		x		x
1317+1318	280		300		x		x		x		x	
1321+1322		x		x		x		x		x		x
1323+1324		350		350		350		350		350		x
1325+1326		x		x		x		x		400		350
1335+1336		x		x		x		x		x		x
1337+1338		x		x		350		x		350		400
1345+1346		x		x		x		x		x		350
1357+1358		x		x		x		x		x		300

Appendix C-1: Raw data from capillary electrophoresis. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
50°C	***609+610***	***110***	***113/96***
52°C	***635+636***	***160***	***145/157***
52°C	***1219+1220***	***90/107/151***	***106/130/139***
50°C	359+360	278	278/288
50°C	***409+410***	***73/78/151***	***143/154***
52°C	855+856	181/241	181/243
50°C	229+230	122/134	122
52°C	771+772	215/226	211/215
52°C	***257+258***	***122/126***	***124***
50°C	***379+380***	***267***	***201/217/232***
50°C	573+574	133/162/166	133/166
52°C	1189+1190	268/275/308	268/275/307
50°C	535+536	137	137
52°C	***749+750***	***198***	***202***
52°C	609+610	116	109/115
50°C	125+126	274	274
50°C	***735+736***	***199***	***197/222***
52°C	991+992	280/288	280
50°C	561+562	124/143	125
52°C	185+186	172/184/191	173/192
52°C	229+230	121	122
50°C	513+514	327/358	328/352
52°C	979+980	285	282/286
50°C	771+772	215/227	211/215
50°C	591+592	146/147	146/147
52°C	127+128	242	198/242
52°C	535+536	136/137	137/138
50°C	559+560	250/256	250/256
50°C	923+924	137	137
52°C	447+448	329/331	329
50°C	257+258	126/127	124/127
52°C	489+490	197/217	196/198/217
52°C	561+562	128/150/198	123/148/150/190
50°C	289+290	290/292	290/291/292
50°C	383+384	272	272/276
52°C	493+494	291/297	291
50°C	407+408	182	182
52°C	133+134	207/223	207/225

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
52°C	591+592	96/102/146	97/102
50°C	***111+112***	***231***	***224/254***
50°C	785+786	207/217	213/217
52°C	943+944	232/237	233
50°C	***639+640***	***150/152***	***146/156***
52°C	409+410	162/168	162/164
52°C	***1247+1248***	***128/132/151***	***120/121***
50°C	***231+232***	***365/366***	***374***
50°C	797+798	151/182/203/207	158/180/207
52°C	149+150	238/288	239/288
50°C	151+152	156	NA
52°C	***143+144***	***233***	***235/237***
52°C	291+292	178/184	178/184
50°C	385+386	303/370	303/370
50°C	801+802	221	221
52°C	289+290	291/293	291/293
50°C	183+184	143/146	143/145
52°C	439+440	242/246	230/242
52°C	477+478	174/176	174/176
50°C	401+402	222	222
50°C	803+804	226/231	226
52°C	199+200	285	284
50°C	377+378	143/154/191/193	144/193/195
52°C	541+542	248/252	246/248
52°C	599+600	148/150	148/150
50°C	***429+430***	***245/251/277***	***256/260***
50°C	929+930	188/191	186/188
52°C	***359+360***	***268/277***	***265/275/286***
50°C	413+414	129/131	129/131
52°C	785+786	207/217/233	212/217/233/238
52°C	643+644	152	151/154
50°C	435+436	276/295	280/295
50°C	171+172	238	238
52°C	379+380	275/277	275
50°C	727+728	185	185/188
52°C	***681+682***	***161***	***206***
50°C	495+496	298	297/298
50°C	369+370	206/210	206

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
52°C	363+364	282	281
50°C	765+766	210/214	209
52°C	801+802	210/214/222	209/221
52°C	377+378	192/194	193/195
50°C	***501+502***	***265***	***263***
50°C	417+418	225	224
52°C	513+514	264/266/328	263/327
50°C	291+292	184/185	177/184/185
52°C	803+804	225/231	225
52°C	413+414	129/131/215	129/131/222
50°C	989+990	312	312
50°C	483+484	215/221	209/221
52°C	563+564	311/317	311
50°C	691+692	175/183	NA
52°C	809+810	231/233	228/230
52°C	733+734	191/192/231	192/193
50°C	1073+1074	340/365	NA
50°C	809+810	230/232	229/231
52°C	231+232	339/365	339/364/366
50°C	635+636	159	159
52°C	163+164	249/250	249/260/266
52°C	765+766	131/209/213	131/208
50°C	469+470	388/433	388/430
50°C	943+944	209/262/264	205/208/262/264
52°C	385+386	262/264/370	260/261/370
50°C	185+186	170/182	170/172
52°C	171+172	274/276	273/274
52°C	175+176	204/213	203/212
50°C	219+220	336	336
50°C	119+120	222	222
52°C	401+402	336/337	335/337
50°C	127+128	205/210	205
52°C	369+370	242	242
50°C	507+508	292/405/437	291/343/351/405
52°C	***691+692***	***137***	***129/131***
50°C	397+398	178/252/256	178/250
52°C	495+496	292/293/405	289/291/405
50°C	489+490	196	196/198

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
52°C	397+398	292	289/291
52°C	695+696	172/173	172/173/193/215
49°C	1317+1318	258/259/328	258/259/329
50°C	651+652	171/172	158/172/176
52°C	501+502	357/359	357
50°C	***653+654***	***168***	***174/195***
52°C	417+418	263/264/304	262
52°C	849+850	203/208/222/237	206/222/237
51°C	1317+1318	328	295/328/329
50°C	117+118	203/237	204
52°C	549+550	NA	294/321
50°C	695+696	177/181	177
52°C	419+420	280/305	280/304
52°C	151+152	156/233	NA
52°C	371+372	360/386	328/360/386
50°C	***143+144***	***232***	***226/234***
52°C	1003+1004	327/347	328
50°C	849+850	193/205/209	193/206/207
52°C	431+432	223/238	223/238
52°C	***183+184***	***142/151***	***145/158***
52°C	219+220	263	262
50°C	***149+150***	***151/182/264/266***	***157/180/262***
52°C	931+932	292/345/347/351	NA
50°C	163+164	214/220	219
52°C	483+484	249/250	249/260/265
52°C	607+608	130/152	130/152
52°C	427+428	388	389
50°C	199+200	286	285
52°C	507+508	381/416/421/438	416/439
54°C	759+760	NA	NA
56°C	849+850	209/225/240	208/225/239
56°C	229+230	121/123	121
54°C	513+514	328/355/360	328
54°C	849+850	207/211/225	NA
56°C	117+118	211/225/240	NA
54°C	257+258	126/151	124/126
56°C	409+410	NA	NA
56°C	409+410	NA	NA

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
56°C	409+410	NA	NA
56°C	257+258	126	124/126
54°C	869+870	251/307	250/252
54°C	163+164	NA	135
56°C	231+232	250	250/261/266
54°C	***605+606***	***147/149***	***152***
56°C	659+660	171	172
56°C	***605+606***	***147/149***	***151***
54°C	969+970	274/276	275/278
54°C	171+172	NA	NA
56°C	513+514	276/278/334	276
54°C	229+230	115/122/124	122
56°C	715+716	189	188
56°C	535+536	137	100/136/137
54°C	361+362	231	230/231
54°C	409+410	151/153	NA
56°C	969+970	NA	NA
54°C	535+535	136	137
56°C	771+772	NA	NA
56°C	561+562	111/123	123
54°C	363+364	190	NA
54°C	143+144	233	NA
56°C	***199+200***	***286***	***284***
54°C	561+562	124	124/125
56°C	991+992	280/287	279
56°C	591+592	146	NA
54°C	433+434	352	322/352
54°C	785+786	111/207/217/233	NA
56°C	363+364	281	NA
54°C	591+592	146/147	NA
56°C	797+798	203/207/239	207/239
56°C	449+450	145	135/145
54°C	501+502	358/360	358
54°C	797+798	NA	207/239
56°C	505+506	327	NA
54°C	407+408	NA	NA
56°C	***801+802***	***222***	***226***
56°C	621+622	159/163	160

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
54°C	829+830	233/235	233
54°C	801+802	222	NA
56°C	433+434	352	NA
54°C	639+640	162/168	160/163
56°C	163+164	250	260/266
56°C	639+640	161/167	161/163
54°C	853+854	241/270	241
54°C	929+930	267	NA
56°C	501+502	358/360	358
54°C	681+682	NA	NA
56°C	417+418	263/265	263
56°C	183+184	142/146	142/144
54°C	989+990	264	NA
54°C	931+932	264/266	266/268
56°C	***563+564***	***312/318***	***314/320***
54°C	151+152	NA	NA
56°C	431+432	360	360
56°C	***377+378***	***191***	***193/195***
54°C	191+192	NA	343/346
54°C	417+418	NA	NA
56°C	989+990	284	283
54°C	183+184	143/146	NA
56°C	483+484	214/220	219
56°C	413+414	129/131	129/131
54°C	371+372	386	386
54°C	419+420	174/175/177	175
56°C	585+586	281/305	NA
54°C	377+378	191/193	193/195
56°C	419+420	191/193/281	193/195/281
56°C	727+728	185	185/188
54°C	401+402	NA	NA
54°C	431+432	194	NA
56°C	107+108	NA	NA
54°C	413+414	129/131	129/131
56°C	979+980	285	281/284
56°C	477+478	176	174/176
54°C	549+550	294	294
54°C	483+484	133	NA

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
56°C	191+192	264/266	NA
54°C	901+902	208	NA
56°C	959+960	208/276	276
56°C	803+804	225/231	174/225/263
54°C	1001+1002	375/376	374/375
54°C	951+952	173	173
56°C	385+386	261	262/266
54°C	727+728	NA	NA
56°C	951+952	262	262/266
56°C	151+152	156/171	NA
54°C	219+220	360	360
54°C	979+980	NA	NA
56°C	401+402	284/344	NA
54°C	765+766	209/214	209
56°C	937+938	209/214	209
56°C	607+608	153/154/235	154/235
54°C	323+324	327	327
54°C	991+992	NA	NA
56°C	525+526	280/287	NA
54°C	607+608	154	154
56°C	397+398	290/292	290/292
56°C	635+636	158/159	125/158
54°C	995+996	356/357	NA
54°C	117+118	264/266	NA
56°C	549+550	NA	294
54°C	635+636	158/159	158/159
56°C	495+496	251	251/252
56°C	***749+750***	***196/198***	***201***
58°C	965+966	NA	NA
54°C	397+398	NA	262/266
56°C	965+966	327	NA
54°C	749+750	197/199	NA
56°C	855+856	241/242	241/242
56°C	185+186	129/144/171	171
58°C	455+456	NA	NA
54°C	943+944	263/264	NA
56°C	219+220	NA	NA
54°C	185+186	NA	125/171

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
56°C	997+998	347	347
56°C	127+128	256	NA
58°C	657+658	344/346	NA
54°C	149+150	NA	131
56°C	427+428	NA	NA
54°C	127+128	152	152
56°C	943+944	242/264	242/262/270
56°C	489+490	NA	158/173
58°C	975+976	343/348	343
54°C	157+158	NA	NA
56°C	737+738	NA	NA
54°C	803+804	124	124
56°C	171+172	226/232/275/276	226/275
56°C	***695+696***	***146/197***	***201***
58°C	219+220	344/346	342/344
54°C	199+200	NA	173/262
59°C	529+530	NA	NA
54°C	809+810	147	147
56°C	993+994	231/233	229/231
56°C	809+810	NA	NA
58°C	459+460	348	NA
54°C	387+388	NA	NA
59°C	881+882	NA	NA
58°C	257+258	NA	148
60°C	195+196	251	NA
60°C	257+258	126	123/126
58°C	163+164	250	NA
58°C	369+370	NA	206
60°C	495+496	NA	NA
58°C	605+606	162/168	162/164/196/198
60°C	489+490	162/168	162/164/196/198
60°C	567+568	124	124
58°C	495+496	NA	NA
58°C	409+410	150/152	NA
60°C	513+514	345/365	326/345
58°C	561+562	153	153/155
60°C	171+172	275	275
60°C	***605+606***	***146/148***	***151***

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
58°C	513+514	326	326
58°C	417+418	NA	NA
60°C	985+986	263/265/281/287	263/282/291
58°C	591+592	NA	NA
60°C	417+418	263/360	263/360
60°C	263+264	142/153	142
58°C	117+118	202/203	NA
58°C	735+736	151/198	151/196
60°C	***149+150***	***198***	***196/221***
58°C	599+600	144	143/145
60°C	185+186	NA	170/171
60°C	229+230	121/123	121
58°C	***149+150***	***150/182***	***156/179***
58°C	773+774	NA	NA
60°C	989+990	285	NA
58°C	639+640	129/131	129/131
60°C	137+138	NA	NA
60°C	413+414	129/131	129/131
58°C	989+990	NA	NA
58°C	951+952	NA	NA
60°C	379+380	344/345	NA
58°C	643+644	185/186	185/189
60°C	797+798	202/207	207
60°C	561+562	123	123/124
58°C	191+192	299/301	NA
58°C	991+992	NA	NA
60°C	***427+428***	***280/287***	***262***
58°C	151+152	193	193
60°C	809+810	231/233	NA
60°C	591+592	146	146
58°C	379+380	347	347
58°C	185+186	171	171
60°C	125+126	399	NA
58°C	183+184	NA	NA
60°C	915+916	261	213
60°C	201+202	186/189	186
58°C	969+970	272/274	274/277
58°C	383+384	NA	NA

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
60°C	117+118	321	272/276
58°C	413+414	NA	NA
60°C	163+164	299	NA
60°C	625+626	156/157	157
58°C	199+200	NA	284
58°C	797+798	202/207	NA
60°C	231+232	238/366	NA
58°C	727+728	210/214	209/210
60°C	397+398	210/214/292	209/210/292
60°C	639+640	132/161/167	160/162
58°C	563+564	312/317	NA
58°C	801+802	221	221
60°C	363+364	282	NA
58°C	***733+734***	***139/141***	***157/162***
60°C	419+420	282	281
60°C	***643+644***	***126/127***	***153/155***
58°C	125+126	398	395/399
58°C	127+128	NA	NA
60°C	549+550	NA	242
58°C	175+176	NA	NA
60°C	431+432	NA	NA
60°C	151+152	NA	NA
58°C	231+232	360/365	343/360
58°C	171+172	275/277	274/275
60°C	673+674	275/277/344	274/275/344
58°C	681+682	221	No peak
60°C	483+484	221	221
60°C	183+184	142/143	NA
58°C	433+434	352	352
58°C	397+398	NA	142/144
60°C	965+966	345/347	345/351/408
58°C	765+766	NA	NA
60°C	991+992	279/287	209/210/279
60°C	733+734	192/193	193
58°C	549+550	294	294
58°C	419+420	192	NA
60°C	199+200	285/286	285
58°C	629+630	NA	NA

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
60°C	951+952	261/262	262/266
60°C	681+682	135/151	NA
58°C	445+446	298/299/324/345	NA
58°C	431+432	NA	NA
60°C	371+372	386	NA
58°C	377+378	192/194	193/196
60°C	943+944	264	NA
60°C	409+410	105/152/174/177	146/156/174/175
58°C	585+586	NA	NA
58°C	483+484	214/220	NA
60°C	401+402	NA	NA
58°C	607+608	154	154
60°C	937+938	NA	275/287
60°C	127+128	142	142/144
58°C	371+372	242/345	242/345/385
58°C	937+938	195	NA
60°C	***415+416***	***277/292***	***275/287/345***
58°C	635+636	159	158/159
60°C	977+978	279	279/280
60°C	629+630	161	157/161
58°C	401+402	346/347	NA
58°C	959+960	118	118
60°C	429+430	276	276
58°C	***749+750***	***198/200***	***202***
60°C	***959+960***	***198/200/277***	***202***
60°C	***143+144***	***195/233***	***200/235***
58°C	415+416	342/347	342/343
58°C	979+980	194/242	NA
60°C	499+500	284/285	NA
58°C	711+712	189	189
60°C	273+274	298	NA
60°C	377+378	191/193	193/195
58°C	499+500	242/308	242
58°C	233+234	264	NA
60°C	501+502	329	329/345
58°C	143+144	NA	NA
60°C	355+356	233	NA
60°C	635+636	158/159/196	158/196

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
58°C	501+502	358/360	NA
58°C	355+356	115/127	NA
60°C	525+526	336/338/345	NA
58°C	531+532	198/200	NA
60°C	487+488	198/200/295/297	297
60°C	749+750	178/182/197/198	178/201
58°C	525+526	328	329
58°C	441+442	295	277/295
60°C	563+564	295/312/318/346	295
58°C	***653+654***	***168***	***174***
60°C	543+544	277/292	NA
60°C	175+176	203/212/231/233	212/229/231
58°C	669+670	338/340/346	341
58°C	541+542	NA	NA
60°C	657+658	343/346/350	NA
56°C	121+122	185	NA
50°C	1139+1140	NA	NA
58°C	***1269+1270***	***338***	***388***
60°C	113+114	NA	146
58°C	1091+1092	186	NA
60°C	***451+452***	***339/341/346***	***385***
56°C	113+114	NA	NA
50°C	397+398	291	NA
58°C	1281+1282	350/356	NA
60°C	***413+414***	***129/132***	***104***
58°C	1147+1148	303/325	304/314
60°C	***597+598***	***345/350***	***343***
56°C	847+848	NA	NA
50°C	1201+1202	337	NA
58°C	***203+204***	***323/359/365***	***448***
60°C	171+172	275	NA
58°C	1183+1184	333/337	NA
60°C	641+642	409	NA
56°C	171+172	204	NA
50°C	1049+1050	NA	307
58°C	1057+1058	NA	340
60°C	***397+398***	***219***	***292***
58°C	1187+1188	219/223	NA

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
60°C	675+676	NA	342/345
56°C	***397+398***	***255***	***224***
50°C	***1089+1090***	***255***	***298***
58°C	***1093+1094***	***281***	***311***
60°C	725+726	NA	193/202
58°C	***1035+1036***	***282/288***	***224/298***
60°C	689+690	NA	344
56°C	725+726	183/185	NA
50°C	1091+1092	305/310/371	305/310
56°C	1101+1102	313/315	313
60°C	891+892	257/259	NA
58°C	1101+1102	241	241
60°C	***855+856***	***313/315***	***344***
50°C	171+172	299/300	299
50°C	1145+1146	299/300/324	299/324
56°C	1297+1298	358/359	358/359
60°C	1187+1188	NA	274/275
58°C	1133+1134	321/322	NA
60°C	1041+1042	344/351	344/347/351
50°C	725+726	NA	NA
52°C	***1251+1252***	***308/348***	***306/346***
56°C	1299+1300	358/362	362/364
60°C	1285+1286	NA	NA
58°C	1165+1166	NA	NA
60°C	1337+1338	344/350/369	344/350
50°C	847+848	305/310	305/310
58°C	427+428	305/310	305/310
56°C	1323+1324	366	359/366
52°C	1049+1050	306/308	NA
58°C	1111+1112	344/351	344/351
60°C	459+460	344/351/424	344/351
50°C	1055+1056	227/229	NA
58°C	451+452	320/322	320
56°C	367+368	323	324
52°C	1055+1056	300	299/300
54°C	***1077+1078***	***241***	***204***
60°C	219+220	344	345/351
56°C	1055+1056	141/177	NA

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	Lab Name	'Football' Allele Size	'Sparrow' Allele Size
58°C	1221+1222	304/340	304/313
60°C	669+670	345/358	345/346
60°C	897+898	NA	NA
54°C	1091+1092	308/310	NA
60°C	453+454	345/351	345/351
56°C	1067+1068	NA	NA
58°C	1139+1140	308	NA
60°C	671+672	NA	NA
60°C	1067+1068	121	NA
54°C	1121+1122	NA	NA
60°C	455+456	344/351	NA
56°C	1091+1092	305/310	306/310
58°C	1297+1298	359	359
60°C	701+702	292	258/292
52°C	397+398	NA	258
54°C	1139+1140	NA	NA
60°C	1325+1326	344	345/351
56°C	1139+1140	320/322	320
58°C	1299+1300	320/322/362	320
60°C	737+738	343/347	299/301/344
54°C	1009+1010	NA	NA
54°C	1145+1146	NA	NA
60°C	1345+1346	324/345/363	324
56°C	1147+1148	304	304/313
58°C	1323+1324	366	NA
60°C	1139+1140	319/345	320/322
54°C	1049+1050	NA	275
54°C	1147+1148	303	304/313
54°C	1299+1300	362	NA
56°C	1183+1184	309	NA
58°C	1337+1338	320/324/329	319/324/329
60°C	1165+1166	NA	NA
54°C	121+122	111	111
54°C	1183+1184	319/323/332	319/320
54°C	1323+1324	366	359/366
56°C	1187+1188	323/333	322/333
58°C	453+454	323/333	322/333
60°C	1199+1200	336	335

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
54°C	725+726	NA	149
54°C	1057+1058	307/311	NA
54°C	1337+1338	369	344/368
56°C	1235+1236	322/333	NA
58°C	1325+1326	322/333	NA
60°C	1221+1222	344/348	343
54°C	847+848	239	177/239
54°C	1089+1090	303/304/313	304/313
50°C	1147+1148	313	313
56°C	1057+1058	307	307
58°C	113+114	149/151	149/151
60°C	1281+1282	298/343/345/391	298/300/345
54°C	1055+1056	180	NA
52°C	1077+1078	312	312
50°C	1297+1298	359/360	359/360
56°C	1089+1090	314/316	314/316
58°C	121+122	197	196/197
60°C	1357+1358	342/344/349	342
52°C	121+122	196	196
52°C	1123+1124	NA	189
50°C	1323+1324	323/325/366	323/360/366
56°C	1105+1106	232	NA
58°C	413+414	129/131	129/131
60°C	***1093+1094***	***343/349***	***311/312***
52°C	725+726	191/193	193/202
52°C	1139+1140	320/321	320/322
52°C	1323+1324	366	360/366
56°C	965+966	409	409
58°C	725+726	193/202	193/202
60°C	203+204	343/349	343/351
52°C	847+848	NA	177/239
52°C	1183+1184	NA	113
52°C	1263+1264	320/324/333	319/324/328
56°C	***1251+1252***	***348***	***346***
58°C	847+848	239	239
54°C	1111+1112	300	298/300
58°C	1055+1056	NA	NA
52°C	1187+1188	NA	NA

Appendix C-1: Raw data from capillary electrophoresis- continued. Exact allele sizes of 'Football' and 'Sparrow' are recorded in the table. A cell marked 'NA' did not display peaks or had peaks too small for detection of the proper allele size. Bolded and italicized rows indicate primers that are possible polymorphic markers and may eventually be used for 'Football' and 'Sparrow' identification.

Annealing Temp.	**Lab Name**	**'Football' Allele Size**	**'Sparrow' Allele Size**
60°C	661+662	346	322/346
56°C	1269+1270	347/349	345/347/349
58°C	1009+1010	NA	234
54°C	1251+1252	308/348	308/345
58°C	1067+1068	308	308
52°C	1201+1202	308/310	NA
60°C	1091+1092	333/337	336
52°C	1091+1092	148/150/306	148/150/155/197
52°C	1089+1090	313/316/148/150	147/150
52°C	1147+1148	191/303/304	191
60°C	1035+1036	298	299
60°C	1101+1102	313/314	NA
60°C	1133+1134	321/322	NA
60°C	***1111+1112***	***424***	***344***
60°C	***1183+1184***	***324/332***	***346***
54°C	989+990	284/285	NA
54°C	1105+1106	316/318	NA

Appendix C-2: Polymorphic markers. Listed lab name, complete forward and reverse primer sequences, as well as the original resource and name for the primer sets in the graph.

Name in Lab	Forward Primer	Reverse Primer	Original Resource
WLLC111-J_N_32820_f	TGTAAAACGACGGCCAGTAGTTTCAGACTTTCCTCGTACATACG	TACCAGCACGAAACTCCAGC	Juglans_nigra_120313_comp32820_c0_seq6
WLLC143-J_N_31873_f	TGTAAAACGACGGCCAGTGGTTTCGTTTCTTCCACGGC	ACCAGTTAACAGGCGTCACC	Juglans_nigra_120313_comp31873_c0_seq1
WLLC149-J_N_7348_f	TGTAAAACGACGGCCAGTATCACAAATGGCAGGGACCC	TGCTTCCAAATAATTCACCGACG	Juglans_nigra_120313_comp7348_c0_seq1
WLLC163-J_N_28449_f	GACGCAGACACAGTCATCC	GAAGAGAGAGATCGCGAGGC	Juglans_nigra_120313_comp28449_c0_seq1
WLLC183-J_N_25195_f	CACATGTCCTGACTCCTGGC	TGTTCCTTGGAAACCAAACCG	Juglans_nigra_120313_comp25195_c0_seq1
WLLC199-J_N_28894_f	GTGGTTCAGGAACGGTCTCC	TGAGAACATCATGTGCATACCC	Juglans_nigra_120313_comp28894_c0_seq2
WLLC203-J_N_24028_f	TCTCAATTCTCACCGCCACC	GAGGGACGAGAACTTGGTGC	Juglans_nigra_120313_comp24028_c0_seq2
WLLC231-J_N_34901_f	GCAACAAGCAAGATAAGCAGG	CAGCAGGTGAAGAACAGTGC	Juglans_nigra_120313_comp34901_c0_seq1
WLLC257-J_N_17055_f	ACAAATCCAAAGGAAAGTGACGC	AAAGCCGATCAAGAGCTCCC	Juglans_nigra_120313_comp17055_c0_seq1
WLLC359-J_N_35305_f	TCATTCATCAAACCATCGCCG	ACTCGGTAGTCGTGTGCTCC	Juglans_nigra_120313_comp35305_c0_seq18
WLLC377-J_N_25293_f	CACTGCCTCCTTCTGTACCG	CTCTCTGCACCTTCCTCTGC	Juglans_nigra_120313_comp25293_c0_seq1
WLLC379-J_N_31522_f	AGTGTCCTCAAGATCCTCCC	CGAATCCGTCAAGGTTGATTCC	Juglans_nigra_120313_comp31522_c0_seq7
WLLC397-J_N_24905_f	CAGTACTTCCAGCTCCTGCC	CACCGTCAAGTCCTGGTACC	Juglans_nigra_120313_comp24905_c0_seq1

Appendix C-2: Polymorphic markers- continued. Listed lab name, complete forward and reverse primer sequences, as well as the original resource and name for the primer sets in the graph.

Name in Lab	Forward Primer	Reverse Primer	Original Resource
WLLC409-J_N_30890_f	AGAAGAGTGCACTGCAGAGC	TCCTTCCTCACAAAGCCTGC	Juglans_nigra_120313_comp30890_c0_seq3
WLLC413-J_N_23725_f	TGCCACAATTCCACAAAGCG	GTCCCTCACTTCTGCTCTGG	Juglans_nigra_120313_comp23725_c0_seq1
WLLC415-J_N_13066_f	AGTTCTCTCGTCTGTGTGCG	TCCACTGCCACAACTTCTCC	Juglans_nigra_120313_comp13066_c0_seq1
WLLC427-J_N_89731_f	TGTTGGGAGTGAGTTGAAGAGG	AGGCAGCAAGGATTTAGGGC	Juglans_nigra_120313_comp89731_c0_seq1
WLLC429-J_N_56153_f	ATGGGTTATCATCGGACGCC	CTTGACTTCCTCCTCCAGGC	Juglans_nigra_120313_comp56153_c0_seq1
WLLC451-J_N_32416_f	TCGGTCACAAGCTGATTCCC	TCCATATCCCTGGCCTCTCG	Juglans_nigra_120313_comp32416_c0_seq1
WLLC501-J_N_24578_f	GACCGATGGGCAAGATAGGG	ACTCGGGCATAGACGAAAGC	Juglans_nigra_120313_comp24578_c0_seq1
WLLC563-J_N_13897_f	TGAGAACCGTCGAGGAAAGC	ACTCTTAGAACGTCCACCGC	Juglans_nigra_120313_comp13897_c0_seq1_ssr126
WLLC597-J_N_9830_f	CCCTCCGCCATTAATTACTTTAGC	CTCATTTGCCGTCCGAATCC	Juglans_nigra_120313_comp9830_c0_seq1_ssr129
WLLC605-J_N_29438_f	TTGTGTTTACGCAGCAAGGC	CGTCTTCCACTCTGCATTTGC	Juglans_nigra_120313_comp29438_c0_seq2_ssr1687
WLLC609-J_N_106830_f	AGAATCCAACGGTGTGCGC	GCGATGGAAGCTTGGAAAGG	Juglans_nigra_120313_comp106830_c0_seq1_ssr25
WLLC635-J_N_28147_f	GCAATTTAGCGACCCACACC	TTTCCCGAGAGAACGTGG	Juglans_nigra_120313_comp28147_c1_seq5_ssr126

Appendix C-2: Polymorphic markers- continued. Listed lab name, complete forward and reverse primer sequences, as well as the original resource and name for the primer sets in the graph.

Name in Lab	Forward Primer	Reverse Primer	Original Resource
WLLC639-J_N_21503_f	GTCTGCATGGTCTCCAGTCC	AGCAACCCATCTGTGAAGGC	Juglans_nigra_120313_comp21503_c0_seq1_ssr512
WLLC643-J_N_74242_f	CTCTGTTGGTCACAATGGCC	GAGAGGTGAGACACAGGTGC	Juglans_nigra_120313_comp74242_c0_seq2_ssr103
WLLC653-J_N_30718_f	CAGAGCACGCAAGCATTAGG	AGGCATGCATGAATGATTCGC	Juglans_nigra_120313_comp30718_c0_seq5_ssr703
WLLC681-J_N_32816_f	TCCATGGATAACGGCGATGG	TTGGTTGCACTGTCTTTGCC	Juglans_nigra_120313_comp32816_c0_seq2_ssr498
WLLC691-J_N_35203_f	CCTGAGGATTGAGGAAGCCG	TGCCAGTGAACCAATCCTCC	Juglans_nigra_120313_comp35203_c0_seq1_ssr3334
WLLC695-J_N_27882_f	AAGCCAATTCCATCGAAACC	GATTGGCAGGATTCAACGGC	Juglans_nigra_120313_comp27882_c0_seq1_ssr277
WLLC733-J_N_35364_f	GAGCAACATGATCTTGGGCC	ACCATTGGTGCACCTTGTGG	Juglans_nigra_120313_comp35364_c1_seq14_ssr843
WLLC735-J_N_26554_f	AACCCTTCTGTGGCCTTTGC	GCTTGGAAACGCAGAATGGG	Juglans_nigra_120313_comp26554_c0_seq1_ssr60
WLLC749-J_N_175580_f	GGGCTTCTTAACGAGGTCCC	CGATCGTTGCAATATCGTCAGG	Juglans_nigra_120313_comp175580_c0_seq1_ssr159

Appendix C-2: Polymorphic markers- continued. Listed lab name, complete forward and reverse primer sequences, as well as the original resource and name for the primer sets in the graph.

Name in Lab	Forward Primer	Reverse Primer	Original Resource
WLLC801-J_N_32497_f	GAGAGCGAGAGACGTGATGG	TCCTCGGCAAGAACCTTTCC	Juglans_nigra_120313_comp32497_c1_seq5_ssr131
WLLC855-J_N_35198_f	CCTAGAACCGGGCGTTATCG	TACTCTTACAGACACCACCG	Juglans_nigra_120313_comp35198_c0_seq2_ssr3789
WLLC1077-J_N_26811_f	ACCTTGCAGTACAGAAGGAGC	GCACTCCACCATGCTAATGC	Juglans_nigra_120313_comp26811_c0_seq2_ssr358
WLLC1089-J_N_28000_f	GCTCCTCCTCAACCTGTTCG	ACCAAGAAACCCAGGAAAGG	Juglans_nigra_120313_comp28000_c0_seq3_ssr1146
WLLC1093-J_N_1424_f	CACACTGTACATGCAAGACAGC	TCCTCTTCCTTGTCATGCCC	Juglans_nigra_120313_comp1424_c0_seq1_ssr492
WLLC1111-J_N_33354_f	CATGCCATCTGCCCATACCC	TGTGGCTGGTGGTTATGTCC	Juglans_nigra_120313_comp33354_c0_seq6_ssr88
WLLC1183-J_N_28186_f	AGCACAAAGACCATAGGCTCC	CCAAAGGTACGCGAGAAAGC	Juglans_nigra_120313_comp28186_c0_seq1_ssr266
WLLC1219-J_N_25152_f	AACTTCAATCACAACCGGCC	AATTCCGACGATGACCCAGC	Juglans_nigra_120313_comp25152_c1_seq1_ssr153
WLLC1247-J_N_34423_f	TCCGAAAGCCTTCAAGGACG	TGATGTTAGCGCTACGTCCC	Juglans_nigra_120313_comp34423_c1_seq12_ssr451
WLLC1251-J_N_34607_f	ACAAACTCATGTACAAGCCTATCC	TAGAGAGGGACAGTCATACG	Juglans_nigra_120313_comp34607_c1_seq3_ssr239
WLLC1269-J_N_24839_f	TTTCCAGCAGTCTCCCATCC	CAGTTCTGCAAAGGAAGCGG	Juglans_nigra_120313_comp24839_c0_seq5_ssr72